Method Validation for Bacterial and Viral Analysis of Geoducks

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Abstract

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Background

The shellfish industry in Western Washington is an integral part of the State economy; with products being distributed in both domestic and international markets. Oysters and geoducks are two widely harvested shellfish in Western Washington, and ensuring clean growing areas with low levels of bacterial and viral contamination is essential for protecting consumers from disease. Microbiological methods for assessing viral and bacterial contamination in oysters have been established; however, similar testing procedures for geoducks have not been validated. Our study adapted viral and bacterial testing methods for oysters to better suit geoduck meat.

Methods

To validate our viral and bacterial testing methods, four trials were performed with MS2 coliphage and five trials were performed with *E. coli* CN-13 seeded into geoduck meat harvested in Western Washington. Each MS2 trial contained four seeding levels for which we calculated plaque forming units (PFU)/100g and percent recovery. MS2 recovery was analyzed using the double agar layer method. The *E. coli* CN-13 trials contained three seeding levels with the most probable number (MPN) calculated for each. MPN was

assessed using the APHA's 5-tube fermentation method. Fermentation on seeded geoduck meat was first performed in Lauryl tryptose broth (LST) and then passaged into EC broth and EC-MUG medium. In addition, six unseeded field samples were also processed using these methods to evaluate the ability of the method to detect naturally occurring levels of *E. coli* and male-specific coliphage.

Results

The average percent recoveries for MS2 trials ranged from 106.7% to 146.6%, with higher recoveries occurring at more concentrated seeding levels. Recoveries of over 100% are likely due to MS2 aggregation. The average recovery ratios for *E. coli* CN-13 trials were greater than 1, with higher recoveries also occurring at more concentrated seeding levels. Recovery ratios greater than 1 are likely due to bacterial aggregation and potential bacterial replication in geoduck meat. All geoduck meat used in the *E. coli* trials was shown to have prior levels of fecal coliforms but tested negative for *E. coli*. In unseeded geoduck, male-specific coliphage levels, though typically low, ranged from 9.55 to 139.39 PFU/100g and *E. coli* levels ranged from .20 to 79.0 MPN/g.

Conclusion

Our findings suggest that, with modifications, the current oyster viral and bacterial testing methods can be applied successfully to geoduck meat. The percent recoveries and MPN ratios indicate that both methods provide good assessments of bacterial and viral contamination in geoducks.

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Chapter I:
Method Validation for the Viral Analysis of Geoducks

Abstract

Oysters and geoducks are two widely harvested shellfish in Western Washington, and ensuring clean growing areas with low levels of viral contamination is essential for protecting consumers from disease. Microbiological methods for assessing viral contamination in oysters have been established; however, similar testing procedures for geoducks have not been validated. Our study adapted viral testing methods for oysters to better suit geoduck meat. To validate our viral testing methods, four trials were performed with MS2 coliphage seeded into geoduck meat harvested in Western Washington. Each MS2 trial contained four seeding levels for which we calculated plaque forming units (PFU)/100g and percent recovery. MS2 recovery was analyzed using the double agar layer (DAL) method. In addition, six unseeded field samples were also processed using these methods to evaluate the ability of the method to detect naturally occurring levels of male-specific coliphage (MSC). The average percent recoveries for MS2 trials ranged from 106.7% to 146.6%, with higher recoveries occurring at more concentrated seeding levels. Recoveries of over 100% are likely due to MS2 aggregation. Our findings suggest that, with modifications, the current oyster viral testing methods can be applied successfully to geoduck meat. The percent recoveries indicate that viral testing methods provide a good assessment of viral contamination in geoducks.

Background

Purpose

Shellfish, particularly bivalve molluscan shellfish, consumption can be a significant public health issue if shellfish are not properly monitored and harvested. Geoduck, a profitable bivalve molluscan shellfish in Washington State, has the potential to cause serious harm to consumers through both viral and bacterial contamination. Currently, the Washington State Department of Health is designated as the state shellfish control authority by the FDA, giving the agency the authority to "enforce the public health based restrictions on harvesting and to obtain meaningful penalties for violation of those harvesting restrictions" (1). However, there is no formally approved method for processing and testing geoducks for bacterial and viral contamination, and the literature on optimal testing methods is severely limited. The purpose of this project is to adapt current approved oyster microbial testing methods to better suit geoduck meat. To accomplish this, we conducted a series of seeding experiments which evaluated MS2 recovery from geoduck tissue using the adapted FDA oyster method by Howell et al. (2). The original FDA method is described in the ISSC Proposal 05-114 (3).

Economic Impact

The shellfish industry is an integral part of the Washington State economy as well as local tribal economies. Currently, shellfish aquaculture takes place in 12 counties in Western Washington with a total of 330 commercial shellfish growers (4). As of 2010, it is estimated that the shellfish industry accounted for 1,840 direct jobs and approximately 390 indirect jobs in Washington State, which translates to approximately 1 employee per

100 farmed acres (4). Additionally, the shellfish aquaculture industry in the state generated approximately $184 million in 2010 (4).

The shellfish aquaculture industry in Washington State affects consumers both nationally and internationally. It is currently estimated that "cultured geoduck production represents 10% of global geoduck production" and that Washington State accounts for 90% of this global production (5). In December of 2013, China imposed a suspension of bivalve molluscan shellfish shipments from Food and Agriculture Organization (FAO) Area 67 due to high levels of inorganic Arsenic (6). The ban included all growing regions in Alaska through the Washington State coast. Since the ban, shellfish, specifically geoducks, have been more carefully studied for arsenic and other environmental contaminants.

Role of the Washington State Department of Health

The Washington State Department of Health is the primary agency responsible for monitoring molluscan shellfish and water quality in the State. In Western Washington, the area stretching from Joint Base Lewis-McChord (JBLM) to Chambers Creek has had previous pollution events, which has restricted shellfish harvesting (7). However, sewage and industrial discharge issues are now being addressed, and the possibility of reopening these areas for shellfish harvest is being investigated (7). Funded through an EPA grant, the Washington State Department of Health has initiated a study to determine if current pollution levels in the JBLM and Chambers Creek growing area allow for harvesting practices to resume (7).

In 2013, the Washington State Department of Health sought to expand the region of the study, and began the process of reviewing the commercial request for classification to allow for subtidal geoduck harvesting in the Three Tree Point Growing Area (8). Currently,

three wastewater plants, Miller Creek, Salmon Creek, and Midway, discharge directly into these tracts, and the impact of bypass events on geoducks in this area is unknown (8). Data collected through viral and bacterial geoduck testing over the coming years will determine the contamination levels of geoducks in this area and will inform decision making on the opening of this growing area. The Washington Department of Fish and Wildlife maintains a complete and current list of active, closed, and "in recovery" beds for consumer safety (9).

Unseeded Environmental Geoduck Sampling

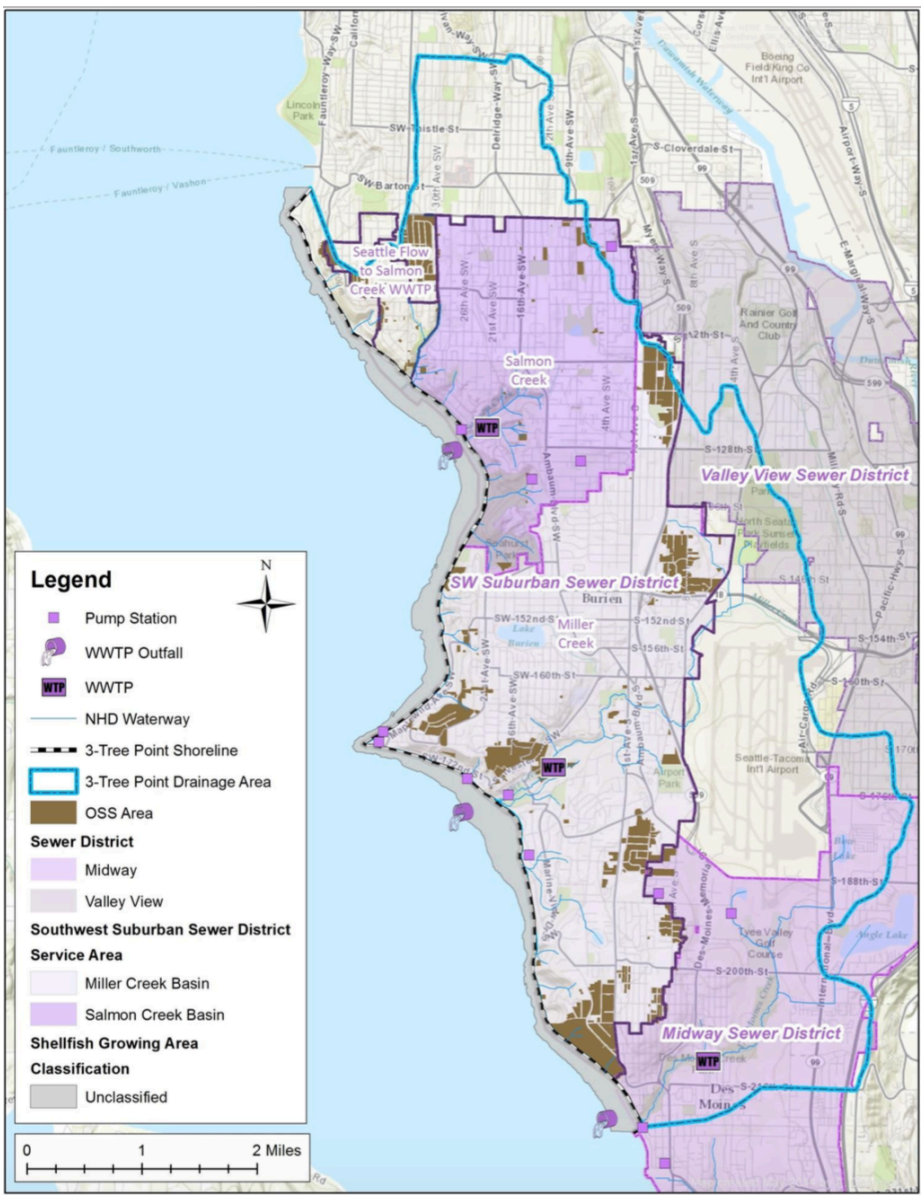
The geoduck provided by the Washington State Department of Health for the seeded experiments originated from the Salmon Creek, Miller Creek, and Midway growing areas. These locations were chosen primarily because the proximity of shellfish beds to waste water treatment plant (WWTP) outfalls are of concern. The locations of WWTP outfalls and WWTP service areas are shown in Figure 1.

Salmon Creek, the most northern location in this study, stretches approximately 1 mile and neighbors relatively undeveloped and residential land (8). The Salmon Creek WWTP is located near the mouth of the Creek, which discharges into Puget Sound (8). The Washington State Department of Health identifies this area as Site 185 and has previously found high levels of arsenic near the mouth of the creek (8).

Miller Creek flows through the Southwest Suburban Sewer District and, "at 4.5 miles in length, is the longest creek within the Three Tree Point Drainage Area" (8). Similar to the Salmon Creek WWTP, the Miller Creek WWTP is located near the mouth of the river and discharges into Puget Sound. The Washington State Department of Health has closely monitored water quality in Miller Creek, and has concluded that "the Creek is affected by erosion, high flows, and a lack of riparian buffer areas" (10). Furthermore, the Washington

State Department of Health found that water in the Creek previously has “exceeded the fecal coliform standard of 50 Most Probable Number (MPN)/100 ml” and has placed it on the 303(d) list (8). Miller Creek was also listed as a “Water of Concern” due to high levels of copper and zinc (10). The Washington State Department of Health identifies this area as Site 258.

Figure 1. Three Tree Point Wastewater Treatment Service Areas and WWTP Infrastructure (8)



Des Moines Creek is the primary creek in the Midway Sewer District and stretches for 2.7 miles South of SeaTac Airport (8). This creek lies in a more urban area and, as a result, has a greater chance of contamination from nearby industries such as the Tyee Valley Golf Course, SeaTac Airport, and I-5 (8). In addition to industrial contamination, the Midway WWTP discharges into Des Moines Creek, adding more potential contamination. Similar to Miller Creek, Des Moines Creek has previously exceeded the fecal coliform limit and has been placed on the 303(d) list (8). The Washington State Department of Health identifies this area as Site 39.

Preliminary Studies

Using geoduck meat from these collection areas, seeded studies were conducted to evaluate the efficacy of approved oyster MS2 testing methods on geoduck meat. To make the most accurate comparison between our seeded method validation and current oyster method validation, our methods closely followed those of a study conducted by Howell et al. that seeded MSC into oyster meat. In 2009, Howell et al. proposed a new analytical method for assessing contamination by using MS2 as a viral indicator in oyster and soft-shelled clams (2). They proposed modifications to the 2004 FDA double agar overlay method with the goal of increasing rates of MSC recovery (2). The proposed modifications include "eluting the shellfish meats with growth broth (2:1), and increasing the blending time to 180 seconds" to create a firm, distinct pellet (2). The proposal, based on EU methods, succeeded in creating a distinct pellet, which allowed for easier removal of the supernatant and reduced the amount of MSC remaining in the pellet. Additionally, a 10 plate minimum replication, with 2.5 ml of supernatant per plate, was proposed to increase the limit of quantitation/sensitivity (LOQ) (2). In this instance, the LOQ can be defined as

the lowest concentration of MSC that can be accurately measured across the 10 plates (11). These modifications were incorporated into our seeded study and were further adapted for geoduck analysis.

Use of Indicator Organisms - Male Specific Coliphage (MS2)

Viral and bacterial indicators can be useful tools for inferring the presence of similar organisms in an environmental sample. Throughout the study, Male Specific Coliphage (MSC), specifically MS2, was used as a viral indicator for human norovirus (NoV). MSC is an F-RNA bacteriophage that serves as a good model for enteric viruses because "like enteric viruses, they almost exclusively originate from the feces of warm-blooded animals; like enteric viruses they fail to multiply in the environment; and in terms of composition, structure and size they closely resemble human enteric viruses" (12). While the presence of MSC does not guarantee the presence of enteric viruses, they are frequently found together in sewage and many investigators believe they are more effective indicators of viral contamination than coliform bacteria (13). Although MS2 has many qualities that make it a good viral indicator for NoV, "some investigators have reported that F-specific RNA coliphages are rarely detected in human feces, suggesting that the presence of these coliphages in water does not necessarily indicate human fecal pollution" (14). While there may be some disadvantages to using MS2 as a viral indicator, it is currently one of the most effective indicator organisms for detecting the presence of enteric viruses.

Methods

Method Modifications

To validate the adapted viral recovery method for geoducks in the seeded trials, a modified double agar layer (DAL) method for oyster viral recovery proposed by Howell et

al. was used. As previously mentioned, Howell et al. made three significant modifications to the original FDA method in order to create a more distinct pellet and allow for more accurate removal of the supernatant. Both increasing the blending time and making a 2:1 ratio of growth broth to shellfish meat helped to create a more distinct pellet according to Howell et al. (2). While these modifications were helpful, the method was further adapted to better suit geoduck meat.

Two significant modifications to the Howell et al. DAL method were used in the geoduck seeding trials. The first modification made was to decrease the amount of geoduck supernatant added to the top agar. As geoduck supernatant is more opaque than oyster supernatant, adding the same amount to the top agar made it difficult to identify plaques. This problem is compounded by the fact that as the supernatant volume is increased, agar concentration must also be increased in order to insure appropriate agar hardening. Increasing the agar concentration also contributed to the lack of ability to accurately identify plaques. Ultimately, instead of adding 2.5 ml of supernatant as prescribed for oysters, the amount was decreased to 2 ml of geoduck supernatant.

To arrive at 2 ml of supernatant, various combinations of supernatant amounts and top agar were assayed. The experimental combinations of supernatant and top agar amounts can be seen in Table 1. Combination E proved to best achieve the balance between plaque readability and supernatant amount. Therefore, 5.7 ml of 1.4X strength top agar and 2 ml of supernatant were used in the DAL method in all geoduck seeding trials. This reduction of the supernatant to agar ratio, from 1:1 to 1:3, and the reduction of the agar concentration, from 7 g/L to 4.9 g/L, allowed for better plaque readability through the geoduck supernatant.

Table 1.

Sample	**A**	**B**	**C**	**D**	**E**	**F**
Agar Concentration (X)	**1.2**	**1.2**	**1.3**	**1.3**	**1.4**	**1.4**
Agar (ml)	6.5	6.7	6	6.2	5.7	5.7
E. coli (ml)	0.2	0.2	0.2	0.2	0.2	0.2
MS2 (-7 dilution)	0.1	0.1	0.1	0.1	0.1	0.1
Supernatant (ml)	1	1.25	1.5	1.75	2	2.25
Total (ml)	7.8	8.25	7.8	8.25	8	8.25

In addition to adjusting the supernatant amount, top agar concentration, and top agar amount, the bottom agar layer was also modified. In the Howell et al. protocol, the bottom agar layer is composed of a combination of tryptone, dextrose, NaCl, and agar (2). This agar, and similarly nutrient agar, proved to be quite transparent in appearance, making it difficult to identify plaques. Therefore, tryptic soy agar (TSA) was used instead. The primary advantage to using TSA was the color contrast it provided. As TSA is more yellow in appearance, plaques were more apparent and more easily identified. Additionally, using TSA is less labor intensive and is more rapidly produced than the bottom agar suggested in Howell et al.

Geoduck MS2 Seeding Method

Day 1

Each geoduck seeding trial took approximately 4 days to complete. A complete methodological protocol detailing preparatory and daily tasks can be found in Appendix C. The first day of a seeding trial is primarily devoted to preparing materials to be used in the following two days of the trial. On day one, the bottom agar was prepared by autoclaving TSA, adding antibiotics, and pouring the mixture into 100X15 mm petri dishes using sterile technique. The *E. coli* in used in the DAL method is *E. coli* F_{amp} (ATCC #: 700891); a strain of

E. coli that is resistant to streptomycin and ampicillin. Therefore, a combination of Streptomycin Sulfate Powder and Ampicillin Sodium Salt (Strep/Amp) was used as antibiotics to minimize the effects of potential unintended bacterial contamination. As recommended by Howell et al., 5 ml per L of a 1% solution of Strep/Amp was added to cooled, yet still liquid, TSA (2). After thoroughly mixing in the Strep/Amp, approximately 20 ml of TSA is then poured into each 100X15 mm petri dish and left to solidify. To maintain the integrity of the antibiotics, the plates were then refrigerated until use.

In addition to preparing the bottom agar layer, Nutrient Broth was also prepared to facilitate the growth of E. coli F_{amp} overnight. After autoclaving and cooling, 5 ml per L of a 1% solution of Strep/Amp was added. In addition, laboratory materials including blenders, beakers, scoopers, stir bars, and glass tubes were autoclaved to be used the following two days. In preparation for MS2 stock enumeration by plaque assay the following day, approximately 20 g of frozen geoduck meat was moved to the refrigerator to slowly defrost overnight. Lastly, approximately 10 µl of *E. coli* F_{amp} from a frozen stock was added to 20 ml of Nutrient Broth and placed in a 37 °C shaker at 100 rpm. This *E. coli* overnight was used in the MS2 stock enumeration by plaque assay the following day.

Day 2

On the second day of the trial, the MS2 stock is enumerated using the DAL method to estimate how many plaque forming units (PFUs) are in the stock. Knowing approximately how many PFUs are in the MS2 stock will provide more accurate information to calculate the amount of the given MS2 dilution going into each seeding level the following day. To prepare for MS2 enumeration by plaque assay, 120 ml of 1.4X top agar was autoclaved. Once cooled, 5 ml per L of a 1% solution of Strep/Amp was added to the top agar.

To extract the supernatant, 20 g of the refrigerated geoduck meat and 40 ml of nutrient broth were blended for 180 seconds. After blending, 33 g of homogenate was aliquoted into a sterile 50 ml conical. The remaining homogenate was aliquoted into another sterile 50 ml conical. Both conicals were then centrifuged at 4°C for 15 min. at 9000 g. After centrifuge was complete, the supernatant was extracted and weighed. The supernatant from the extra homogenate was then used to create a 10-fold MS2 dilution series.

In earlier MS2 enumeration by plaque assay experiments, MS2 aggregation significantly impacted recovery results, and was a challenge to combat. In an attempt to break up MS2 aggregation, the dilution series was first filtered through a sterile PES 0.45 micron filter and then through a sterile PES 0.22 micron filter. While this filtration helped to break up some aggregates, it was not completely effective. The effects of remaining MS2 aggregation are further discussed in the results section.

Previous MS2 enumeration by plaque assay experiments, revealed that the MS2 stock that was to be used in the seeding experiments contained approximately 10^9 PFU/ml. To make the first dilution (-1), 900 µl of a 1X Phosphate Buffer Solution (PBS) was combined with 100 µl of the frozen MS2 stock. This 1:10 dilution contained approximately $4.00x10^8$ PFU/ml. To make the second 1:10 dilution (-2), 4.5 ml of 1X PBS was combined with 500 µl of the -1 solution. The volume was increased in the second dilution to account for loss during filtration and to save enough of the filtered product to be used for seeding the following day. The remaining filtered product was refrigerated overnight and was used the following day in the geoduck seeding. The following seven 1:10 dilutions were

prepared using geoduck supernatant instead of PBS. This was done to further reduce MS2 aggregation.

To achieve plates with countable plaques, the sixth dilution (10^3 PFU/ml) through the ninth dilution (1 PFU/ml) was plated in duplicate. In the DAL method for MS2 enumeration by plaque assay, each plate contained 5.7 ml of 1.4X top agar, 1.9 ml of geoduck supernatant, 100 µl of dilutions (-6 through -9) and 200 µl of *E. coli* F_{amp} overnight. After all plates dried, they were inverted and incubated at 36 °C for 16-20 hours. After the completion of the MS2 enumeration by plaque assay DAL, an *E. coli* F_{amp} overnight was placed in a 37 °C shaker at 100 rpm. Additionally, 180 g of frozen geoduck meat was put in the refrigerator to slowly defrost.

Day 3

After overnight incubation, plaques and a weighted titer from the MS2 enumeration by plaque assay the previous day were calculated. A weighted titer places more importance on the highest countable dilution and is considered to be a more accurate representation of total PFUs/ml. Therefore, weighted titers were used in all titer calculations. The calculated weighted titer shows approximately how many PFUs/ml are currently in the saved filtered product that will be used for the seeding trial.

The calculated weighted titer determines the amount of the MS2 filtered product added to each seeding level. In each seeding trial, four target seeding levels were used: 100 PFU/100 g, 500 PFU/100 g, 2,000 PFU/100 g, and 10,000 PFU/100 g. The amount of MS2 filtered product added to achieve the desired PFU for each level was then calculated.

The total number of MS2 needed for each seeding level was calculated using the following formula (2):

$$\frac{\text{Total number of MS2 (N)}}{\text{Total supernatant plated (20g)}} \times \frac{\text{Weight of supernatant extracted (25g estimate)}}{\text{Grams of sample used (11g)}} \times 100 = \text{PFU of MS2/100g}$$

Once the total number of MS2 required for a given level is calculated, that number is then multiplied by 3.33 to estimate the amount needed per 110 g of geoduck homogenate. Each seeding level contains exactly 110 g of homogenate. The product of the total number of MS2 and 3.33 is then divided by the weighted MS2 titer and the most appropriate amount of a certain MS2 dilution is then determined.

To prepare for the seeding DAL, 800 ml of 1.4X top agar is autoclaved and cooled. Antibiotics were added just before the DAL took place. To extract the supernatant, 180g of geoduck meat and 360 ml of nutrient broth were blended for 180 seconds. The geoduck homogenate was then aliquoted into 4 beakers with 110g in each, with each beaker representing a different seeding level. The extra homogenate was then aliquoted into a sterile 50 ml conical and centrifuged at 4°C for 15 min. at 9000 g. The extra supernatant was then divided into 900 µl aliquots to prepare the remainder 10-fold serial dilutions (3 through 9). 100 µl of the remaining filtered product from day 2 was then used to make the third dilution. Similarly, the remaining serial dilutions were made.

After a sterile stir bar was placed in each beaker, the appropriate amount of MS2, as determined by the previous calculation, was seeded into each level. Each level's homogenate was then stirred for 15 min. at 300 rpm. After 15 minutes, the homogenate from each beaker was portioned into three 33 g aliquots. Each aliquot was labeled L1 A, L1 B, etc. to achieve triplicate samples for each level. All aliquots were then centrifuged at 4°C for 15 min. at 9000 g. The supernatant from each aliquot was removed and allowed to cool to room temperature.

In the DAL method for MS2 seeding, each plate contained 5.7 ml of 1.4X top agar, 2 ml of geoduck supernatant, and 200 µl of *E. coli* F_{amp} overnight. After all plates dried, they were inverted and incubated at 36 °C for 16-20 hours. The positive and negative controls were produced in the same manner as the DAL for MS2 enumeration by plaque assay on day 2. To achieve countable plaques, dilutions 6 through 9 were plated.

Day 4

After overnight incubation, plaques from all four levels of the MS2 seeding the previous day were calculated. Additionally, a weighted titer from the control plates was calculated. To calculate the seeded concentration for a given level, the amount of MS2 seeded in was multiplied by the weighted titer from day 3. This product was then multiplied by 99 g to account for the 3 aliquots of 33 g of geoduck homogenate at the given level. This product was then divided by 110 g to account for the actual amount of homogenate that was aliquoted for each level originally. This value represents the total number of MS2.

Similar to calculating the estimated seeding levels in day 3, the calculated total number of MS2 is then used in the following formula (2):

$$\frac{\text{Total number of MS2 (N)}}{\text{Total supernatant plated (20g)}} \times \frac{\text{Weight of supernatant extracted (25g estimate)}}{\text{Grams of sample used (11g)}} \times 100 = \text{PFU of MS2/100g}$$

To account for triplicates in each level, the weight of all three supernatants in a given level is summed, the amount of sample used is increased to 33 g, and the total supernatant plated is increased to 60 g. The product, expressed as PFU of MSC/100g, is the seeded concentration for a given level.

To calculate the replicate plate concentration, the number of plaques over ten plates in a triplicate replication are summed and used in the formula above as total number of MS2. The percent recovery is then calculated by dividing the replicate plate concentration by the given seeding level.

Results

A total of four MS2 trials with four seeding levels per trial were analyzed. The same four target seeding levels were used in each trial: 100 PFU/100 g (level 1), 500 PFU/100 g (level 2), 2,000 PFU/100 g (level 3), and 10,000 PFU/100 g (level 4). Within each trial, each seeding level was assayed in triplicate, with percent recoveries for each seeding level represented as the average of the triplicate percent recoveries within that seeding level.

The average percent recoveries for all MS2 seeding levels ranged from 106.7% to 146.6%, with higher recoveries occurring at more concentrated seeding levels. This trend can be seen in Figure 2 and Table 2. Detailed data tables containing percent recoveries and standard deviations for all seeding levels by trial can be found in Appendix A.

Figure 2. Average Percent Recoveries by Seeding Level

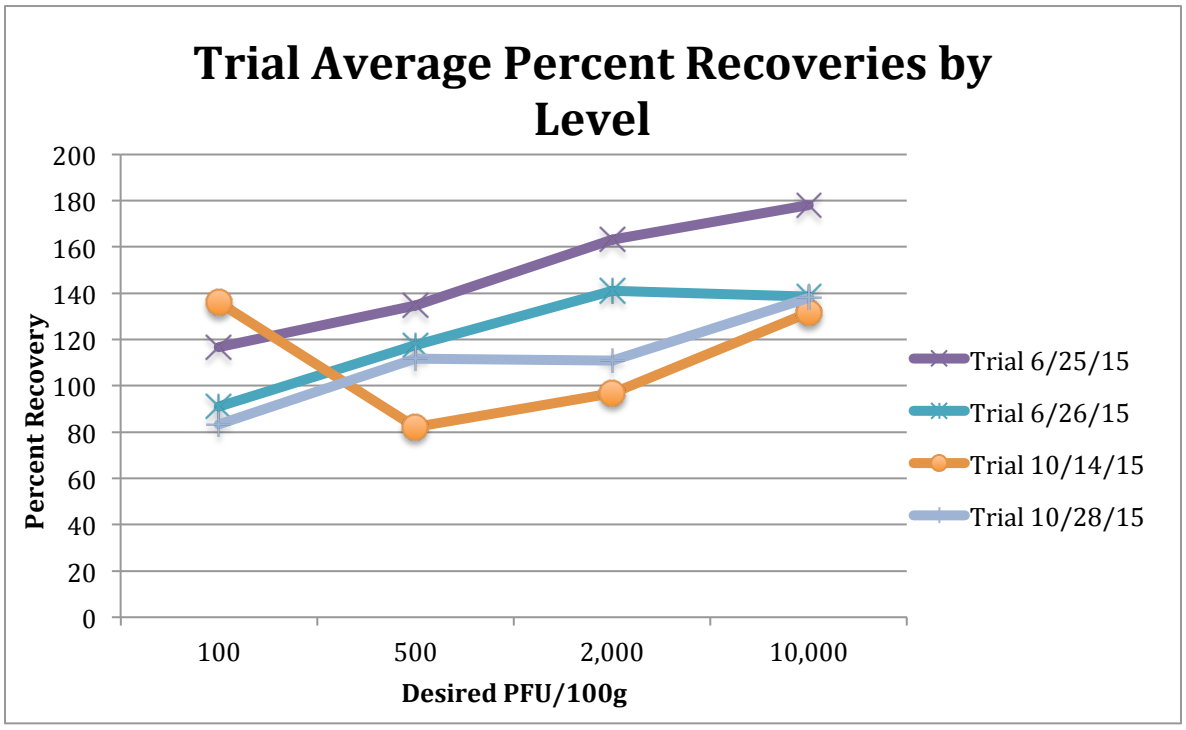


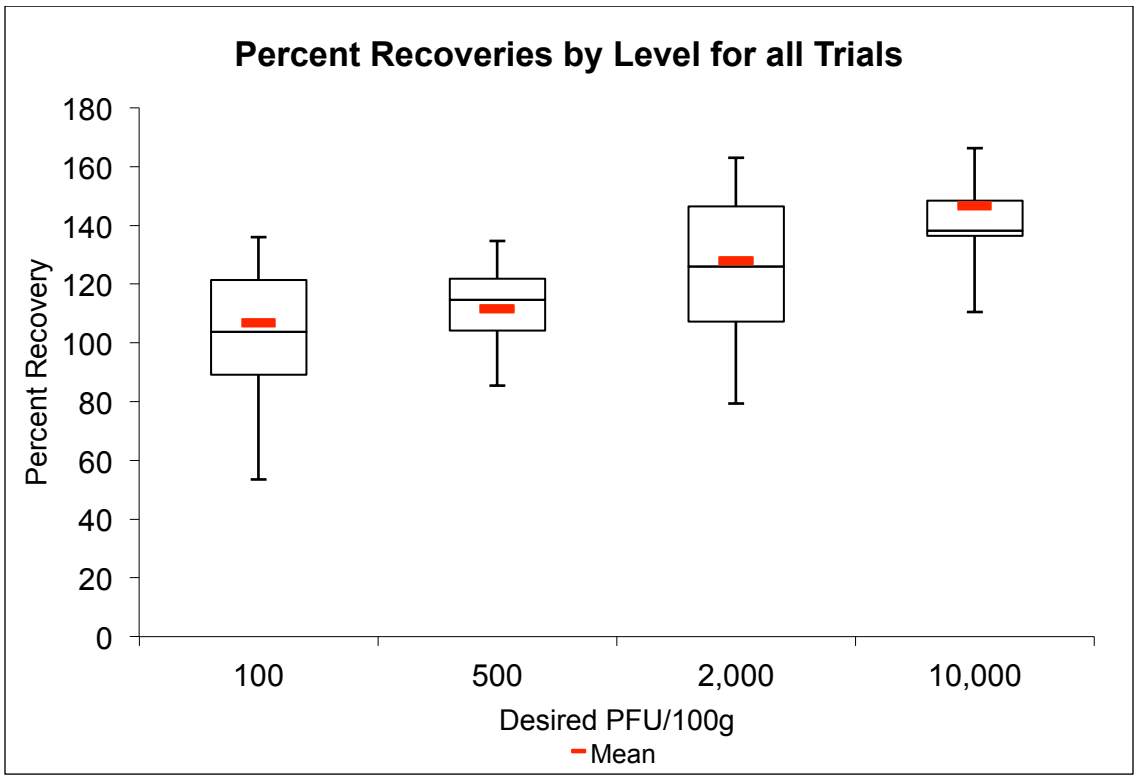
Table 2. Average Percent Recoveries by Seeding Level

Trial Average Percent Recoveries				
	Level 1	**Level 2**	**Level 3**	**Level 4**
Trial 6/25/15	116.61	134.74	163.11	178.02
Trial 6/26/15	91.00	117.59	141.04	138.50
Trial 10/14/15	135.94	82.17	96.69	131.74
Trial 10/28/15	83.33	111.59	110.82	138.01
Average % Recovery	106.72	111.52	127.91	146.57
Std. Dev.	24.12	21.89	29.88	21.19

Additionally, the distribution of the percent recoveries varied by seeding level. As shown in Figure 3, the percent recoveries in seeding levels 2 and 4 maintained a relatively narrow range, whereas the percent recoveries in seeding levels 1 and 3 had larger ranges.

The wide range of recoveries seen in seeding level 1 could be, in part, due to seeding at a level near the limit of detection (LOD).

Figure 3. Percent Recoveries by Seeding Level for all Trials



Discussion

This method validation study was undertaken to investigate the efficacy of adaptations made to the current oyster viral testing method produced by Howell et al. These adaptations were vital, as current oyster viral testing methods are not suitable for geoduck meat. As all average percent recoveries were above 106%, it can be concluded that this new viral testing method provides a good assessment of viral contamination in geoducks.

As previously mentioned, percent MS2 recovery typically increased at more concentrated seeding levels, frequently over 100%. This increase can be predominantly

explained by higher MS2 aggregation at more concentrated seeding levels. As noted in Dika et al., "the presence of the RNA genome within MS2 likely contributes to significant interparticular attractive forces (van der Waals and hydrophobic) that overwhelm the repulsive electrostatic contribution and thus lead to undifferentiated aggregation behavior at low and high salt concentrations" (15).

To minimize aggregation, the MS2 used in the seeding experiments was first filtered through a sterile PES 0.45 micron filter and then through a sterile PES 0.22 micron filter. The decision to filter the MS2 before seeding into geoduck meat was made after initial seeding experiments yielded even higher average percent recoveries. While filtration did eliminate some aggregation, it was not completely effective, as seen by the recoveries of over 100%.

In all trials, the MS2 was filtered at the 1:100 dilution from the undiluted MS2 stock. While filtering at this dilution did eliminate some aggregation, it may have been more effective to filter the MS2 stock at the 1:1,000 or the 1:10,000 dilution (16). In future, filtering at these lower concentrations may help prevent additional aggregation by creating more of a buffer space between individual phage particles, likely reducing the strength of the interparticular attractive forces (16).

Limitations

One potential limitation of this study was the use of MS2 as an indicator organism for human norovirus (NoV). While MS2 has proven to be an effective indicator organism for enteric viruses, it may not act in the same way as NoV in seeding trials. Additionally, it is unknown if human norovirus aggregates in the same manner as MS2, potentially making direct comparison in this seeding study difficult.

While taking into account MS2 aggregation in the seeded trials, our findings suggest that, with modifications, the current oyster viral testing methods can be applied successfully to geoduck meat. As all percent recoveries were over 106%, it can be concluded that the viral testing methods described in the seeded trials provide good assessment of viral contamination in geoducks. Finally, further method validation studies are needed to characterize MS2 recovery in geoduck meat with a focus on reducing MS2 aggregation.

Laboratory Materials

Item	**Amount**	**Product Identification**	**Notes**
Autoclavable Blender with lid	1 blender, 1 lid		Blender needs to hold at least 500 ml
Nutrient Broth	400 ml	Company: Difco Ref. 234000	Need for seeding and overnight
Tryptic Soy Agar (TSA)	2.8 L	Company: Difco Ref. 236920	
Sterile 100 x 15 mm Petridish	130 plates	Company: Falcon Ref. 351029	
Streptomycin Sulfate Powder	0.5 g	Company: Cellgro Cat. No. 61-088-RM	Needs to be kept refrigerated in powder form and frozen for stock
Ampicillin, Sodium Salt	0.5 g	Company: Fisher Scientific Ref. BP 1760-25	Needs to be kept refrigerated in powder form and frozen for stock
Disposable Glass Culture Tubes 13 x 100 mm	130 tubes	Company: Fisher Scientific Cat. No. 14-961-27	Need to be sterilized
Laboratory Scale	1 scale	Company: VWR Model CLW 2000	
Magnetic Stir Plates	2 stir plates	Company: Fisher Scientific Cat. No. 11-600-49SH	Do not need to have heating capabilities
Tryptone	11.2 g	Company: Fisher Scientific Ref. BP1421-500	
Dextrose	1.12 g	Company: Fisher Scientific Ref. D14-500	
Sodium Chloride (NaCl)	12.56 g	Company: Fisher Scientific Cat. No. 7647145	Used for top agar and PBS
1 M $CaCl_2$	0.56 ml	Company: Fisher Scientific Ref. BP510-500	
Bacto Agar	7.84 g	Company: BD Ref. 214010	
Sterile 50 ml conicals	26 conicals	Company: Thermo Scientific	

		Mfr #: 339653	
500 ml glass flask	4 flasks	Company: Pyrex Cat. No. 4980	Need to be sterilized
Potassium Chloride (KCl)	0.2 g	Company: EMD Omnipur Cat. No. 7447407	
Sodium Phosphate dibasic, anhydrous (Na_2HPO_4)	1.44 g	Company: Fisher Scientific Cat. No. 7558794	
Potassium Phosphate monobasic, crystal (KH_2PO_4)	0.24 g	Company: J.T. Baker Cat. No. 778770	
Magnetic stir bar	4		Need to be sterilized and must fit into bottom of glass flask
Sterile PES .45 micron filter	1 filter	Company: Millex Ref. SLHP033NS	
Sterile PES .22 micron filter	1 filter	Company: Millex Ref. SLGP033RS	
Sterile syringe	3 syringes	Company: BD Ref. 309653	1 syringe for antibiotics 2 syringes for MS2 filtration
1.5 ml microcentrifuge tubes	Approximately 40 tubes	Company: VWR Cat. No. 20170-333	Need to be sterilized
Aluminum Foil	Approximately 1 x 1’		
E. coli F_{amp}	10 microliters	ATCC #: 700891	Used in overnight
MS2	Amount dependent on MS2 titer	ATCC #: 15597-B1	

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Chapter II:
Method Validation for the Bacterial Analysis of Geoducks

Abstract

The shellfish industry in Western Washington is an integral part of the State economy; with products being distributed in both domestic and international markets. Oysters and geoducks are two widely harvested shellfish in Western Washington, and ensuring clean growing areas with low levels of bacterial and viral contamination is essential for protecting consumers from disease. Microbiological methods for assessing bacterial contamination in oysters have been established; however, similar testing procedures for geoducks have not been validated. Our study adapted bacterial testing methods for oysters to better suit geoduck meat. To validate our bacterial testing methods, five trials were performed with E. coli CN-13 seeded into geoduck meat harvested in Western Washington. The E. coli CN-13 trials contained three seeding levels with the most probable number (MPN) calculated for each. MPN was assessed using the APHA's 5-tube fermentation method. Fermentation on seeded geoduck meat was first performed in Lauryl tryptose broth (LST) and then passaged into EC broth and EC-MUG medium. In addition, six unseeded field samples were also processed using these methods to evaluate the ability of the method to detect naturally occurring levels of E. coli and fecal coliforms. The average recovery ratios between seeded and control groups for E. coli CN-13 trials were greater than 1, with higher recoveries also occurring at more

concentrated seeding levels. Recovery ratios greater than 1 were likely due to bacterial aggregation and potential bacterial replication in geoduck meat. All geoduck meat used in the E. coli trials was shown to have prior levels of fecal coliforms but tested negative for E. coli. Our findings suggest that, with modifications, the current oyster bacterial testing methods can be applied successfully to geoduck meat. The percent recoveries indicate that both methods provide a good assessment of bacterial contamination in geoducks.

Background

Purpose

Shellfish, particularly bivalve molluscan shellfish, consumption can be a significant public health issue if shellfish are not properly monitored and harvested. Geoduck, a profitable bivalve molluscan shellfish in Washington State, has the potential to cause serious harm to consumers through both viral and bacterial contamination. Currently, the Washington State Department of Health is designated as the state shellfish control authority by the FDA, giving the agency the authority to "enforce the public health based restrictions on harvesting and to obtain meaningful penalties for violation of those harvesting restrictions" (1). However, there is no formally approved method for processing and testing geoducks for bacterial and viral contamination, and the literature on optimal testing methods is severely limited. The purpose of this project is to adapt current approved oyster microbial testing methods to better suit geoduck meat. To accomplish this, we conducted a series of seeding experiments which evaluated *E. coli* CN-13 recovery from geoduck tissue using an adapted FDA BAM 5-tube fermentation method (2).

Economic Impact

The shellfish industry is an integral part of the Washington State economy as well as local tribal economies. Currently, shellfish aquaculture takes place in 12 counties in Western Washington with a total of 330 commercial shellfish growers (3). As of 2010, it is estimated that the shellfish industry accounted for 1,840 direct jobs and approximately 390 indirect jobs in Washington State, which translates to approximately 1 employee per 100 farmed acres (3). Additionally, the shellfish aquaculture industry in the state generated approximately $184 million in 2010 (3).

The shellfish aquaculture industry in Washington State affects consumers both nationally and internationally. It is currently estimated that "cultured geoduck production represents 10% of global geoduck production" and that Washington State accounts for 90% of this global production (4). In December of 2013, China imposed a suspension of bivalve molluscan shellfish shipments from Food and Agriculture Organization (FAO) Area 67 due to high levels of inorganic Arsenic (5). The ban included all growing regions in Alaska through the Washington State coast. Since the ban, shellfish, specifically geoducks, have been more carefully studied for arsenic and other environmental contaminants.

Role of the Washington State Department of Health

The Washington State Department of Health is the primary agency responsible for monitoring molluscan shellfish and water quality in the State. In Western Washington, the area stretching from Joint Base Lewis-McChord (JBLM) to Chambers Creek has had previous pollution events, which has restricted shellfish harvesting (6). However, sewage and industrial discharge issues are now being addressed, and the possibility of reopening these areas for shellfish harvest is being investigated (6). Funded through an EPA grant, the Washington State Department of Health has initiated a study to determine if current pollution levels in the JBLM and Chambers Creek growing area allow for harvesting practices to resume (6).

In 2013, the Washington State Department of Health sought to expand the region of the study, and began the process of reviewing the commercial request for classification to allow for subtidal geoduck harvesting in the Three Tree Point Growing Area (7). Currently, three wastewater plants, Miller Creek, Salmon Creek, and Midway, discharge directly into these tracts, and the impact of bypass events on geoducks in this area is unknown (7). Data

collected through viral and bacterial geoduck testing over the coming years will determine the contamination levels of geoducks in this area and will inform decision making on the opening of this growing area. The Washington Department of Fish and Wildlife maintains a complete and current list of active, closed, and "in recovery" beds for consumer safety (8).

Unseeded Environmental Geoduck Sampling

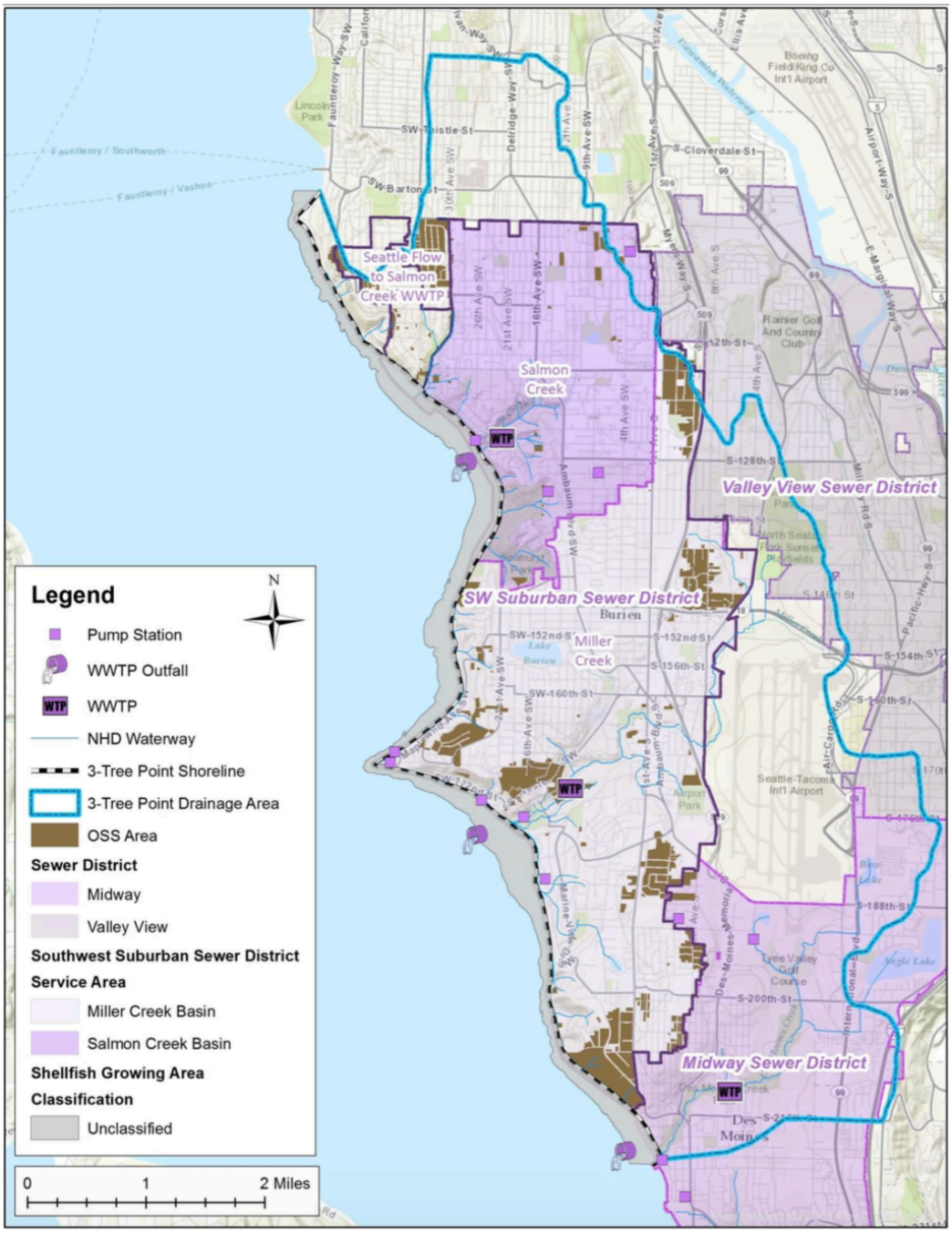
The geoduck provided by the Washington State Department of Health for the seeded experiments originated from the Salmon Creek, Miller Creek, and Midway growing areas. These locations were chosen primarily because the proximity of shellfish beds to waste water treatment plant (WWTP) outfalls are of concern. The locations of WWTP outfalls and WWTP service areas are shown in Figure 1.

Salmon Creek, the most northern location in this study, stretches approximately 1 mile and neighbors relatively undeveloped and residential land (7). The Salmon Creek WWTP is located near the mouth of the Creek, which discharges into Puget Sound (7). The Washington State Department of Health identifies this area as Site 185 and has previously found high levels of arsenic near the mouth of the creek (7).

Miller Creek flows through the Southwest Suburban Sewer District and, "at 4.5 miles in length, is the longest creek within the Three Tree Point Drainage Area" (7). Similar to the Salmon Creek WWTP, the Miller Creek WWTP is located near the mouth of the river and discharges into Puget Sound. The Washington State Department of Health has closely monitored water quality in Miller Creek, and has concluded that "the Creek is affected by erosion, high flows, and a lack of riparian buffer areas" (9). Furthermore, the Washington State Department of Health found that water in the Creek previously has "exceeded the fecal coliform standard of 50 Most Probable Number (MPN)/100 ml" and has placed it on

the 303(d) list (7). Miller Creek was also listed as a “Water of Concern” due to high levels of copper and zinc (9). The Washington State Department of Health identifies this area as Site 258.

Figure 1. Three Tree Point Wastewater Treatment Service Areas and WWTP Infrastructure (7)



Des Moines Creek is the primary creek in the Midway Sewer District and stretches for 2.7 miles South of SeaTac Airport (7). This creek lies in a more urban area and, as a result, has a greater chance of contamination from nearby industries such as the Tyee Valley Golf Course, SeaTac Airport, and I-5 (7). In addition to industrial contamination, the Midway WWTP discharges into Des Moines Creek, adding more potential contamination. Similar to Miller Creek, Des Moines Creek has previously exceeded the fecal coliform limit and has been placed on the 303(d) list (7). The Washington State Department of Health identifies this area as Site 39.

Fecal Coliforms and E. coli

As filter feeders, geoducks can accumulate fecal coliforms and *E. coli* from surrounding water in their digestive tracts. The presence of fecal coliforms and *E. coli* in geoduck meat is often a good indicator of fecal contamination in surrounding waters. The relationship between *E. coli* and fecal contamination was first described in the late 19th century, and has continued to be used as a metric for measuring fecal contamination (10). Since then, other organisms, such as Klebsiella, have been found to exhibit similar characteristics as *E. coli*, namely the ability to ferment lactose (10). This ability to ferment lactose in experimental trials made distinguishing between the two organisms difficult (10). As a result, scientists refer to the group of enteric bacteria that share these similar characteristics as "coliforms," and define the group as being "Gram-negative, facultative anaerobic rod-shaped bacteria that ferment lactose and gas within 48 hours at 35° C" (10).

While *E. coli* and fecal coliforms can be good indicators of the presence of viral contamination, these organisms themselves can also cause mild to severe gastroenteritis (11). Bacteria that have been associated with molluscan shellfish contamination include

Salmonella spp., Shigella spp., Campylobacter spp., Plesiomonas spp., Aeromonas spp., and *E. coli* (11). Although bacterial agents can be the cause of serious disease, it is estimated that, historically, viral agents have proportionately accounted for more instances of disease from consumption of molluscan shellfish (11). Nevertheless, bacterial agents in molluscan shellfish pose a serious threat to Washington State consumers.

The Washington State Department of Health routinely measures *E. coli* and fecal coliform concentrations in the Puget Sound and assesses the impact on local shellfish aquaculture, such as geoduck growth and production. *E. coli* and fecal coliform contamination can originate from a variety of sources throughout the Sound. Some of the sources the Department of Health is most concerned with include "freshwater shoreline discharges, stormwater, illicit discharges, leaking wastewater treatment plants (WWTPs), pet waste, wildlife, and on-site sewage systems" (7). Understanding and rapidly addressing these sources of contamination is imperative to the health and safety of geoduck consumers domestically and internationally.

Most Probable Number (MPN)

In all five seeded trials, the most probable number (MPN) was calculated based on Appendix 2 from the *FDA Bacteriological Analytical Manual (BAM)* (12). This appendix describes the MPN calculation process based on a serial dilution method described in the *FDA BAM: Enumeration of Escherichia coli and the Coliform Bacteria* (10). A modified version of this FDA BAM serial dilution method was used in all five seeded trials.

Unlike other forms of bacterial enumeration, calculating the MPN does not result in a precise bacterial count. Instead, the MPN is used to "measure the concentration of a target microbe in a sample" (12). This method is meant to combat limit of detection inaccuracies

associated with calculating the precise bacterial load in a sample with a low bacterial count. The FDA defines a sample as having a low bacterial count when there are fewer than 100 bacteria per gram of sample (12). This method allows for a better bacterial analysis of geoduck meat in particular, as it is a medium "whose particulate matter may interfere with accurate colony counts" (12). Lastly, one of the primary advantages to the serial dilution method and MPN calculation is that it measures viable bacteria (12). Unlike RT-PCR, which merely detects the presence of an organism of interest, the serial dilution method provides data that could be used for a more direct analysis on the potential health impact of shellfish on consumers.

As previously mentioned, the MPN is a calculation based on a serial dilution analysis of a sample of interest. All five trials using seeded geoduck meat used a 5-tube, 3-dilution experimental structure. Throughout all trials, the MPN was calculated by totaling the positive and negative tubes in each dilution after the LST, EC and EC-MUG incubation periods. To calculate the MPN, the number of positive tubes per dilution was used as a component of the formula (Figure 2) developed by M.H. McCrady in 1915 (12).

Figure 2. MPN Formula (12)

$$\sum_{j=1}^{k} \frac{g_j m_j}{1-\exp(-\lambda m_j)} = \sum_{j=1}^{k} t_j m_j$$

where exp(x) means e^x, and

K denotes the number of dilutions,
g_j denotes the number of positive (or growth) tubes in the *j*th dilution,
m_j denotes the amount of the original sample put in each tube in the *j*th dilution,
t_j denotes the number of tubes in the *j*th dilution.

The FDA has released an online calculation tool, using Microsoft Excel, that allows for quicker MPN calculation (12). This tool was used to calculate MPN's throughout all five

trials, as it provides an accurate MPN calculation based on the original equation by McCrady. In addition to MPN calculations, this tool can also be used to calculate the 95% confidence intervals (CIs) for each MPN value. While there are various approaches to CI calculation, the FDA utilizes a method based on a log normal distribution, which was first proposed for a similar purpose by J.B.S. Haldane in 1939 (12, 13).

While there are many advantages to using an MPN to assess the bacterial concentration in a sample, there are several disadvantages. These disadvantages are further discussed in the limitations section.

Methods

Method Modifications

To validate the adapted bacterial recovery method for geoducks in seeded trials, a modified version of the *FDA Bacteriological Analytical Manual (BAM): Enumeration of Escherichia coli and the Coliform Bactria* was used (10). As the FDA BAM method is intended for detection of *E. coli* in unseeded environmental samples, the method was modified to allow for *E. coli* CN-13 seeding. Additionally, the FDA BAM method was adapted to better suit geoduck meat, as geoduck meat was not compatible with multiple steps of the method. Both of these fundamental modifications had significant impacts on the existing method and allowed for more successful processing of geoduck meat.

To create realistic *E. coli* seeding conditions, the laboratory-grown *E. coli* CN-13 was conditioned in Puget Sound water collected from Shilshole Beach. After a 14-hour growth period in Tryptic Soy Broth (TSB), the *E. coli* CN-13 cells were transferred into refrigerated sound water that mimicked the average water temperature in the Puget Sound - approximately 10° C (14). This process was undertaken to mimic conditions that *E. coli*

would face when in the same environment as geoducks. Conditioning the *E. coli* CN-13 cells in this cold, saline environment for three hours was predicted to cause a decline in *E. coli* CN-13 population and, therefore, more accurately represent environmental *E. coli* characteristics.

The change in *E. coli* population was measured using spectrophotometry. Readings were performed both immediately after the *E. coli* cells entered the sound water and after three hours of conditioning. The paired spectrometry readings showed little to no change in *E. coli* population throughout the five trials. Once read, the conditioned *E. coli* in sound water was then diluted and seeded into the geoduck meat.

Aside from modifying the method for seeding, the FDA BAM method was also modified in other critical ways. The current FDA BAM method is written for the processing of small bi-valve molluscan shellfish such as oysters and, therefore, calls for the blending of 10-12 individual shellfish liquors and meats. As geoduck concentrate most of their pollutants in their gutball, only the gutballs were used throughout the seeded trials (15). Therefore, the first modification to the FDA BAM method was to dissect out and blend three geoduck gutballs. Prior environmental sampling efforts showed that a geoduck gutball typically weighed approximately 150-200g. Three blended gutballs provided enough geoduck meat to complete the *E. coli* assay. Additionally, the blending time was increased from two to three minutes to ensure thorough homogenization. Lastly, a standard isotemp incubator was used instead of the FDA recommended covered water bath to incubate EC broth and MUG broth tubes.

The other key modification to the current FDA BAM method was the inclusion of a final verification step to identify positive EC-MUG tubes. As stated in the current method, a

positive EC-MUG tube is indicated by gas production and by fluorescence under longwave UV (10). While this method works well for oyster meat assays, it is not effective for geoduck meat assays, as geoduck meat particulates naturally fluoresce and can result in false positives. To combat this, a loopful of each gas producing EC-MUG sample was streaked onto agar and then checked for fluorescence under longwave UV. The agar used in all five trials contained a mixture of EC-MUG Broth, to allow fluorescence, and Bacto agar as a thickening agent. Streaking gas-producing samples and examining the plates under longwave UV allowed for a more definitive identification of positive EC-MUG tubes.

Geoduck E. coli Seeding Method

Before the trials began, the *E. coli* CN-13 stock was enumerated to determine strength after 14 hours of growth. It was estimated that *E. coli* CN-13 would reach peak growth at approximately 14 hours based on a series of periodic spectrophotometry readings performed on a similar *E. coli* F_{amp} strain. After 14 hours of growth in TSB, the *E. coli* CN-13 stock was centrifuged for 15 min at 5,000 g at 4 ° C to get a distinct pellet. The TSB supernatant was then discarded and 50 ml of refrigerated sound water was added to the pellet. The *E. coli* CN-13 cells and sound water were then vortexed thoroughly to re-suspend the cells. After vortexing, the seeded sound water was placed in the refrigerator for three hours to condition the *E. coli* CN-13 cells.

Once the *E. coli* CN-13 cells were conditioned, the seeded sound water was enumerated using a spot-titer culture based method developed by Beck et al. (16). First, ten-fold serial dilutions were made from the conditioned sound water and each dilution was spot titered onto MacConkey agar. Each dilution was evaluated using ten ten-microliter drops, with the average number of colonies per drop used as a metric for

calculating an overall weighted titer. The weighted titer based on the spot titering experiment was $6.09*10^8$ CFU/ml. This weighted titer was used as a baseline estimate of *E. coli* CN-13 stock strength for all five *E. coli* CN-13 seeding trials.

Based on this weighted titer and experimental parameters, it was decided that each seeding level would contain 60g of geoduck meat. Table 1 outlines the estimated number of *E. coli* CN-13 cells per gram of geoduck meat for each level based on the initial weighted titer. The highlighted dilutions in Table 1 represent the dilutions most likely to produce countable results and were, therefore, used as the three seeding levels in all trials.

Table 1. *E. coli* CN-13 Seeding Estimates

CFU/ml	CFU/g of geoduck	Dilution	Level
$6*10^8$	10,000,000	0 (undiluted)	
$6*10^7$	1,000,000	-1	
$6*10^6$	100,000	-2	
$6*10^5$	10,000	-3	
$6*10^4$	1,000	-4	
$6*10^3$	**100**	**-5**	**Level 1**
$6*10^2$	**10**	**-6**	**Level 2**
$6*10^1$	**1**	**-7**	**Level 3**
$6*10^0$	.1	-8	
$6*10^{-1}$	.01	-9	

Day 1

Each *E. coli* CN-13 seeding trial took approximately 7 days to complete. A complete methodological protocol detailing preparatory and daily tasks can be found in Appendix C. The first day of the trial largely consisted of preparatory work that involved preparing TSB, LST tubes, EC Broth tubes, and EC-MUG tubes. Additionally, a 1% nalidixic acid solution was made, as described in EPA method 1601, to act as an antibiotic throughout all five trials (17).

First, 360 20 ml glass tubes were sterilized and fitted with inverted Durham glass culture tubes. The sterilized tubes were then divided into three groups of 120 tubes. Each group of tubes was filled with 9 ml of Lauryl Tryptose Broth (LST), EC Broth, or EC-MUG medium. In all three media types, the inverted Durham culture tubes were used to show gas production from *E. coli* and fecal coliforms.

In the evening, an *E. coli* CN-13 overnight was started. This overnight consisted of 10 microliters of *E. coli* CN-13 stock added to 50 ml of TSB containing a 1% nalidixic acid solution. As previously mentioned, the *E. coli* CN-13 overnight was allowed to grow for 14 hours in a 37 °C shaker at 100 rpm.

Day 2

In the second day of the trial, the *E. coli* CN-13 was seeded into geoduck meat. To prepare the *E. coli* CN-13 for seeding, it was first conditioned in Puget Sound water to better represent environmental conditions for growth. The conditioning process began after 14 hours of growth, when the *E. coli* CN-13 overnight was centrifuged for 15 minutes at 5,000 g at 4° C to create a distinct pellet. Once the pellet was formed, the supernatant was pipetted off and discarded. Next, 50 ml of refrigerated sound water was added to the pellet, and the mixture was vortexed until the pellet completely dissolved. Once thoroughly vortexed, a spectrophotometry reading was taken and the seeded sound water was refrigerated for three hours.

While the *E. coli* CN-13 was being conditioned, 90 microliters of a 1% nalidixic acid solution was individually added to all sterile LST tubes. After three hours of conditioning, another spectrophotometry reading was taken to assess the change in *E. coli* population. After re-vortexing the seeded sound water, a ten-fold dilution series was made for seeding

into geoduck meat and controls. The geoduck meat was divided into three 60 g aliquots and 1 ml of the appropriate dilution (shown in in Table 1) was seeded into each aliquot. After seeding, each aliquot was blended for three minutes. Similarly, PBS was divided into three 60 g aliquots and 1 ml of the appropriate dilution (shown in in Table 1) was seeded into each aliquot. The seeded PBS aliquots were then vortexed for three minutes.

Once the conditioned the *E. coli* CN-13 was seeded into the geoduck meat and PBS, dilutions of both the seeded meat and controls were made using sterile PBS. The dilutions used at each level are shown in Table 2. All trials utilized the 5-tube MPN analysis framework outlined in the FDA BAM method (10). Using this method, 5 LST tubes for each dilution were seeded with 1 ml of either seeded geoduck meat or seeded PBS for each level. To calculate the most probable number (MPN) for each level, positive and negative tubes were considered for three dilutions per level. Because levels 1 and 2 had four dilutions associated with each, only the three dilutions that had countable tubes were considered. In this context, "countable tubes" refer to instances where there is at least one negative and one positive tube per dilution. It was determined that these levels would contain four dilutions in order to insure the inclusion of the countable range.

Table 2. *E. coli* CN-13 Seeding Levels

	Experimental (geoduck meat)	**Controls (PBS)**
Level 1 (using the $6*10^3$ CFU/ml dilution)	1:10	1:10
	1:100	1:100
	1:1,000	1:1,000
	1:10,000	1:10,000
Level 2 (using the $6*10^2$ CFU/ml dilution)	Undiluted	Undiluted
	1:10	1:10
	1:100	1:100
	1:1,000	1:1,000
Level 3 (using the $6*10^1$ CFU/ml dilution)	Undiluted	Undiluted
	1:10	1:10
	1:100	1:100

After all seeded geoduck meat and PBS was passaged into the LST tubes, all tubes were incubated for 48 hours at 35°C.

Day 3

No activity.

Day 4

After 48 hours of incubation, the positive and negative LST tubes were recorded. A loopful of media from all positive tubes was transferred to EC and EC-MUG tubes, all containing 90 microliters of a 1% nalidixic acid solution. The experimental and control EC and EC-MUG tubes were then incubated for 48 hours at 44°C.

Day 5

No activity.

Day 6

After 48 hours of incubation, the positive and negative EC and EC-MUG tubes were recorded. In order to insure accuracy when assessing fluorescence in the EC-MUG tubes, a loopful from each positive EC-MUG media tube was streaked onto EC-MUG agar. The EC-MUG agar was then incubated for 20 hours at 44°C.

Day 7

After 20 hours of incubation, each plate was evaluated for fluorescence under a longwave UV lamp. All positive and negative samples were recorded, and the MPN values for LST, EC, and EC-MUG tubes were calculated based on the methods described in the *FDA Bacteriological Analytical Manual (BAM)* Appendix 2 (12).

Results

A total of five *E. coli* CN-13 seeding trials with three seeding levels per trial were analyzed. Within each seeding level, three dilutions were analyzed using the FDA multiple tube fermentation method. The same three target seeding levels were used in each trial: $6*10^3$ CFU/ml (level 1), $6*10^2$ CFU/ml (level 2), and $6*10^1$ CFU/ml (level 3). The MPN and 95% confidence intervals were calculated for all seeding levels in each trial and for all three media types: LST, EC broth, and EC-MUG broth.

Additionally, the geometric mean MPN values were calculated for all seeding levels across trials. The geometric means and 95% CI's by media type are summarized in Table 3. One reason the geometric mean, instead of the arithmetic mean, was chosen to represent the data was because the data contained some significant outliers that heavily skewed the arithmetic mean (18). As the geometric mean is less sensitive to outliers, it proved to be a more accurate representation of the center of the data (18). Another reason the geometric mean was chosen was because the confidence intervals for the MPN calculations were

calculated using the normal approximation to the log (12). As the geometric mean also uses the log scale, it proved to be a better fit for data representation than the arithmetic mean (18). The individual MPN results and 95% CI's for each trial can be found in Appendix B.

Table 3. Geometric Mean MPN's and 95% Confidence Intervals by Media Type (18)

LST 1X: Geometric Mean and Estimated 95% Confidence Intervals			
Level	**Condition**	**Geometric Mean (MPN/g)**	**Estimated 95% CI**
1	Seeded geoduck	109	(66, 180)
	Control (PBS)	34	(21, 55)
2	Seeded geoduck	14	(9, 23)
	Control (PBS)	12	(7, 19)
3	Seeded geoduck	2.1	(1.3, 3.5)
	Control (PBS)	0.6	(0.3, 1.1)

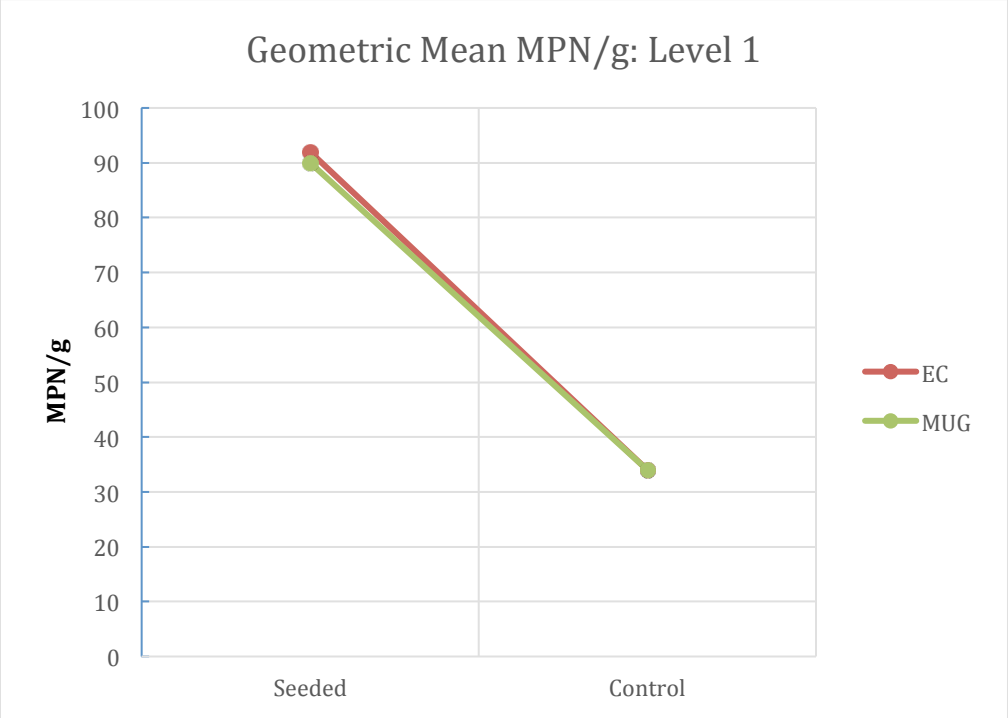
EC Broth: Geometric Mean and Estimated 95% Confidence Intervals			
Level	**Condition**	**Geometric Mean (MPN/g)**	**Estimated 95% CI**
1	Seeded geoduck	92	(56, 150)
	Control (PBS)	34	(21, 55)
2	Seeded geoduck	5	(3, 8)
	Control (PBS)	7	(4, 11)
3	Seeded geoduck	Unable to calculate	Unable to calculate
	Control (PBS)	0.5	(0.3, 1.0)

EC-MUG Broth: Geometric Mean and Estimated 95% Confidence Intervals			
Level	**Condition**	**Geometric Mean (MPN/g)**	**Estimated 95% CI**
1	Seeded geoduck	90	(54, 150)
	Control (PBS)	34	(21, 55)
2	Seeded geoduck	6	(4, 10)
	Control (PBS)	11	(7, 17)
3	Seeded geoduck	Unable to calculate	Unable to calculate
	Control (PBS)	Unable to calculate	Unable to calculate

Throughout all five trials, the MPN values from the seeded geoduck and the MPN values from the corresponding control group were analyzed. In Level 1, the highest seeding

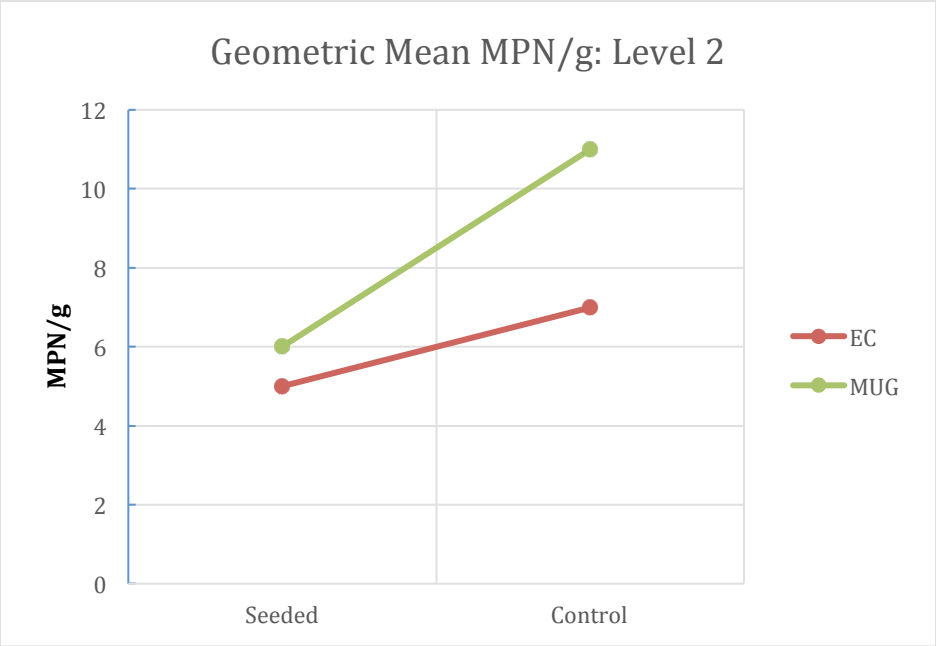
group, there was a clear trend of a higher MPN in the seeded geoduck than the control group, which can be seen in Figure 3. While the exact reason for this result is unknown, there are some potential explanations including *E. coli* CN-13 stock aggregation and potential bacterial replication in the geoduck meat after seeding.

Figure 3. Level 1 Geometric Mean MPN/g



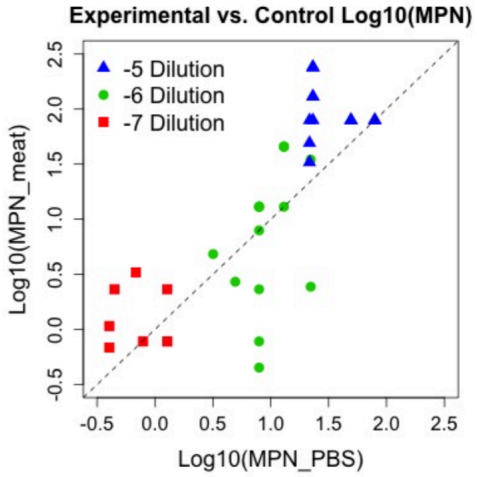
In the second highest seeding group, Level 2, there is much less of a defined trend, as seen in Figure 4. MPN values for level 3, the lowest seeding group, could only be calculated for the LST 1X media. As level 3 was the lowest seeding group, there were instances where there were no positive tubes in the EC and EC-MUG media. This result was not surprising as the amount seeded in was likely close to the limit of detection.

Figure 4. Level 2 Geometric Mean MPN/g



Additionally, the MPN ratios of seeded meat to controls were calculated in an attempt to show the efficacy of the FDA multiple tube fermentation method. As seen in Figure 5, the ratios frequently exceed 1:1, which is represented by the dotted line in the figure. This trend is seen primarily in the highest and lowest seeding levels.

Figure 5. Experimental vs. Control Log10 MPN (18)



(For the sake of correlative analysis, LST seeding data was included in Figure 5)

While the MPN ratios showcase the relationship between the seeded and control groups, it was not possible to calculate confidence intervals for these ratios. This was because in order to calculate 95% CI's for these ratios, independence or multivariate normality between the seeded and control groups needed to be assumed (18). For this experimental design, assuming independence between the seeded and control group when both were seeded from the same bacterial source at the same time would not be accurate. Nevertheless, the ratios presented in Figure 5 give preliminary insight into the relationship between the seeded and control groups.

Discussion

This method validation study was performed to characterize the efficacy of adaptations made to the current FDA BAM multiple tube fermentation method. As this method is widely accepted as the most effective way to test for *E. coli* and fecal coliforms in shellfish, adaptations to this method to better suit geoduck meat could have a significant impact. While many of the MPN ratios between the seeded geoduck and control groups exceeded 1, it can still be concluded that the modified FDA BAM multiple tube fermentation method provides a good assessment of bacterial contamination in geoducks.

In an effort to minimize the effect of background coliforms on the seeding results, antibiotics were added to all media before seeding took place. The antibiotic added was a 1% nalidixic acid solution, as recommended in EPA Method 1601 for use with *E. coli* CN-13 (17). By adding nalidixic acid, the intent was to eliminate all background levels of *E. coli* and fecal coliforms while still allowing the seeded *E. coli* CN-13 to survive. While adding the nalidixic acid did lower background levels of fecal coliforms, it was not completely effective in the first round of the trials in the LST media. However, after the seeded and control

groups were transferred into the EC and EC-MUG media, background levels of fecal coliforms were not present.

In addition to background levels of fecal coliforms, bacterial growth, before and after seeding, could have contributed to the higher levels of fecal coliforms detected in the geoduck meat experimental groups. Nutrients in the geoduck meat could have helped facilitate this growth, as it could have created a more hospitable growth environment than the PBS provided the bacteria in the control seeding groups. While this effect was not quantified, it remains an important factor to consider when analyzing the results of the experimental trials.

Due to this differential effect on the seeded *E. coli* CN-13 between the experimental and control groups, it could be beneficial to analyze the results using a direct CFU to MPN comparison. Assuming a 1:1 relationship between CFU and MPN, it is possible to analyze percent recovery comparing the resulting MPN/g to the CFU/g seeded into the experimental geoduck group (Appendix B). This method showed that the arithmetic mean percent recovery in the highest seeded level was 105.37% with a median percent recovery of 79.24%. In the highest seeded level, the percent recoveries ranged from 49.32% to 239.79%. This analysis also showed that the arithmetic mean percent recovery in the second highest seeded level was 139.51% with a median percent recovery of 79.24%. In the second highest seeded level the percent recoveries ranged from 7.78% to 456.19%. While this comparison does provide further insight into the experimental results, a 1:1 relationship between MPN and CFU has not been established. Therefore, these results should be interpreted with caution.

Limitations

While the FDA BAM serial dilution method has been shown to be an effective approach to measure bacterial concentration, there are still limitations associated with the method. One of the fundamental limitations associated with the method is that the resulting MPN value is only an estimation of bacterial concentration and, therefore, does not provide a specific enumeration of bacteria in the sample. As this level of specificity can be important when analyzing environmental samples, there have been studies that have sought to classify the relationship between colony forming units (CFUs) and MPN. One study, conducted by Gronewold and Wolpert, produced a probabilistic model that characterized this relationship in water (19). While there is still inherent uncertainty in the model, this type of tool can begin to help scientists gain a clearer estimate of bacterial load in a sample, beyond what an MPN value can provide (19).

Another limitation associated with the FDA BAM serial dilution method is the limited capacity to detect *E. coli* and fecal coliforms at very low concentrations (12). As in many bacterial and viral assays, attempting to recover organisms that are under the limit of detection can produce false negative results. For this reason, it is important to sample at multiple dilutions to increase the probability of including positive and negative tubes in an MPN calculation.

While taking into account the limitations of the FDA BAM serial dilution method and the bacterial aggregation seen in the experimental trials, our findings suggest that, with modifications, the current FDA BAM serial dilution method can be applied successfully to geoduck meat. Although not as precise as CFU, calculating an MPN can provide valuable information about bacterial concentration in a geoduck sample. To completely understand the efficacy of the modified FDA BAM serial dilution method on geoducks, further seeded

studies are needed. Additionally, further studies are needed aimed at characterizing the relationship between CFU and MPN in geoduck meat samples to gain a better understanding of how to more accurately quantify bacterial load.

Laboratory Materials

Item	**Amount**	**Product Identification**	**Notes**
Nalidixic Acid Sodium Salt	1 g	Company: MP Biomedicals, LLC Cat. No. 151724	Must be kept at -20 °C
Tryptic Soy Broth (TSB)	50 ml	Company: EMD Ref. 1.05459	
Sound Water	50 ml		Collected in Shilshole, WA
Blender, blades, rubber rings, metal rings	3 of each	Oster mason jar blender	
Disposable culture glass tubes 16 x 150 mm	360 tubes	Company: Fisher Scientific Cat. No. 14-961-31	Need to be sterilized
Borosilicate glass disposable culture tubes	360 tubes	Company: Kimble Cat. No. 73500-650	
Laboratory Scale	1 scale	Company: VWR Model CLW 2000	
Sterile 50 ml conicals	11 conicals	Company: Thermo Scientific Mfr #: 339653	
Potassium Chloride (KCl)	.08 g	Company: EMD Omnipur Cat. No. 7447407	
Sodium Phosphate dibasic, anhydrous (Na_2HPO_4)	0.576 g	Company: Fisher Scientific Cat. No. 7558794	
Potassium Phosphate monobasic, crystal (KH_2PO_4)	0.096 g	Company: J.T. Baker Cat. No. 778770	
Sodium Chloride (NaCl)	3.2 g	Company: Fisher Scientific Cat. No. 7647145	
Lauryl Tryptose Broth (LST)	1.2 L	Company: Difco Ref. 224150	
EC Broth	1.2 L	Company: Himedia Ref. MI27I-500G	
EC Medium MUG	1.2 L for tubes Variable for plates	Company: Difco Ref. 222200	
Bacto Agar	Variable for plates	Company: BD	

		Ref. 214010	
E. coli CN-13		ATCC #: 700609	

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Chapter III: Discussion

The shellfish industry in Washington State is an important part of the state economy and produces shellfish that is consumed both domestically and internationally. As filter feeders, bi-valve molluscan shellfish can pose a serious threat to consumers if not properly tested for viral and bacterial contamination. Adequate testing methods for oysters have been established, however, these testing methods are not suitable for geoduck meat. The purpose of this project was to adapt current approved oyster methods to better suit the unique characteristics of geoduck meat with the goal of establishing concrete testing methods that could be employed by regulatory agencies.

This project had two primary aims, the first being to adapt approved viral testing methods and the second to adapt approved bacterial testing methods. Viral and bacterial method adaptations were validated using seeded studies, with viral and bacterial recovery recorded as the outcomes of interest. In both the viral and bacterial experimental trials, different amounts of either MS2 or *E. coli* CN-13 were seeded into blended geoduck meat. The various seeding amounts were represented as seeding levels, and recoveries were compared across all levels to determine validity of the adaptations made to the viral and bacterial testing methods.

MS2 Seeding Results and Conclusions

In the MS2 seeded study, MS2 percent recovery was used as a metric to evaluate the efficacy of method adaptations. The average percent recoveries observed for MS2 trials ranged from 106.7% to 146.6%, with higher recoveries occurring at more concentrated seeding levels. As noted in Chapter I, recoveries of over 100% were likely due to MS2 aggregation. While taking into account MS2 aggregation, our findings suggest that, with modifications, the current oyster viral testing methods can be applied successfully to geoduck meat. As all percent recoveries were over 106%, it can be concluded that the adapted viral testing methods described in the seeded trials provide good assessment of viral contamination in geoducks.

E. coli CN-13 Seeding Results and Conclusions

In the *E. coli* CN-13 seeded study, the ratios of the Most Probable Number (MPN) between the experimental and control groups were used as a metric to evaluate the efficacy of adaptations to the current FDA BAM multiple tube fermentation method. Similar to the MS2 seeded trials, many recovery ratios for *E. coli* CN-13 trials were greater than 1, with higher recoveries also occurring at more concentrated seeding levels. Recovery ratios greater than 1 were likely due to background coliform levels and potential bacterial replication in geoduck meat. While many of the MPN ratios between the seeded geoduck and control groups exceeded 1, it can still be concluded that the modified FDA BAM multiple tube fermentation method provides a good assessment of bacterial contamination in geoducks.

MS2 Seeding Limitations

MS2 aggregation proved to be a significant challenge in the viral method validation portion of the project. To combat aggregation, a two-step filtration method using a PES 0.45 micron filter and a PES 0.22 micron filter was employed to separate MS2 particles before seeding occurred. While this helped to address the problem, significant aggregation did occur in all seeded trials.

Throughout all trials, the MS2 was filtered at the 1:100 dilution from the undiluted MS2 stock. While filtering at this dilution did eliminate some aggregation, it may have been more effective to filter the MS2 stock at the 1:1,000 or the 1:10,000 dilution (1). In future, filtering at these lower concentrations may help prevent additional aggregation by creating more of a buffer space between individual phage particles, likely reducing the strength of the interparticular attractive forces (1). While studies on MS2 aggregation in water are abundant, further studies on MS2 aggregation in shellfish meat, particularly geoduck meat, are needed to fully characterize how this indicator organism behaves during testing. This vital information on MS2 aggregation could greatly influence the method adaptations discussed in Chapter I.

Another limitation of the viral seeded studies was the use of MS2 as an indicator organism for human norovirus (NoV). While viral indicators, such as MS2, can be useful tools for inferring the presence of similar organisms in an environmental sample, they are not perfect surrogates. MS2 coliphage was used throughout the study because "like enteric viruses they almost exclusively originate from the feces of warm-blooded animals; like enteric viruses they fail to multiply in the environment; and in terms of composition, structure and size they closely resemble human enteric viruses" (2). While the presence of

MS2 does not guarantee the presence of enteric viruses, they are frequently found together in sewage and many investigators believe they are more effective indicators of viral contamination than coliform bacteria (3). Although MS2 has many qualities that make it a good viral indicator for NoV, "some investigators have reported that F-specific RNA coliphages are rarely detected in human feces, suggesting that the presence of these coliphages in water does not necessarily indicate human fecal pollution" (4). While there may be some disadvantages to using MS2 as a viral indicator, it is currently one of the most effective indicator organisms for detecting the presence of enteric viruses.

E. coli CN-13 Seeding Limitations

Throughout the five bacterial seeded trials, bacterial aggregation and growth presented a significant challenge. While MPN calculations made it difficult to accurately quantify the amount of aggregation or growth, it was present in the majority of seeding levels throughout the trials. The exact reason for this effect is unknown, however, it can be hypothesized that nutrients in the geoduck meat could have helped facilitate bacterial growth. The nutrients in the geoduck meat could have created a more hospitable growth environment than the PBS provided the bacteria in the control seeding groups, therefore resulting in an inflated level of bacteria and fecal coliforms in the experimental seeding groups.

Another limitation to this study was the background level of fecal coliforms present in the geoduck meat used. While the geoduck meat used throughout all trials tested negative for background levels of *E. coli*, it did test positive for background gas-producing coliforms. Though a 1% nalidixic acid solution was added to minimize this effect, it was not completely effective in the first round of the trials in the LST media; therefore, caution

should be taken when analyzing the LST MPN values. However, after the seeded and control groups were transferred into the EC and EC-MUG media, background levels of fecal coliforms were not present.

While the FDA BAM multiple tube fermentation method has been shown to be an effective approach to measure bacterial concentration, there are still limitations associated with the method itself. One of the fundamental limitations associated with the method is that the resulting MPN is only an estimation of bacterial concentration and, therefore, does not provide a specific enumeration of bacteria in the sample. As this level of specificity can be important when analyzing environmental samples, studies have been performed that have sought to classify the relationship between colony forming units (CFUs) and MPN. While these studies have provided a solid foundation, more studies are needed that characterize this relationship, particularly in geoducks and other shellfish.

Project Impact

Geoducks harvested in Washington State are an important food source both domestically and abroad. As filter feeders, geoducks have the ability to concentrate harmful viruses and bacteria in their gut and meat, potentially harming consumers and posing a significant public health risk. Because geoduck harvesting in the U.S. is limited to the Pacific Northwest and Alaska shorelines, limited attention has been paid to optimizing current viral and bacterial testing methods to better suit geoduck meat. As such, there is currently no approved regulatory framework for the viral and bacterial testing of geoduck meat. The primary aim of this project was to move toward filling this gap by exploring adaptations to current microbiological methods that provide a solid foundation for further studies in this field.

The viral and bacterial method adaptations, outlined in Chapters I and II respectively, have the potential to impact the way the Washington State Department of Health and other regulatory agencies test their harvested geoducks. While there were notable limitations in both the viral and bacterial trials, the adaptations presented are a step toward developing more advanced microbiological testing methods unique to geoduck meat.

The literature on geoduck microbiological testing is very limited, and there are many topics that still need to be addressed. MS2 and bacterial aggregation in seeded studies are particularly important topics, as they can greatly affect recovery results and method validation conclusions. Additionally, further studies are needed that are aimed at characterizing the relationship between CFU and MPN in geoduck meat samples to gain a better understanding of how to more accurately quantify bacterial load. Lastly, additional viral and bacterial seeding trials are needed to gain a better understanding of how the adaptations proposed throughout this project build on the current approved testing methods.

In conclusion, our findings suggest that, with modifications, the current oyster viral and bacterial testing frameworks can be applied successfully to geoduck meat. The percent recoveries in both the viral and bacterial trials indicate that the adapted methods provide a good baseline assessment of bacterial and viral contamination in geoducks.

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Appendix A:
MS2 Seeding Data

Tabulated Results of Geoduck Seeding Trials:

1	Tabulated Results Trial 6/25/15						
	Spike Concentration	MS2 Replicate Plate Concentration	Percent Recovery	Average Percent Recovery	Log of Replicate MS2 Plates	Average log Replicate MS2 Plates	Relative Standard Deviation
	28	36.82	129.38	116.61	1.57	1.51	0.07
		24.55	86.26		1.39		
		38.18	134.18		1.58		
	146	208.64	142.83	134.74	2.32	2.29	0.02
		178.18	121.98		2.25		
		203.64	139.41		2.31		
	525	840.91	160.11	163.11	2.92	2.93	0.01
		921.82	175.52		2.96		
		807.27	153.71		2.91		
	2579	4200.00	162.88	178.02	3.62	3.66	0.01
		4570.91	177.27		3.66		
		5000.00	193.91		3.70		

2	Tabulated Results Trial 6/26/15						
	Spike Concentration	MS2 Replicate Plate Concentration	Percent Recovery	Average Percent Recovery	Log of Replicate MS2 Plates	Average log Replicate MS2 Plates	Relative Standard Deviation
	52	35.45	68.25	91.00	1.55	1.67	0.07
		59.09	113.75		1.77		
		47.27	91.00		1.67		
	260	319.09	122.66	117.59	2.50	2.47	0.06
		208.64	80.20		2.32		
		390.00	149.92		2.59		
	990	1375.00	138.92	141.04	3.14	3.14	0.01
		1536.36	155.23		3.19		
		1276.36	128.96		3.11		
	4772	5760.00	120.71	138.50	3.76	3.82	0.01
		6850.91	143.57		3.84		
		7215.91	151.22		3.86		

3	Tabulated Results Trial 10/14/15						
	Spike Concentration	MS2 Replicate Plate Concentration	Percent Recovery	Average Percent Recovery	Log of Replicate MS2 Plates	Average log Replicate MS2 Plates	Relative Standard Deviation
	27	36.82	135.94	135.94	1.57	1.57	-
		12.27	45.31		1.09		
		12.73	46.99		1.10		
	137	98.18	71.86	82.17	1.99	2.04	0.06
		85.91	62.88		1.93		
		152.73	111.78		2.18		
	548	470.91	85.88	96.69	2.67	2.71	0.04
		420.00	76.60		2.62		
		699.55	127.58		2.84		
	2841	3730.45	131.33	131.74	3.57	3.57	0.00
		3690.91	129.93		3.57		
		3805.45	133.97		3.58		

*Note: red = no recovered plaques. Calculations assuming 1 plaque recovered.

4	Tabulated Results 10/28/15						
	Spike Concentration	MS2 Replicate Plate Concentration	Percent Recovery	Average Percent Recovery	Log of Replicate MS2 Plates	Average log Replicate MS2 Plates	Relative Standard Deviation
	87	76.36	88.24	83.33	1.88	1.86	0.02
		63.64	73.53		1.80		
		76.36	88.24		1.88		
	427	490.91	114.94	111.59	2.69	2.67	0.05
		337.50	79.02		2.53		
		601.36	140.80		2.78		
	1765	1985.45	112.50	110.82	3.30	3.29	0.02
		1590.91	90.14		3.20		
		2290.91	129.81		3.36		
	9244	13295.45	143.82	138.01	4.12	4.11	0.01
		13010.45	140.74		4.11		
		11969.09	129.47		4.08		

Appendix B:
E. coli CN-13 Seeding Data

Seeding Trial #1: 11-20-15

LST 1X: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	79.242	25.39	247.704
-6	2.312	0.781	6.843
-7	0.778	0.244	2.482

LST 1X: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	49.322	15.456	157.611
-6	7.924	2.538	24.76
-7	0.788	0.244	2.482

EC: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	79.242	25.39	79.242
-6	0.45	0.111	0.45
-7	No positive tubes		

EC: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	49.322	15.456	157.611
-6	7.924	2.538	24.76
-7	0.778	0.244	2.482

EC-MUG: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	79.242	25.39	247.704
-6	0.778	0.244	2.482
-7	No positive tubes		

EC-MUG: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	49.322	15.456	157.611
-6	7.924	2.538	24.76
-7	0.778	0.244	2.482

Seeding Trial #2: 11-30-15

LST 1X: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	239.789	79.725	722.636
-6	12.993	4.548	37.152
-7	2.312	0.781	6.843

LST 1X: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	23.116	7.814	68.461
-6	7.924	2.538	24.76
-7	0.447	0.11	1.807

EC: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	239.789	79.725	722.636
-6	12.993	4.548	37.152
-7	No positive tubes		

EC: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	23.116	7.814	68.461
-6	7.924	2.538	24.76
-7	0.447	0.11	1.807

EC-MUG: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	239.789	79.725	722.636
-6	12.993	4.548	37.152
-7	No positive tubes		

EC-MUG: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	23.116	7.814	68.461
-6	7.924	2.538	24.76
-7	0.447	0.11	1.807

Seeding Trial #3: 12-4-15

LST 1X: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	79.242	25.39	247.704
-6	12.993	4.548	37.152
-7	2.312	0.781	6.843

LST 1X: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	21.609	8.789	53.187
-6	12.993	4.548	37.152
-7	1.276	0.447	3.637

EC: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	32.906	10.943	99.074
-6	2.708	1.149	6.385
-7	0.778	0.244	2.482

EC: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	21.609	8.789	53.187
-6	4.932	1.545	15.754
-7	1.276	0.447	3.637

EC-MUG: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	49.322	15.456	157.611
-6	7.924	2.538	24.76
-7	0.778	0.244	2.482

EC-MUG: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	21.609	8.789	53.187
-6	7.924	2.538	24.76
-7	1.276	0.447	3.637

Seeding Trial #4: 12-7-15

LST 1X: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	79.242	25.39	247.704
-6	45.619	14.976	139.158
-7	3.291	1.094	9.903

LST 1X: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	79.242	25.39	247.704
-6	12.993	4.548	37.152
-7	0.199	0.028	1.415

EC: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	79.242	25.39	247.704
-6	45.619	14.976	139.158
-7	3.291	1.094	9.903

EC: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	79.242	25.39	247.704
-6	12.993	4.548	37.152
-7	0.199	0.028	1.415

EC-MUG: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	79.242	25.39	247.704
-6	45.619	14.976	139.158
-7	3.291	1.094	9.903

EC-MUG: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	79.242	25.39	247.704
-6	12.993	4.548	37.152
-7	No positive tubes		

Seeding Trial #5: 12-11-15

LST 1X: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	129.933	45.503	371.674
-6	34.767	11.176	108.295
-7	3.291	1.094	9.903

LST 1X: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	23.116	7.814	68.461
-6	22.116	8.955	54.68
-7	0.684	0.217	2.152

EC: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	129.933	45.503	371.674
-6	4.828	2.26	10.321
-7	1.071	0.388	2.954

EC: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	23.116	7.814	68.461
-6	3.174	1.409	7.155
-7	0.403	0.1	1.618

EC-MUG: Spiked geoduck meat (experimental)			
		Confidence Limit 95%	
	MPN/g	Low	MPN/g
-5	79.242	25.39	247.704
-6	2.439	1.153	5.158
-7	0.684	0.217	2.152

EC-MUG: Spiked PBS (controls)			
		Confidence Limit 95%	
	MPN/g	Low	High
-5	23.116	7.814	68.461
-6	22.116	8.955	54.68
-7	0.403	0.1	1.618

Appendix C:
Viral and Bacterial Analysis Laboratory Protocols

MS2 Seeding Protocol

Materials:

- 2 blenders, 2 lids
- 460 ml nutrient broth
    - 40 for MS2 stock titering (blender)
    - 20 ml for overnight #1
    - 360 for MS2 recovery trial (blender)
    - 40 for overnight #2
- ~150 TSA plates with Strep/Amp (100X15 mm petri dishes)
- ~150 sterile glass tubes
- Scale (with plastic bag cover)
- 120 ml 1.4X top agar for MS2 stock titering
- 800 ml 1.4X top agar for MS2 recovery trial (4 200ml bottles)
- 20 g geoduck meat for MS2 stock titering
- 180 g geoduck meat for MS2 recovery trial
- 4 sterile beakers
- 2 scoopers
- 2 ml of 1X PBS for MS2 stock titering
- 2 ml of 1X PBS for MS2 recovery trial
- 1 sterile 0.45 micron filter for MS2 stock titering
- 1 sterile 0.22 micron filter for MS2 stock titering
- 2 sterile syringes
- Aluminum foil
- 2 stir plates
- 4 sterile stir bars
- 26 50 ml conicals (for 4 levels)
    - 12 for 33g of homogenate
    - 12 for supernatant
    - 1 for extra homogenate
    - 1 for extra supernatant

Day 1:

- **Start overnight (20 ml)**
- Make TSA, add antibiotics, and pour plates (need 30 plates per level)
    - Total of ~ 150
- Autoclave
    - Blenders & lids (2 of each)

    - Beakers (4)
    - Scoopers (2)
    - Stir bars (4)
    - Glass tubes ~150
- Make nutrient broth (at minimum 460 ml)
- Put geoduck in the refrigerator to defrost (20 g)

Day 2:

Prep

- Make and autoclave 120 ml of 1.4X top agar
- Turn on water bath
- UV hood
- Dry and label plates (ex: -6A, -6B)
- Take geoduck meat out of field lab refrigerator & bring nutrient broth to field lab
- Add antibiotics to top agar

Assay Prep

- Blend 20 g geoduck meat with 40 ml nutrient broth
- Aliquot 33 g of homogenate into conical
- Aliquot extra homogenate into another 50 ml conical
- Turn on centrifuge and cool to 4°C
- Balance homogenate (whatever is left over) and water and centrifuge for 15 min @ 9000 g with J.S. 7.5 rotor
- Pipette off supernatant, weigh and record
- Dilution Series
    - Use 900 µl of 1X PBS & 100 µl of MS2 stock for -1 dilution
    - Use 4.5 ml of 1X PBS & 500 µl -1 for -2 dilution
    - Filter all 1 ml of -2 dilution through .45 micron filter
    - Filter all of this filtered product through .22 micron filter
        - Save ~300 microliters of filtered -2 product
    - Aliquot 900 µl of supernatant for dilutions -3 through -9
    - Use 100 µl of -2 dilution for -3. Continue through -9.
    - **Save dilutions for DAL (-6 through -9 & NC).**
    - **Save remainder of -2 filtered product for MS2 recovery trial tomorrow.**
- Pipette overnight into a 50 ml conical

Double Agar Layer Assay

- Make sure water bath is ~ 37-39 °C
- Vortex all dilutions before using
- Positive controls:
    - 5.7 ml 1.4X top agar
    - 1.9 ml of clean supernatant
    - 100 µl of dilution (-6 through -9)
    - 200 µl of E. coli
- Negative control (2 plates):

    - 5.7 ml 1.4X top agar
    - 2 ml of clean supernatant
    - 200 µl of E. coli
- Put all plates into 36 °C incubator inverted for 16-20 hours

Prep for Day 3

- **Start overnight (40 ml)**
- Label conicals
    - For each level: 3 for 33 g homogenate, 3 supernatant
    - 1 for extra homogenate, 1 for extra supernatant
- Put geoduck in the refrigerator to defrost (need 45 g per level)
    - 4 levels: 180 g geoduck
- Locate 2 spin plates and put in 2nd bay

Day 3:

- Count and record plaques from MS2 titering

Calculate MS2 titer from day 2

- Weighted titer = (sum of plaques on all countable plates)/(sum of amount plated)*first countable dilution

    Example:
    - -6: TNTC, TNTC
    - -7: 65, 66
    - -8: 7, 9
    - -9: 0,0

    weighted titer = $[(65+66+7+9)/.22]*10^7$
    weighted titer = $6.68*10^9$

    Multiply by 10^7 because these were the first countable plates. Because of this, we are going to weight the titer on 10^7.

    Divide by .22 because:
    - 100 µl for each -7 plate = 200 µl
    - 10 µl for each -8 plate (10X lower than -7) = 20 µl
    - 200 µl + 20 µl = 220 µl = .22 ml

- If -9 plates had countable plaques you would divide by .222 because you would add 1 µl for each -9 plate (10 X lower than -8). 200 µl+ 20 µl + 2 µl = 222 µl = .222 ml

Calculate how much MS2 to spike into each level

MS2 Calculation Example

$$\frac{\text{Total number of MSC (N)}}{\text{Total supernatant plated (g)}} * \frac{\text{Weight of supernatant}}{\text{grams of sample used}} = \text{PFU of MSC/100g}$$

Example levels (desired PFU/100g):

1. 100 PFU of MSC/100g
2. 500 PFU of MSC/100g
3. 2,000 PFU of MSC/100g
4. 10,000 PFU of MSC/100g

Example titer: $5.73*10^9$ (This should be the titer calculated this morning from the DAL yesterday)

Level 1:

$$\frac{X}{20g} * \frac{23g\ (estimate)}{11g} * 100 = 100\ \text{PFU of MSC/100g}$$

X = 9.5652*3.33 = 31.8521 ~ 32

If 0 dilution of titer = $5.73*10^9$ PFU/ml then:

- -6 dilution = 5730 PFU/ml
- -7 dilution = 573 PFU/ml

32/5730 = .00558*1000= 5.584 µl of -6
32/573 = 0.0558*1000 = 55.84 µl of -7

In this instance you want to use 55.84 µl of -7 because it is more reasonable to pipette this amount into homogenate.

Level 2:

$$\frac{X}{20g} * \frac{23g\ (estimate)}{11g} * 100 = 500\ \text{PFU of MSC/100g}$$

X = 47.82608*3.33 = 159.2608 ~160

If 0 dilution of titer = $5.73*10^9$ PFU/ml then:

- -6 dilution = 5730 PFU/ml
- -7 dilution = 573 PFU/ml

160/5730 = .00558*1000= 27.923 µl of -6
160/573 = 0.0558*1000 = 279.23 µl of -7

In this instance you want to use 279 µl of -7 because it is more reasonable to pipette this amount into homogenate.

Prep

- Make and autoclave 800 ml of 1.4X top agar (in 4 200 ml portions)
    - Tryptone: 2.8 g
    - Dextrose: .28 g
    - NaCl: 1.4 g
    - 1 M $CaCl_2$: .14 ml (140 µl)
    - Bacto Agar: 1.96 g
    - DI Water: 200 ml
- Turn on water bath
- UV hood
- Dry and label plates (ex: L1 A, L1 B…)
- Take geoduck meat out of field lab refrigerator & bring nutrient broth to field lab
- Add antibiotics to top agar

Assay Prep

- Blend 180 g geoduck meat with 360 ml nutrient broth (for 4 levels)
- Aliquot 110 g of homogenate into each of the 4 beakers (for 4 levels)
- Aliquot extra homogenate into 50 ml conical(s)
- Bring beakers back into lab and cover with foil
- Turn on centrifuge and cool to 4°C
- Balance extra homogenate (whatever is left over) and water and centrifuge for 15 min @ 9000 g with J.S. 7.5 rotor
- Pipette off supernatant, weigh and record
- Dilution Series
    - Aliquot 900 µl of supernatant for dilutions -3 through -9
    - Use 100 µl of -2 dilution (from yesterday) for -3. Continue dilution series through -9.
    - Save dilutions for controls (-6 through -9 & NC)
- Spike into each beaker the correct amount of MS2 dilution for that level
- Put sterile stir bar into levels 1 & 2 and stir for 15 min. @ 300 rpm
- After 15 minutes, portion homogenate from each beaker into 3 33g aliquots
    - Label L1 A, L1 B, etc.
- Balance levels 1 & 2 and centrifuge @ 4°C for 15 min @ 9000 g with J.S. 7.5 rotor
- Put sterile stir bar into levels 3 & 4 and stir for 15 min. @ 300 rpm
- After 15 minutes, portion homogenate from each beaker into 3 33g aliquots
    - Label L3 A, L3 B, etc.
- Balance levels 3 & 4 and centrifuge @ 4°C for 15 min @ 9000 g with J.S. 7.5 rotor
- Pipette off supernatant from all levels (12 conicals) and weigh and record
- Allow supernatant to warm to room temperature (~20 min)
- Pipette overnight into a 50 ml conical

Double Agar Layer Assay

- Make sure water bath is ~ 37-39 °C
- Vortex all supernatant conicals before using
- For each plate:
    - 5.7 ml 1.4X top agar
    - 2 ml of supernatant
    - 200 µl of E. coli
- Positive controls:
    - 5.7 ml 1.4X top agar
    - 1.9 ml of clean supernatant
    - 100 µl of dilution (-6 through -9)
    - 200 µl of E. coli
- Negative control (2 plates):
    - 5.7 ml 1.4X top agar
    - 2 ml of clean supernatant
    - 200 µl of E. coli
- Put all plates into 36 °C incubator inverted for 16-20 hours

Day 4:

- Count and record plaques from MS2 recovery trial

Calculate MS2 titer from day 3

- Weighted titer = (sum of plaques on all countable plates)/(sum of amount plated)*first countable dilution

  Example:
    - -6: TNTC, TNTC
    - -7: 65, 66
    - -8: 7, 9
    - -9: 0,0

  weighted titer = $[(65+66+7+9)/.22]*10^7$
  weighted titer = $6.68*10^9$

  Multiply by 10^7 because these were the first countable plates. Because of this, we are going to weight the titer on 10^7.

  Divide by .22 because:
    - 100 µl for each -7 plate = 200 µl
    - 10 µl for each -8 plate (10X lower than -7) = 20 µl
    - 200 µl + 20 µl = 220 µl = .22 ml

- If -9 plates had countable plaques you would divide by .222 because you would add 1 µl for each -9 plate (10 X lower than -8). 200 µl+ 20 µl + 2 µl = 222 µl = .222 ml

Calculate the Spike Concentration for each level

a. Calculate: how much you put in * the weighted titer for that day
b. Multiply this number by 99 (33 g * 3) and then divide by 110 g (the amount actually in the beaker)
c. Plug this number into the formula for total number of MSC

$$\frac{\text{Total number of MSC (N)}}{\text{Total supernatant plated (g)}} * \frac{\text{Weight of supernatant}}{\text{grams of sample used}}$$

total supernatant plated = 60 g (assuming all plates went well)
weight of supernatant = sum of all 3 supernatants for that level
grams of sample used = 33 g

Example: Added 183 µl of -7 to level 1, real weighted titer for that day = $4.73*10^9$

$4.73*10^2*.183 = 8.66*10^1$
$(8.66*10^1*99g)/110g = 7.79*10^1$

$$[\frac{7.79*10^1}{60\ (g)} * \frac{72}{33g} * 100] / 3 = 94.4280$$

Calculate Replicate Plate Concentration

d. Sum up the number of plaques per 10 plates
e. Plug this number into the formula for total number of MSC

Example: L1 A had 4 plaques total

$$\frac{4}{20g} * \frac{23}{11g} = 83.6364$$

Calculate Percent Recovery

f. Percent Recovery = Replicate Plate Concentration/Spike Concentration

Example: 83.6364/94.4280= 88.5716

- Autoclave waste from day 2 & 3

E. coli **CN-13 Seeding Protocol**

Materials:

- 3 small blenders, 3 blades, 3 foam rings, 3 metal rings
- 50 ml tryptic soy broth (TSB) with N.A. for overnight
- 50 ml refrigerated sound water
- Experimental: 60 autoclaved 20 ml glass tubes with caps & 60 Durham culture tubes
- Control: 60 autoclaved 20 ml glass tubes with caps & 60 Durham culture tubes
- LST 1X ~ 120 tubes minimum ~1200 ml
    - 9 ml per tube
    - 90 microliters of N.A. per tube
- EC Broth ~ 120 tubes minimum ~1200 ml
    - 9 ml per tube
    - 90 microliters of N.A per tube
- EC MUG Broth ~ 120 tubes minimum ~1200 ml
    - 9 ml per tube
    - 90 microliters of N.A per tube
- Scale (with plastic bag cover)
- 180 g geoduck meat
- 11 sterile 50 ml conicals
- At least 400 ml of 1X PBS
- 3 Sterile Scoopers
- EC-MUG Broth & Bacto Agar for plates (with N.A.)
- 15 MacConkey Agar plates (with N.A.)

Nalidixic Acid

- EPA Method 1601
    - 7.2.1.2: Dissolve 1 g of nalidixic acid sodium salt in 100 mL reagent water. Filter through a sterile, 0.22-µm-pore-size membrane filter assembly. Dispense 5 mL per 5-mL freezer vial, date vial, and store frozen at -20°C for up to one year. Thaw at room temperature or rapidly in a 36°C ± 1.0°C water bath. Mix well prior to use.
    - 7.3.1.2: TSB with nalidixic acid (for growth of E. coli CN-13)—Aseptically add 10 mL of stock nalidixic acid (Section 7.2.1) to 1 L of autoclaved, cooled (48°C ± 1.0°C) TSB (Section 7.3.1.1) and mix. Please note: Antibiotics must always be added to medium after the medium has been autoclaved and cooled.

Day 1 (Thursday 11/19):

Pre-conditioning E. coli CN-13 titer from November 16th, 2015: $4.86*10^8$
Post-conditioning E. coli CN-13 titer from November 16th, 2015: **$6.09*10^8$**

CFU/ml	CFU/ml	CFU/g of geoduck	Dilution
$6*10^8$	600,000,000	10,000,000	0
$6*10^7$	60,000,000	1,000,000	-1
$6*10^6$	6,000,000	100,000	-2
$6*10^5$	600,000	10,000	-3
$6*10^4$	60,000	1,000	-4
$6*10^3$	**6,000**	**100**	**-5**
$6*10^2$	**600**	**10**	**-6**
$6*10^1$	**60**	**1**	**-7**
$6*10^0$	6	.1	-8
$6*10^{-1}$	0.6	.01	-9

Want to put dilutions -5 through -7 into tubes in order to get in the countable range.

Start an overnight with 10 microliters of E. coli CN-13 and 50 ml of tryptic soy broth (TSB) with N.A. Grow up overnight for 14 hours.

Put geoduck meat in field lab refrigerator to defrost.

Day 2 (Friday 11/20):

Condition overnight in refrigerated sound water for an additional 3 hours. Expected weighted titer = $6.09*10^8$ CFU/ml.

- Centrifuge overnight for 15 min at 5,000 g at 4 ° C to get a distinct pellet.
- Pipette off supernatant
- Add 50 ml of refrigerated sound water of the 50 ml conical with E. coli cells to re-suspend pellet. Vortex for 30 sec to re-suspend cells.
- Perform an initial spec reading after the E. coli has entered sound water
- Spot titer E. coli with sound water onto MacConkey agar with N.A.
    - 10 microliters per spot, 10 spots

Allow E. coli to sit in sound water in the dirty fridge for 3 hours.

Add nalidixic acid to each LST tube.

After 3 hours:

- Perform spec reading
- Spot titer E. coli with sound water onto MacConkey agar with N.A.
    - 10 microliters per spot, 10 spots

Dilute E. coli in sound water through -7 using:

- -1: 5.4 ml 1X PBS & 600 microliters of E. coli in sound water.
- -2: 5.4 ml 1X PBS & 600 microliters of -1.
- -3: 5.4 ml 1X PBS & 600 microliters of -2.
- -4: 5.4 ml 1X PBS & 600 microliters of -3.
- -5: 5.4 ml 1X PBS & 600 microliters of -4.
- -6: 5.4 ml 1X PBS & 600 microliters of -5.
- -7: 5.4 ml 1X PBS & 600 microliters of -6.

Portion 60g of geoduck meat into 3 containers (180 g total needed).

Level 1 (using -5 dilution):

- Spike 1 ml of -5 dilution into 60g of geoduck meat and blend for 180 seconds
    - Spiked in 6,000 CFU total
        - 100 CFU/g
- 1:10
    - Weigh 1 g of spiked geoduck meat into 9 ml of 1X PBS
        - Inserting a total of 100 CFU
        - Should expect 10 CFU tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:10 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:100
    - Pipet 1 ml of 1:10 dilution into 9 ml of 1X PBS
        - Inserting a total of 10 CFU
        - Should expect 1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:100 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:1,000
    - Pipet 1 ml of 1:100 dilution into 9 ml of 1X PBS
        - Inserting a total of 1 CFU into dilution
        - Should expect .1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:1,000 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:10,000
    - Pipet 1 ml of 1:1,000 dilution into 9 ml of 1X PBS
        - Inserting a total of .1 CFU into dilution
        - Should expect .01 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:10,000 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)

Level 1 Controls (using -5 dilution):

- Spike 1 ml of -5 dilution into 60ml of 1X PBS and vortex for 180 seconds
    - Spiked in 6,000 CFU total
        - 100 CFU/g
- 1:10
    - Pipet 1 ml of spiked PBS into 9 ml of 1X PBS
        - Inserting a total of 100 CFU into dilution
        - Should expect 10 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:10 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:100
    - Pipet 1 ml of 1:10 dilution into 9 ml of 1X PBS
        - Inserting a total of 10 CFU into dilution
        - Should expect 1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:100 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:1,000
    - Pipet 1 ml of 1:100 dilution into 9 ml of 1X PBS
        - Inserting a total of 1 CFU into dilution
        - Should expect .1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:1,000 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:10,000
    - Pipet 1 ml of 1:1,000 dilution into 9 ml of 1X PBS
        - Inserting a total of .1 CFU into dilution
        - Should expect .01 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:10,000 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)

Level 2 (using -6 dilution):

- Spike 1 ml of -6 dilution into 60g of geoduck meat and blend for 180 seconds
    - Spiked in 600 CFU total
        - 10 CFU/g
- Undiluted
    - Weigh 1 g of spiked geoduck meat directly into 1X LST tube (5 total)
        - Should expect 10 CFU per tube
- 1:10
    - Weigh 1 g of spiked geoduck meat into 9 ml of 1X PBS
        - Inserting a total of 10 CFU into dilution
        - Should expect 1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:10 dilution into each 1X LST tube (5 total)

    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:100
    - Pipet 1 ml of 1:10 dilution into 9 ml of 1X PBS
        - Inserting a total of 1 CFU into dilution
        - Should expect .1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:100 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:1,000
    - Pipet 1 ml of 1:100 dilution into 9 ml of 1X PBS
        - Inserting a total of .1 CFU into dilution
        - Should expect .01 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:1,000 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)

Level 2 Controls (using -6 dilution):

- Spike 1 ml of -6 dilution into 60ml of 1X PBS and vortex for 180 seconds
    - Spiked in 600 CFU total
        - 10 CFU/g
- Undiluted
    - Pipet 1 ml of spiked PBS directly into 1X LST tube (5 total)
        - Should expect 10 CFU per tube
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)

- 1:10
    - Pipet 1 ml of spiked PBS into 9 ml of 1X PBS
        - Inserting a total of 10 CFU into dilution
        - Should expect 1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:10 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:100
    - Pipet 1 ml of 1:10 dilution into 9 ml of 1X PBS
        - Inserting a total of 1 CFU into dilution
        - Should expect .1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:100 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:1,000
    - Pipet 1 ml of 1:100 dilution into 9 ml of 1X PBS
        - Inserting a total of .1 CFU into dilution
        - Should expect .01 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:1,000 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)

Level 3 (Using -7 dilution):

- Spike 1 ml of -7 dilution into 60g of geoduck meat and blend for 180 seconds
    - Spiked in 60 CFU total
        - 1 CFU/g
- Undiluted
    - Weigh 1 g of spiked geoduck meat directly into 1X LST tube (5 total)
        - Should expect 1 CFU per tube
- 1:10
    - Weigh 1 g of spiked geoduck meat into 9 ml of 1X PBS
        - Inserting a total of .1 CFU into dilution
        - Should expect 1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:10 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:100
    - Pipet 1 ml of 1:10 dilution into 9 ml of 1X PBS
        - Inserting a total of .1 CFU into dilution
        - Should expect .01 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:100 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
    -

Level 3 Controls (Using -7 dilution):

- Spike 1 ml of -7 dilution into 60 ml of 1X PBS and vortex for 180 seconds
    - Spiked in 60 CFU total
        - 1 CFU/g
- Undiluted
    - Pipet 1 ml of spiked PBS directly into 1X LST tube (5 total)
        - Should expect 1 CFU per tube
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:10
    - Pipet 1 ml of spiked PBS into 9 ml of 1X PBS
        - Inserting a total of 1 CFU into dilution
        - Should expect .1 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:10 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)
- 1:100
    - Pipet 1 ml of 1:10 dilution into 9 ml of 1X PBS
        - Inserting a total of .1 CFU into dilution
        - Should expect .01 CFU per tube
    - Vortex/shake well
    - Pipette 1 ml of this 1:100 dilution into each 1X LST tube (5 total)
    - Spot titer onto MacConkey agar plate (10 microliters per spot, 10 spots)

Incubate LST tubes for 48 hours at 35°C.
Incubate MacConkey plates at 37°C for 16-20 hours.

Day 3 (Saturday 11/21):
Count colonies on MacConkey plates and calculate titer.

Day 4 (Sunday 11/22):
Check and record positive LST tubes. Transfer a loopful of positive LST tubes into EC and EC-MUG tubes. Incubate for 48 hours at 44°C.

- Do this for both the experimental (with geoduck) and control group (with PBS).

Make EC-MUG agar for plating tomorrow:

- EC MUG media: 37.1 g/L
- Bacto agar: 15 g/L
- N.A. antibiotics 10 ml/L

Day 6 (Tuesday 11/24):
Streak positive MUG tubes onto EC MUG agar. Incubate for 20 hours at 44°C.

Day 7 (Wednesday 11/25):
Look for fluorescence on MUG plates and record.

Calculate MPN based on FDA BAM table.

- Do this for both the experimental (with geoduck) and control group (with PBS).

Appendix D:
Extra Notes and Observations

Chapter I: MS2 Method Validation

One goal when performing the MS2 seeded trials was to create MS2 seeding conditions that would mimic environmental contamination in harvested geoduck. This was done to more accurately show how the proposed seeded study method would function when assessing environmental contamination in geoducks. In an effort to mimic environmental contamination, the majority of the MS2 dilution series was made using geoduck supernatant, instead of PBS.

After observing significant MS2 aggregation in early trials, it was evident that MS2 filtration, at the dilution series production step, was needed prior to seeding. As the filter pore sizes were 0.45 and 0.22 microns, filtering supernatant through the filters was not possible. To combat this problem, the first two MS2 dilutions (1:10 and 1:100) were made using a 1X PBS, which was able to pass easily through the filter pores. After filtration, the filtered product was then used to make the 1:1,000 dilution, and further dilutions, in geoduck supernatant. As noted in Chapter I, future seeded studies may benefit from continuing the dilution series in PBS and filtering the 1:1,000 or the 1:10,000 dilutions to prevent additional aggregation.

Another challenge that was encountered throughout the trials was maintaining appropriate top agar temperature. Namely, the top agar needed to be warm enough so it did not solidify and cool enough that it did not inactivate the antibiotics or kill biological

organisms of interest. As each seeded trial required 800 ml of top agar, it was often difficult to maintain temperature control if the entire volume of top agar was in the same container. Furthermore, in a standard sized waterbath, much of the 800 ml of liquid in a 1 L container would sit above the waterline and cool too rapidly. Therefore, in the seeded trials described in Chapter I, the top agar was split into four smaller containers each holding 200 ml of top agar. This allowed the top agar to remain below the waterline and not solidify. In addition, should accidental contamination occur, keeping the top agar in separate containers allows for immediate continuation of the trial after the contaminated portion is discarded.

Chapter II: Bacterial Method Validation

As described in Chapter II, the relationship between MPN and CFU in geoduck meat has not been established. In an attempt to characterize this relationship throughout the seeded trials, geoduck homogenate at each dilution in all levels was spot tittered onto MacConkey agar containing a 1% nalidixic acid solution. It was observed that *E. coli* CN-13 did not grow on MacConkey agar at the concentrations that were seeded into the LST media. Further studies are needed to characterize this relationship in geoduck meat, as it can provide more applicable information than the current studies that focus on this relationship in water.

E. coli growth in early seeded experiments also proved to be a significant challenge. Originally, the seeded trials were to be conducted using *E. coli* F_{amp}, not *E. coli* CN-13. However, early experiments proved that *E. coli* F_{amp} did not grow well in the LST media and was resulting in uncharacteristically low MPN values. In contrast, *E. coli* CN-13 grew well in all media types and provided more accurate seeding results. The reason for this outcome is unclear, and further studies are needed to understand the mechanism by which this result

occurs if *E. coli* F_{amp} continues to be used as a control in environmental sampling.