Comparative Microbial Dynamics in *Crassostrea virginica* and *Crassostrea ariakensis*

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Comparative Microbial Dynamics in *Crassostrea virginica* and *Crassostrea ariakensis*

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Abstract

Considerations to introduce the Suminoe or Asian oyster *Crassostrea ariakensis* along the East Coast have raised many questions regarding ecology, economics, and human health. To date, research has focused primarily on the ecological and socioeconomic implications of this initiative, yet few studies have assessed its potential impact on public health. Our work compares the rates of bioaccumulation, depuration and post harvest decay of indicator organisms (such as *E. coli)* and *Vibrio sp.* between *Crassostrea virginica* and *Crassostrea ariakensis* in the laboratory. Preliminary results suggest that the rates of bioaccumulation of *E. coli* in *Crassostrea ariakensis* were significantly lower than those for *Crassostrea virginica*, depuration of *E. coli* was variable between the two species, and *Crassostrea ariakensis* post harvest decay rates of *Vibrio sp*. were significantly lower than *Crassostrea virginica*. This research provides coastal managers with insight into the response of *Crassostrea ariakensis* to bacteria, an important consideration for determining appropriate management strategies for this species. Further field-based studies will be necessary to elucidate the mechanisms responsible for the differences in rates of bioaccumulation and depuration.

Introduction

The Eastern Oyster, *Crassostrea virginica*, was once one of the most heavily exploited marine organisms on the eastern seaboard, particularly in Chesapeake Bay. Historical records show that in the peak oyster harvest era (mid to late 1800's), over 600,000 metric tons per year were harvested from Chesapeake Bay (Jackson et al., 2001). During this time, oysters were so widespread in the area that boats would ground on oyster reefs and oysters had the potential to filter the entire water column in just a few days (Newell, 1988). Since the development of modern fishing techniques (e.g., mechanical dredges), oyster landings have declined and the incidence of eutrophication and poor water quality in mid-Atlantic coastal waters has risen dramatically (Rothschild et al., 1994; Jackson et al., 2001). Average oyster densities in the bay are estimated to be 96% lower today than they were in 1884. Researchers estimate that it would take approximately 325 days for oysters to filter the water column (Newell, 1988; Rothschild et al., 1994). Oyster restoration efforts in Chesapeake Bay have been further hindered by a relentless increase in watershed development, accompanied by increased pollutant-laden runoff, and the spread of parasitic diseases (Virginia Institute of Marine Science, 1996).

In an effort to improve estuarine water quality and boost the local economy, Maryland and Virginia are investigating ways to revitalize oyster populations in Chesapeake Bay. Previous attempts to restore oysters were focused on economics and the maintenance of harvestable oyster stocks. Only recently have restoration efforts focused on ecology and depuration of oyster populations and reefs (Committee on Nonnative Oysters in the Chesapeake Bay, 2004). At present, the main question

surrounding oyster restoration is whether the native *Crassostrea virginica* or a non-native oyster can be a successful component of restoration of ecological functionality of the bay (Committee on Nonnative Oysters in the Chesapeake Bay, 2004).

Previous attempts to restore *Crassostrea virginica* in Chesapeake Bay have been severely limited by persistent parasite infections by *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) (Committee on Nonnative Oysters in the Chesapeake Bay, 2004). With the widespread distribution of these protozoa in the bay, researchers have been working to identify the genes involved with infection and to selectively breed a strain of *Crassostrea virginica* that is resistant to MSX and Dermo. This is a time consuming and involved process that has had limited success thus far; consequently, it appears that it will be quite some time before a disease resistant strain will be ready for widespread dissemination (Ford and Haskin, 1987; Encomio et al., 2005; Goedken et al., 2005).

Another proposal to restock Chesapeake Bay oyster populations involves the introduction of a non-native oyster species. The main obstacle faced by advocates of this initiative is to find a species that is resistant to the dominant parasitic infections that plague the estuary (namely MSX and Dermo), that is well suited for local physical-chemical parameters, and that is commercially harvestable/marketable. Oyster species, including *Crassostrea gigas* and *C. ariakensis* have been proposed as candidates. The oyster industry of the west coast of the U.S. has flourished since the introduction of the non-native *C. gigas*. In light of the successes of this introduction, *C. gigas* was the first to be evaluated for introduction into the Chesapeake Bay region. Field comparisons of *C. gigas* and *C. virginica* in the Chesapeake Bay region have shown that *C. gigas* is more

resistant to parasitic infections, but does not grow as well as the native oyster in eastern seaboard environments (Calvo, 1999). Consequently, efforts to introduce *C. gigas* to Chesapeake Bay region have been delayed while other prospects are considered. At present, the species with the greatest potential for successful introduction in the mid-Atlantic is *C. ariakensis.* This species is of particular interest due to tolerance to oyster pathogens such as MSX and Dermo, rapid growth, and potential for marketability (Calvo, 2001; Bishop, 2005).

While increasing oyster populations may be ecologically and economically beneficial, human health concerns remain largely unknown. Shellfishing waters can be contaminated with a wide range of bacterial and viral pathogens, including Hepatitis A, *Salmonella, Campylobacter, E. coli* O157:H7, enteroviruses, noroviruses, and adenoviruses (Rippey, 1994; Hurst, 2002; Griffin et al., 2003). The danger of contact with these and other pathogens is substantiated by the frequent occurrence of illness linked to waterborne pathogens and shellfish. There is evidence of human disease resulting from the consumption of bivalve shellfish dating from medieval times (Lees, 2000). In the United States, over 400 outbreaks and 14,000 recorded cases of infectious disease have been attributed to shellfish consumption since the late 1800's. Filter feeding bivalve shellfish (particularly oysters and clams) are known vectors of human disease, as they concentrate microbial pathogens, via the feeding process, from overlying waters and are routinely eaten raw or lightly cooked. The infectious dose of shellfish associated pathogens, especially those viral in nature, is believed to be low, and fatal infections have been reported after the ingestion of a single oyster (Oliver, 2006). Under-reporting of shellfish illness is likely, as nearly all shellfish-associated infections manifest themselves

as gastrointestinal discomfort, which is often treated at home without a visit to health care professionals (Rippey, 1994).

Indicator bacteria such as total coliforms, fecal coliforms (including *E. coli*), and enteroccoci are used by regulatory agencies as proxies of fecal contamination found in both recreational and shellfish harvesting waters. Indicator bacteria are generally not pathogenic and are abundant in the feces of warm-blooded organisms. In addition, these bacteria are not found naturally in aquatic systems and can be easily detected. These organisms have proven useful to coastal managers as a tool to recognize the occurrence of fecal contamination so that preventive measures can be taken to protect public health (Griffin et al., 2001; Noble et al., 2003). State regulatory agencies are responsible for ensuring the protection of public health via the appropriate closure of contaminated shellfish harvesting waters. Historically, the regulations used to control shellfish waters have been successful at controlling foodborne illness vectored by *Crassostrea virginica*. However, it is unknown whether the present regulations will be sufficient to protect consumers from pathogens vectored by *C. ariakensis.*

The goal of this study was to measure and compare the rates of bioaccumulation and depuration and post harvest (storage) growth of indicator bacteria (*E. coli*) and *Vibrio sp.* in the native and Suminoe oyster. To our knowledge, this is the first report to address these issues. Such research is necessary to determine the vulnerability and response of *Crassostrea ariakensis* to fecal bacteria so public health officials can either accept the present management strategies as sufficient or establish new, more appropriate strategies for this species.

Methods

Oyster husbandry

All trials were conducted with one year old diploid *Crassostrea virginica* and one year old triploid (reproductively sterile) *Crassostrea ariakensis,* except where noted. *Crassostrea virginica* were obtained from an aquaculture facility in North Carolina. *Crassostrea ariakensis* were obtained from the Virginia Institute of Marine Sciences (VIMS), Gloucester Point, Virginia and were certified as disease-free in accordance with the shellfish importation requirements of the North Carolina Department of the Environment and Natural Resources. Oysters were transported in coolers to indoor laboratory facilities at the National Oceanic and Atmospheric Administration (NOAA), Center for Coastal Fisheries and Habitat Research, Beaufort, North Carolina. Upon arrival, oysters were scrubbed with sterile brushes and marked according to species, using permanent markers or fingernail polish, before introduction into recirculating tanks. All oysters were divided randomly among six 315 L seawater systems comprised of a 40 watt ultraviolet sterilizer (UV), biofiltration, and a recirculating pump. Recirculating tanks were comprised of two polyethylene tanks, one where the oysters were housed (rearing tank) and one where the pumps and biofiltration were contained (tank reservoir). These units were maintained in an enclosed building with salinity and temperature at 23.8 ±2 ppt and 23.4 ±1.5°C, respectively (unless otherwise noted). Oyster tanks were filled with water from shellfishing areas open to harvest in the Newport River, North Carolina (34.770569° N, 76.736099° W). Throughout the experiment, oysters were fed Shellfish Diet 1800® (Reed Mariculture, Campbell, California) at 1-3% total wet meat weight per

day via an automated dosing system every 4-6 hours. Water exchange rate in each rearing tank was calibrated to achieve complete turnover every 10 minutes.

All methods for holding non-native oysters were in compliance with the NOAA Environmental Assessment and Biosecurity Requirements identified for all non-native oyster research projects supported by NOAA funds (see Federal Register Vol. 69, No. 107, Thursday, June 3, 2004, pp 31359-31361). All seawater effluent was pumped into a holding tank where it was sterilized through chlorination using a minimal concentration of 5 ppm of free chlorine per liter of seawater. Once chlorine was added to the sterilization tank, the seawater was mixed and left stagnant for a minimum of 5 days. Following the five day sterilization period, sodium thiosulphate was added and mixed until chlorine levels were reduced to zero. Chlorine levels were monitored and once they were reduced to a nominal concentration, the sterilized seawater was released into the estuary.

Bacteria

Bacteria used for tank inoculations were acquired from American Type Culture Collection (ATCC), Manassas, Virginia (*E. coli K12* ATCC # 47076). This strain of *E. coli* has been well studied and the entire genome has been sequenced and characterized (Blattner et al., 1997). Selection of a strain that was a BioSafety level 1 agent was of the highest priority for this project, as oysters were housed in a multiple-use building and contaminated samples were tested at multiple labs.

The original ATCC bacterial cells arrived lyophilized and were reconstituted according to the ATCC recommended methods. To ensure bacteria were healthy and

replicating at expected rates, bacteria were subcultured a minimum of three times from the original ATCC strain before use in the experiment. All cell culture was performed in a biological safety cabinet under sterile conditions. Bacterial cultures were grown in a 37°C shaking incubator at 200 rpm until they reached log phase. Aliquots of bacterial culture were pipetted into sterile tubes and transported at room temperature to oyster tanks. Tanks were inoculated with 10^3 to 10^4 *E. coli* cells/100 ml culture by diluting the culture with seawater then adding the mixture into the tank reservoir; ensuring equal distribution of inoculum over the oysters.

Oysters were monitored for total *Vibrio sp*. levels throughout all experiments. The oysters were not artificially contaminated with laboratory strains of *Vibrio sp.;* therefore, all *Vibrio sp.* were part of a natural assemblage found in the oyster guts or in the Newport River Estuary water used in the tanks.

Sample collection

All water samples were collected in bottles that were sterilized by means of triple rinsing with 5% HCl followed by steam sterilization at 121°C for 20 minutes. At the sample site, bottles were rinsed three times with a full volume of the water that was to be analyzed. Samples were collected just below the surface of the water. Samples were transported, in the dark, on ice, to the University of North Carolina at Chapel Hill's Institute of Marine Sciences (IMS), Morehead City, North Carolina and the North Carolina Department of Environment and Natural Resources, Division of Environmental Health Shellfish Sanitation and Recreational Water Quality Section (NCDENR), Morehead City, NC for analysis.

Oyster processing

Oysters were removed from tanks, by hand, within 30 minutes of processing. Oyster samples were collected at discrete times and were homogenized according to sampling standards currently being employed by NCDENR shellfish monitoring programs, based on the guidelines set forth in Recommended Procedures for the Examination of Sea Water and Shellfish (American Public Health Association, 1970). These guidelines are approved by the Interstate Shellfish Sanitation Conference along with the National Shellfish Sanitation Program and are the approved methods for shellfish testing in regulatory agency laboratories. In general, the protocol entails: scrubbing the exterior of oysters with a sterile brush, removal of shell contents using a sterile shucking knife, transfer of meat and mantle liquor (minimum sample size n=10-12 to account for oyster variability) to a sterile tared beaker, addition of an equal volume of 0.5% sterile peptone water, transfer to a sterile blender jar, and storage at 4°C until all samples were processed in this manner (less than one hour). This last storage step was not in the standard method; however, it was necessary in this situation, as several oyster samples were processed at once and oysters needed to be kept intact for as long as possible to reduce enzymatic sample degradation. Once all samples were shucked and diluted with peptone water, they were homogenized for 60 seconds on low speed as described in standard methods. Oyster homogenate was then poured into separate sterile containers and transported immediately, on ice, to IMS and NCDENR. A subset of samples were processed at Duke University Marine Laboratory for confirmation of *E. coli* enumeration. Microbial analyses were performed on samples immediately upon arrival and all samples were processed within two hours of homogenization.

Microbial analyses

Water samples and oysters were analyzed for *Vibrio sp*., fecal coliform, and *E. coli* concentrations. *Vibrio sp.* were enumerated using membrane filtration on thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Kobayashi 1963). Homogenate and water samples were split between two labs and analyzed independently for *E. coli* using most probably number (MPN) enumeration. Fecal coliform and *E. coli* determinations were conducted in an FDA certified lab (NCDENR) using conventional techniques (multiple tube fermentation (MTF) with 4-methylumbelliferyl-β-D-glucuronide (MUG) verification. Additional *E. coli* MPN enumeration was performed using the chromogenic, fluorogenic substrate test Colilert-18® (IDEXX Laboratories, Inc.); however, all results presented for the depuration, bioaccumulation, and storage studies in this chapter are based on results using the MTF approach.

Experimental design

Depuration of naturally contaminated bacteria

Upon arrival from aquaculture facilities, a random sample of oysters was tested for *E. coli* and *Vibrio sp.* The remaining oysters were randomly distributed in each of the six recirculating rearing systems. Analysis of the meat samples taken on arrival showed that both species of oyster had high levels of *E. coli*. On the day of these results (3rd day after the oysters were introduced to the recirculating system), the ultraviolet lights were turned on and the tank water was sterilized. Initially, tank temperatures were set at

different temperatures, three of the tanks were held at 15°C, and three tanks were held at 22 ^{o}C, with all tanks reporting the same salinity (23.8 ±2 ppt). Six days after the oysters were distributed in the tanks, the water temperatures in all tanks were adjusted to 23.4 ±1.5^{o}C. Meat samples were collected and analyzed as previously described at days 7 (before tank temperature change), 10, 17 and 22, following introduction to the recirculating holding tanks (Table 1). Water samples were collected throughout this depuration experiment to ensure the efficiency of the ultraviolet sterilizer light. Statistical analyses were performed using data from day 0 to day 17. Even though tank temperatures differed between day 0 and day 7, these data are included in the statistical analyses because the effect of temperature on depuration was evenly distributed between the two species of oyster. Statistical analyses of the depuration rates only includes data collected up to 17 days as this was the sample date when the concentration of *E. coli* in *Crassostrea virginica* was first detected as nominal.

Table 1. Experimental design

Experiment	**Dates**	**Duration (hours)**	**Sampling frequency (hours post inoculation)**	**Microorganism Analyzed**
Depuration of Natural Bacteria	19 July-3 August 2005	358	0, 74, 245, 358	*E. coli* *Vibrio sp.*
Bioaccumulation	3 August 2005	4	0, 4	*E. coli* *Vibrio sp.*
Storage	3 August-15 August 2005	288	0, 288	*E. coli*
Depuration of Cultured Bacteria	3 August-17 August 2005	332	0, 332	*E. coli* *Vibrio sp.*

Bioaccumulation

Following depuration (day 22 from arrival), the oysters were artificially contaminated by inoculating the tanks with *E. coli*. At time 0, meat samples were collected to establish baseline levels of bacteria concentrations and then each tank was seeded with *E. coli K12* to a final concentration of 10^3 to 10^4 cells/100 ml. Oyster samples were collected and analyzed for *E. coli* and total *Vibrio sp.* four hours after inoculation (Table 1).

Storage

At the end of the bioaccumulation experiment, 10 oysters of each species were harvested from each of the six tanks, and stored in mesh bags in temperature controlled storage systems at 4°C. During the storage trial the oysters were jostled daily to mimic handling in a commercial setting. After 12 days, the oysters were removed from storage and analyzed for *E. coli* and *Vibrio species* (Table 1). The *Crassostrea ariakensis* sample from tank 2 was mistakenly discarded during sample processing; therefore, data for this tank is not presented.

Depuration of cultured bacteria

At the end of the bioaccumulation experiment, 10 oysters of each species remained in each of the six tanks. These oysters were allowed to depurate naturally in water that was not UV sterilized for 14 days. On day 14 the oysters were removed and bacteria were enumerated as described (Table 1).

Statistical analyses

Concentration of each bacterium was calculated (as either a most probable number (MPN) or as a colony forming unit (CFU) per 100 grams of oyster meat), samples that were at the upper or lower limit of test sensitivity were increased by one significant figure or decreased by 50% respectively (i.e., >100,000 became 100,001 and <18 became 9). Concentration data were log transformed and the slope of the linear best fit line was determined for each replicate (each tank). Error bars represent the standard deviation of replicates for each sample point. In experiments where only two time points exist (bioaccumulation, storage), the net increase or decrease in bacterium were calculated and compared using a one-way repeated ANOVA using SigmaStat 3.0 (SPSS). For the depuration experiments, the rate of depuration was determined by calculating the slope of the log transformed data. For species and bacterium specific comparisons, a one-way repeated measure ANOVA was again tested using SigmaStat 3.0 (SPSS). Statistical significance was determined based on a p-value of 0.05 and power analysis conducted was based on an alpha of 0.05.

Results

Depuration of naturally contaminated bacteria

Oysters arrived from aquaculture facilities naturally contaminated with mixed bacterial assemblages including both *Vibrio sp.* and *E. coli*. Initial concentrations of *Vibrio sp.* were above 10^7 CFU/100 grams in *Crassostrea ariakensis*, as well as in *Crassostrea virginica* (Figure 1). Within one week of depuration in UV sterilized water,

concentrations of *Vibrio sp.* in oysters had been reduced by one order of magnitude. After this time, there were no significant changes in the concentrations of *Vibrio sp.* in either *C. ariakensis* or *C. virginica*. This trend continued throughout the depuration, as both species maintained concentrations on the order of 10^6 CFU/100g through the duration of the experiment (Figure 1, ANOVA, p=0.071). Concentrations of *Vibrio sp.* in the water were never reduced below 1000 CFU/100 ml, even though the water was continually sterilized with UV irradiation (Figure 1).

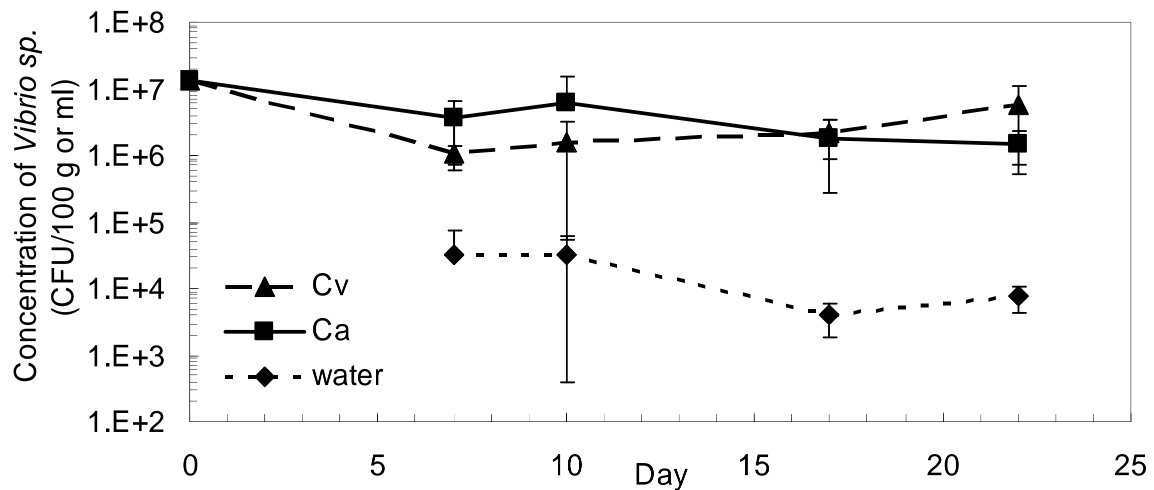

Figure 1. Depuration of *Vibrio sp*. in *Crassostrea ariakensis* meats (Ca) and *Crassostrea virginica* meats (Cv) during 22 day depuration in UV sterilized water (mean, n = 2, 2, 6, 6, 6 for days 0, 7, 10, 17, and 22, respectively). Error bars represent one standard deviation.

Depuration rates of *E. coli* differed greatly from *Vibrio sp*. depuration results. *E. coli* concentrations were two orders of magnitude higher in *Crassostrea ariakensis* than *Crassostrea virginica* when the oysters arrived (Figure 2). After one week in tanks treated with UV irradiation, *C. ariakensis* had dropped 97.9% to 3350 MPN/100 g; whereas, *C. virginica* had been reduced by 98.7% to 64 MPN/100 g (Figure 2, mean, n=6). Even though the concentration of bacteria was lower in *C. virginica*, the rate of

depuration for natural *E. coli* was significantly higher in *C. ariakensis* (Table 2, ANOVA, P<0.05). However, the rate constant (defined as the depuration rate divided by the initial concentration) did not show a statistically significant difference for the depuration of *E. coli* in the two oyster species. Concentrations of *E. coli* in *C. virginica* were below the limit of detection (<18 MPN/100 g) by day 17; however, by day 22 *C. ariakensis* still contained *E. coli* at concentrations of 64 MPN/100 g. As expected, the concentration of *E. coli* in the UV sterilized water remained <1.8 MPN/100 ml for the duration of the depuration period (Figure 2).

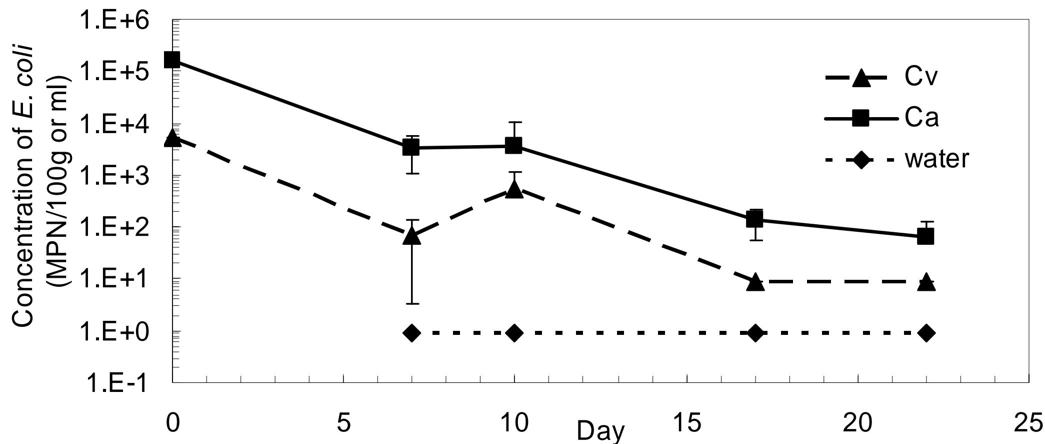

Figure 2. Depuration of *E. coli* in *Crassostrea ariakensis* meats (Ca) and *Crassostrea virginica* meats (Cv) during 22 day depuration in UV sterilized water (mean, n = 2, 2, 6, 6, 6 for days 0, 7, 10, 17, and 22, respectively). Error bars represent one standard deviation.

Table 2. Results of ANOVA statistical analysis for each experiment. Membrane Filtration (MF) and Multiple Tube Fermentation (MTF).

Experiment	Microorganism analyzed (method)	Mean rate/net difference	n	Standard deviation	P value	Alpha value
Depuration of Natural Bacteria	*E.coli* (MTF)	*C. ariakensis* = -0.41 *C. virginica* = -0.35	6 6	0.046 0.023	0.02	0.05
Bioaccumulation	*E.coli* (MTF)	*C. ariakensis* = 2.08 *C. virginica* = 3.79	5 6	0.25 0.18	<0.001	0.05
Storage	*Vibrio* (MF)	*C. ariakensis* = -0.51 *C. virginica* = -1.26	6 6	0.069 0.092	0.009	0.05
Depuration of Cultured Bacteria	*E.coli* (MTF)	*C. ariakensis* = -0.32 *C. virginica* = -0.60	6 6	0.069 0.069	<0.001	0.05

Bioaccumulation

Over the course of this experiment (4 h), *E. coli* was enumerated in the tank water and in the meats of *Crassostrea ariakensis* and *Crassostrea virginica* (Figure 3, mean, n=6). *C. ariakensis* had higher baseline concentrations of *E. coli* at the start of the experiment. After four hours, concentrations of *E. coli* in *C. virginica* were more than an order of magnitude higher than *C. ariakensis*. Concentrations of *E. coli* in the water were inversely related to concentrations in the meat, where there was a three fold decrease in bacteria concentrations over the four hour time period (Figure 3). There was a statistically significant difference in the rates of bacterial uptake, where *C. virginica* uptake rates were nearly ten fold higher than *C. ariakensis* (Table 2, ANOVA, $p<0.001$). *Vibrio sp*. concentrations in both species remained constant throughout the uptake experiment (data not shown).

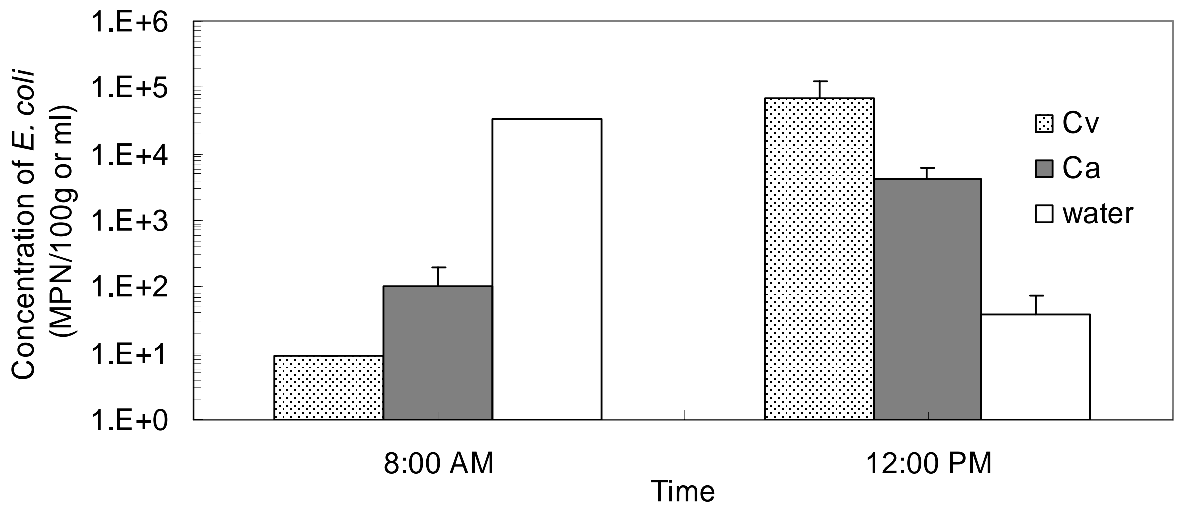

Figure 3. Concentrations of cultured *E. coli* in water, *Crassostrea ariakensis* meats (Ca), and *Crassostrea virginica* meats (Cv) during bioaccumulation experiment (mean, n=6). Error bars represent one standard deviation.

Storage

After 12 days of storage at 4°C, concentrations of *E. coli* in shellfish meats were reduced by 77% and 86% in *Crassostrea virginica* and *Crassostrea ariakensis* respectively (Figure 4). Both *C. ariakensis* and *C. virginica* exhibited a large amount of sample variability. This variability confounded the ability to detect significant differences between the decay rates of *E. coli* between the two species of oyster (ANOVA, p=0.677).

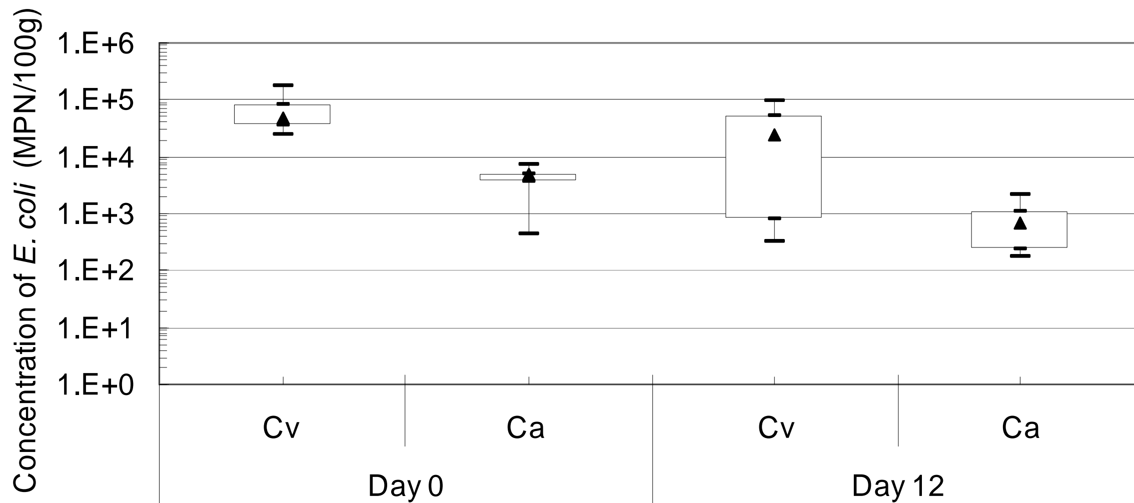

Figure 4. Effect of 12 day storage at 4°C on the concentration of *E. coli* in *Crassostrea ariakensis* meats (Ca) and *Crassostrea virginica* meats (Cv) represented using box and whisker plots. Range of results for the 6 replicates are represented in each box plot as minimum (long dash), 25% quartile (short dash), median (triangle), 75% quartile (short dash), and maximum (long dash).

Vibrio sp. concentrations during the storage experiment were reduced by 92% in *Crassostrea virginica* and 69% in *Crassostrea ariakensis* (Figure 5, mean, n=5). There was a statistically significant difference in the reduction of *Vibrio sp.* between the two species during storage, whereby the rate of *Vibrio sp.* decay was 2.5 times greater in *C. virginica* than *C. ariakensis* (Table 2, ANOVA, $p<0.05$).

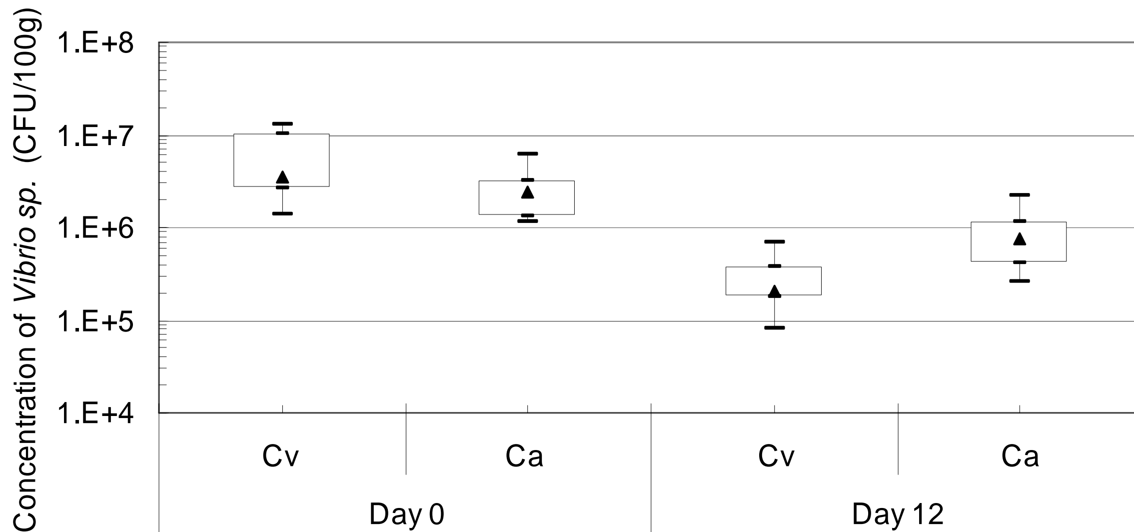

Figure 5. Effect of 12 day storage at 4°C on the concentration of *Vibrio sp.* in *Crassostrea ariakensis* meats (Ca) and *Crassostrea virginica* meats (Cv). Range of results for the 6 replicates are represented in each box plot as minimum, (long dash), 25% quartile (short dash), median (triangle), 75% quartile (short dash), and maximum (long dash).

Depuration of cultured bacteria

E. coli was found to depurate significantly faster in *C. virginica;* when compared to *C. ariakensis* (Figure 6, Table 2, ANOVA, p value <0.001). The concentration of *Vibrio sp.* remained on the order of 10^6 CFU/100 ml in both species of oysters, and 10^4 CFU/100 ml in untreated water during the depuration period (Figure 7). Concentrations of *Vibrio sp.* in the water and tanks were nearly identical to the levels measured during the first depuration trial, where *Vibrio sp*. was consistently detected at concentrations near 10^6-10^7 CFU/100 ml. Levels of *Vibrio sp.* in tank water were also similar during both depuration experiments (~10^4 CFU/100 ml), although tank water was treated with UV irradiation for the first depuration but not for the second.

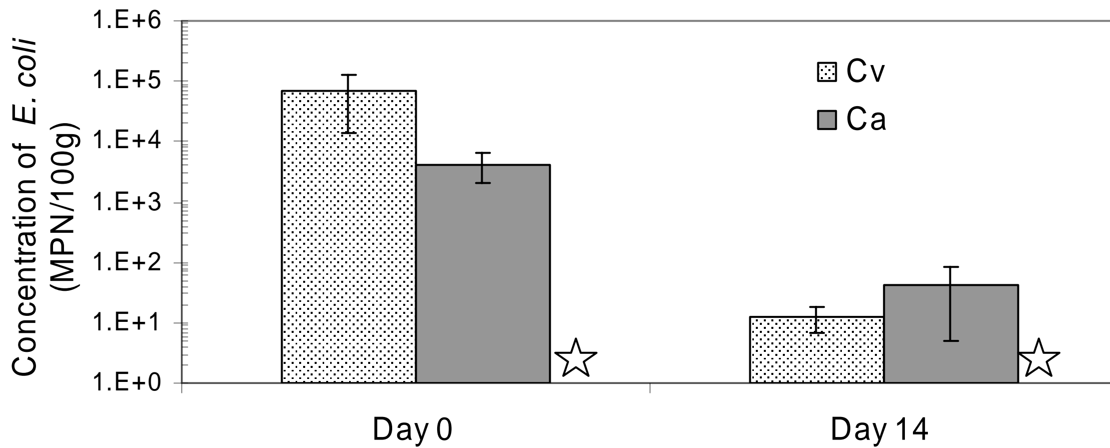

Figure 6. Depuration of *E. coli* in *Crassostrea ariakensis* meats (Ca) and *Crassostrea virginica* meats (Cv) during 12 day depuration in non - UV sterilized water (mean, n=6) Stars represent negligible concentration of *E. coli* in water. Error bars represent one standard deviation.

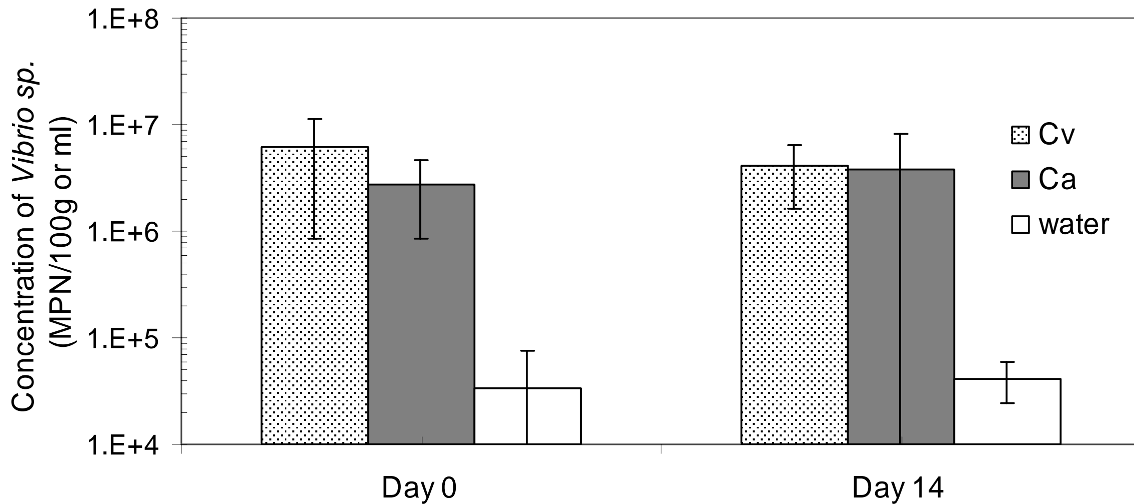

Figure 7. Depuration of *Vibrio sp.* in *Crassostrea ariakensis* meats (Ca) and *Crassostrea virginica* meats (Cv) during 12 day depuration in non-UV sterilized water (mean, n=6). Error bars represent one standard deviation.

Discussion

Depuration of naturally contaminated bacteria

Vibrio sp.

Several studies have reported a commensal, resident population of *Vibrio sp.* in oyster meats (e.g. (Colwell and Liston, 1960; Tamplin and Capers, 1992). Our results show that *Crassostrea ariakensis*, like other oyster species, maintains stable populations of *Vibrio sp*. in gut tissue. Total *Vibrio sp*. concentrations in oyster meats were reduced after seven days in UV sterilized water; however, levels in both *C. ariakensis* and *C. virginica* remained comparable and relatively constant from day 7 to day 22. Similar results were found by Tamplin and Capers (1992) where initially oysters released large quantities of *Vibrio sp.* to surrounding waters followed by a period of consistently low depuration rates.

Water samples were collected throughout the depuration trial to ensure that tank water was being effectively sterilized by ultraviolet light exposure. Use of UV irradiation for tank water sterilization was shown to be successful at reducing *E. coli* to nominal levels; however, total *Vibrio sp.* levels in the water were never reduced to less than 10^3 CFU/100 ml. Although these results are based on total *Vibrio sp.,* they are again similar to the previous research of Tamplin and Capers (1992), who found that, when naturally contaminated oysters were placed in depuration tanks, *Vibrio vulnificus* levels in the UV irradiated water never fell below 10^3 organisms/ ml. These authors concluded that *V. vulnificus* was multiplying in the oyster tissue at a greater rate than UV sterilization was capable of eliminating this bacterium.

E. coli

Previous studies have reported that *E. coli* depuration is controlled by water temperature, and that *E. coli* is rapidly depurated from oysters in the first few days after introduction to clean water (Haven et al., 1978). In our study, there was a noticeable difference in depuration rates for both oyster species when held at 15°C and 22°C (data not shown). However, no samples were collected before day seven of the depuration trial, therefore, no conclusions can be made on the differential rate of depuration between *Crassostrea ariakensis* and *Crassostrea virginica* on this time scale.

Analysis of the depuration rates of *C. virginica* and *C. ariakensis* show that *C. ariakensis* depurated significantly faster than *C. virginica* between day 0 and day 17 (ANOVA, $p=0.02$). There was not a statistically significant difference, however, between the rate constants for the two oyster species ($p=0.3$). This indicates that the initial concentration of bacteria in the oyster meats was a significant factor in the depuration rate of *E. coli* in the oyster meats. These results are consistent with the results of Haven et al. (1978), who found that the initial concentration of *E. coli* in *C. virginica* was a significant factor in the rate of depuration, as oysters with high concentrations depurated faster than oysters with low concentrations.

Given that depuration is a function of filtration rate, it is expected that differential filtration rates could play a role in the respective depuration rates of each species. Preliminary studies indicated no difference in the clearance rates of adult diploid *C. virginica* and adult triploid *C. ariakensis* at ambient seston levels of 8 to 12 mg/L and ~23°C (R. Newell, pers. comm., University of Maryland, Center for Environmental Science, Horn Point Laboratory). Even though we did not measure and quantify filtration

rates specifically, we assume that they are comparable for both species. We recognize that individual oysters will have varying filtration rates due to differences in size, maturity, and well-being. The present experiment reduced such bias through the use of pooled oyster samples containing a minimum of 10 shellfish per homogenate.

It is important to recognize that the two oyster populations used were from different aquaculture facilities. The different rearing locations impose several confounding factors on this experiment, since the oysters were contaminated by different bacterial assemblages, from different sources, and under different environmental parameters. These factors could have impacts on the rates of depuration from the oyster species. More research is recommended to determine if there is a true difference in the responses of the two species. Future studies should use oysters grown in the same waters (i.e., conduct long term field trials), therefore eliminating the source bias and possibly reducing the difference in initial bacterial concentrations in each oyster species.

Bioaccumulation

Bioaccumulation studies, using laboratory cultures of *E. coli,* show that *Crassostrea virginica* accumulate bacteria significantly faster than *Crassostrea ariakensis* over a 4-hour period. In an attempt to resolve fine scale differences in bacterial uptake between the two species, an attempt was made to repeat the previous bioaccumulation experiment with the addition of timepoints at 1, 3, and 5 hours post-inoculation. Unfortunately, the data generated from this experiment was not usable due to laboratory error and we could not repeat the failed experiment due to *C. ariakensis* availability. *C. ariakensis* availability is limited as they are only cultured on small scales

and the facilities that supply these oysters only maintain enough oysters for ongoing research. The bioaccumulation data strongly suggests that there is a difference in the response of *C. ariakensis* and *C. virginica* to bacterial contamination. Experiments should be repeated with finer temporal resolution to determine if rates of *E. coli* uptake are indeed higher in *C. virginica* than in *C. ariakensis*. There may be small scale changes (short time scales) that could cause the oysters to respond in a variable way to bacterial uptake, and experiments should be conducted to address these changes.

Storage

The results of the storage trial show that the natural decay of *Vibrio sp*. in *Crassostrea virginica* is statistically greater than the loss of *Vibrio sp.* during cold storage in *Crassostrea ariakensis* ($p<0.05$). Average loss of naturally contaminated *Vibrio sp.* in *C. virginica* was one fold, a value comparable to reported values for *Vibrio vulnificus* in shellstock under similar storage times and conditions (Hood et al., 1983; Kaysner et al., 1989; Kaspar and Tamplin, 1993). Further research is necessary to determine if pathogenic strains of *Vibrio sp*. such as *V. vulnificus* and *V. parahaemolyticus* will follow the same patterns.

During the storage trial, *Crassostrea ariakensis* became noticeably more desiccated than *C. virginica* (J. Morris pers. observation). This observation may indicate that *C. ariakensis* has a shorter shelf life during post-harvest storage than *C. virginica*. While there are several factors that likely contribute to the observed desiccation (e.g., shell morphology), the persistence of high concentrations of *Vibrio sp.* in *C. ariakensis* cannot be discounted as a contributing factor. Other researchers (e.g., Colwell and

Liston, 1960) have implicated commensal *Vibrio sp.* populations as contributing factors in oyster shellstock spoilage. These scientists found that nearly 50% of the organisms isolated from shellstock were able to ferment glucose anaerobically. They hypothesized that during oyster storage, these organisms were able to metabolize glycogen from the oyster tissue and thus facilitate oyster tissue deterioration. Glucose levels in oysters were not monitored during this study, though it is possible that the desiccation of *C. ariakensis* is related to the observed perpetuation of high levels of *Vibrio sp.* in oyster tissue. More research is necessary to determine the role of desiccation and high *Vibrio sp*. levels on the shelf life and marketability of *C. ariakensis* in post-harvest markets.

Depuration of cultured bacteria

The depuration of pure laboratory strains of *E. coli* was assessed as part of the present research study comparing *Crassostrea ariakensis* and *C. virginica*. In the experiment assessing depuration rates in oysters that had been inoculated with laboratory cultured strains of *E. c*oli, *C. virginica* had statistically higher rates of depuration (ANOVA, $p<0.001$) than *C. ariakensis*. Depuration experiments using oysters that were contaminated with naturally found strains of *E. coli*; however, did not yield a significant difference in the rates of depuration between the two oyster species. These results are confounding, but we speculate that the differences in the rates of depuration of bacteria between the two species may depend upon the source and types of bacteria in the gut, i.e., natural versus laboratory acquired strains of *E. coli*. Several studies have reported that bacteria are eliminated faster from bivalves when they are contaminated artificially than when the oysters are contaminated in natural waters (e.g. Heffernan and Cabelli, 1971;

Tamplin and Capers, 1992). Oysters are selective filter feeders, which actively differentiate between food and non-food items (Newell and Jordan, 1983). It is possible that, when filtered out of the water column, phytoplankton-attached bacteria are directed to the oyster gut, whereas laboratory cultured strains of bacteria would not be attached onto food items and would be directed to the pseudofeces. As a result, naturally derived bacteria could become well-established in the gut of the oyster, whereas cultured bacteria would not be recognized as a food source by the oyster and would be expelled rapidly.

Conclusion

The proposed introduction of *Crassostrea ariakensis* into the Chesapeake Bay has profound implications for the future of the shellfishing industry on the mid-Atlantic seaboard. If approved, introduction of this species may require a re-evaluation of the present standards used to assess shellfish safety and the classification of waters approved for harvest. Comparative studies, such as those presented here, are a critical first step for state regulatory agencies that will be charged with protecting the public health of *C. ariakensis* consumers. Results suggest that *C. ariakensis* depurates naturally contaminated fecal indicator bacteria faster and uptakes bacteria slower than *C. virginica*. These findings suggest that the present standards for oyster harvest may be acceptable for the protection of public health for *C. ariakensis*.

The finding that *Crassostrea ariakensis* retains high levels of *Vibrio sp.* during cold storage is a concern. *Vibrio sp.* are autochthonous estuarine bacteria, and, while they are not indicative of human fecal pollution, some species are human pathogens. Future research should focus on the isolation and speciation of *Vibrio sp.* in *C. ariakensis* meats to determine if there is a sufficient human health risk associated with post storage consumption. Such research is necessary to assess whether the present regulations for post harvest storage of oysters are applicable to this species and will adequately protect public health. Further field-based studies are also warranted to elucidate the mechanisms responsible for the differences in rates of bioaccumulation and depuration.

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