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Bioaccumulation, Retention, and Depuration of Enteric Viruses by *Crassostrea virginica* and *Crassostrea ariakensis* Oysters▽

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***Crassostrea ariakensis* oysters are under review for introduction into the Chesapeake Bay. However, the human health implications of the introduction have not been fully addressed. This study evaluated rates of bioaccumulation, retention, and depuration of viruses by *Crassostrea virginica* and *C. ariakensis* when the two oyster species were maintained in separate tanks containing synthetic seawater of various salinities (8, 12, or 20 ppt). Oyster bioaccumulation tanks were seeded with 10^3 PFU/ml of hepatitis A virus (HAV), poliovirus, male-specific bacteriophage (MS2), and murine norovirus 1 (MNV-1) and 10^3 PCR units/ml of human norovirus (NoV). After 24 h, depuration commenced as oysters ($n = 255$) were placed in pathogen-free seawater under continuous filtration. Oysters ($n = 6$) were sampled weekly for 1 month from each tank. Viral RNA was recovered using a modified proteinase K, guanidine, and glassmilk method and analyzed by quantitative reverse transcription-PCR. The odds of *C. ariakensis* oysters harboring NoV, MNV-1, or HAV were statistically greater than the odds of *C. virginica* oysters harboring the same viruses (MNV-1 odds ratio [OR], 4.5; $P = 0.01$; NoV OR, 8.4; $P < 0.001$; HAV OR, 11.4; $P < 0.001$). Unlike *C. virginica*, *C. ariakensis* bioaccumulated and retained NoV, MNV-1, and HAV for 1 month at all salinities. Additionally, the odds of an oyster testing positive for NoV was 25.5 times greater ($P < 0.001$) when the oyster also tested positive for MNV-1. This research helps assess the threat of *C. ariakensis* as a vehicle for viral pathogens due to the consumption of raw oysters and validates the role for MNV-1 as a surrogate for NoV.**

Prior to the mid-1980s, the Chesapeake Bay region furnished one of the most prominent oyster industries in the United States, accounting for nearly 50% of the U.S. harvest (20). However, the combination of overharvesting and the appearance of two oyster parasites, *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo), led to a rapid decline in native *Crassostrea virginica* oyster health and population (20). In efforts to ameliorate the Chesapeake's commercial oyster industry, the states of Maryland and Virginia are currently considering the introduction of a reproductively sterile triploid nonnative oyster species, *Crassostrea ariakensis* (20). While *C. ariakensis* oysters have been harvested for more than 300 years in Asia, there is a lack of understanding regarding the proclivity of the oyster to harbor human pathogens. This is a great public health concern since these oysters will be harvested for human consumption.

Consumption of raw oysters frequently causes outbreaks of viral gastroenteritis (16), but little is understood about the rate of uptake and retention of viruses by *C. ariakensis*. Viral pathogens can accumulate in oysters when they are adsorbed to food or suspended particles and transported into the oysters' digestive system during feeding (25). Rates of bioaccumulation can be affected by environmental factors, such as temperature and salinity, or oyster physiological factors, such as size and species (25). Larger oysters have the capacity to filter more water (10), thus potentially exposing these oysters to greater numbers of pathogenic viruses in polluted waters (25). *C. ariakensis* oysters grow faster, are larger overall, and tolerate a broader range of water salinities than *C. virginica* (3). A minimum salinity of 5 to 7 ppt is necessary for *C. virginica* survival (11), whereas *C. ariakensis* can thrive in low-salinity water (3). Additionally, *C. ariakensis* oysters are able retain protozoan pathogens longer than *C. virginica* (8). Thus, *C. ariakensis* oysters have the potential to bioaccumulate and retain viral pathogens at a greater rate than *C. virginica* across a wider range of salinities within the Chesapeake Bay.

Human noroviruses (NoVs) and hepatitis A virus (HAV) contribute to many cases of oyster-associated enteric diseases (13, 21). However, unlike laboratory strains of HAV, NoV is not easily replicated in cultured cells (1, 6), and few studies have assessed NoV in oyster bioaccumulation and depuration trials (23, 30). Previous studies have employed the male-specific coliphage MS2, a virus that infects bacteria, and poliovirus (PV) as surrogates for NoV in oyster bioaccumulation, retention, and depuration evaluations (18, 25). Murine norovirus 1 (MNV-1), a culturable mammalian virus within the *Caliciviridae* family, is genetically similar to NoV (32, 33) and may be a more useful surrogate for modeling NoV in oysters. Recent studies indicate that NoV binds preferentially to type A-like histo-blood group antigens (HBGA) on oyster gastrointestinal cells (14, 28, 29)—thus increasing the need to evaluate a more genetically similar NoV surrogate, such as MNV-1, in oyster studies.

Most early bioaccumulation and depuration research also assessed only the fate of one virus type and lacked multiple virus comparisons (25). The objectives of this study were to evaluate and contrast the abilities of two oyster species, *C. ariakensis* and *C. virginica*, to bioaccumulate, retain, and depu-

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rate five viral pathogens across relevant salinity ranges representing oyster harvesting regions of the Chesapeake Bay.

MATERIALS AND METHODS

Oysters ($n = 255$) in sets of 45 were placed in bioaccumulation tanks (14 liters each) for 24 h, followed by depuration for 29 days, with weekly sampling. The human enteric viruses NoV and HAV and three enteric virus surrogates, PV, MNV-1, and MS2, were simultaneously evaluated in order to assess the following: (i) the effect of salinity and time on the rates of viral bioaccumulation, retention, and depuration by two oyster species; (ii) the abilities of the two oyster species to harbor multiple virus types; (iii) the use of a novel norovirus surrogate, MNV-1, to predict human NoV in oysters; and (iv) the potential public health threats associated with the introduction of *C. ariakensis* into the Chesapeake Bay.

Virus preparations. A diarrheal stool sample containing Norwalk virus GI-1 (Norwalk/1968/US), commonly denoted substrain 8fIIb (kindly provided by Christine Moe, Emory University, Atlanta, GA), was diluted 10-fold in Dulbecco's phosphate-buffered saline (pH 7.4, without calcium chloride or magnesium chloride; Invitrogen, Inc.) and emulsified with an equal volume of Vertrel XF hydrofluorocarbon fluid (DuPont, Wilmington, DE) by homogenization. Virus-containing supernatant was recovered by centrifugation at 5,000 × *g* for 15 min at 4°C.

Mammalian viral stocks including MNV-1 (kindly provided by Herbert Skip Virgin, Washington University, St. Louis, MO), HAV HM175, and PV type 1 LSc were generated by inoculation onto confluent monolayers of appropriate cell lines (mouse leukemic monocyte macrophage [RAW 267.4], fetal rhesus monkey kidney [FRhK4], and buffalo green monkey kidney [BGMK], respectively) as described previously (2, 24). MS2 (ATCC 15597-B1) was propagated with *Escherichia coli* C3000 host cells (2).

Oysters. Viable *C. virginica* oysters were obtained from Baltimore, MD. The Virginia Institute of Marine Sciences shipped triploid *C. ariakensis* oysters overnight from the Aquaculture Genetics and Breeding Technology Center, Gloucester Point, VA. Upon arrival at the laboratory, all oysters were rinsed and immediately placed in tanks containing approximately 80 liters of sterile 8-, 12-, or 20-ppt-salinity InstantOcean (Marine Enterprises International, Inc., Baltimore, MD) water. Throughout the study, oysters were monitored daily for mortality. When an oyster's bivalves were continuously open while submersed, the oyster was monitored for 15 min to observe respiration and feeding activities. If the oyster did not move after 15 min, it was removed from the tank, inspected to confirm lack of viability and subsequently discarded following autoclaving.

Bioaccumulation of viruses by oysters. Three aerated 14-liter tanks of water and marine salts (InstantOcean, Marine Enterprises International, Inc., Baltimore, MD), maintained between 20 and 23°C at a salinity of 8, 12, or 20 ppt, were inoculated with five different viruses, MNV-1, NoV, HAV, PV, and MS2, at approximately 1,000 PFU/ml for each virus, with the exception of NoV (1,000 PCR units/ml). After 1 h of viral circulation in the aerated, saline tanks, *C. ariakensis* oysters (45 oysters per salinity for a total of 135 oysters) were added and bioaccumulation commenced with continued aeration. Within the first 2 h of bioaccumulation, oysters were fed 1 ml/tank of concentrated shellfish diet 1800 (ReedMarine, Inc., Campbell, CA), administered according to the manufacturer's instructions. Following 24 h of viral bioaccumulation by the oysters (i.e., day 1), the exteriors of the oysters were rinsed with deionized water and placed in a clean depuration tank containing approximately 80 liters of sterile InstantOcean seawater adjusted to the appropriate salinity and temperature (Fig. 1). For each depuration tank, water was circulated, constantly aerated, and filtered for 5 to 8 h per day using a flowthrough dual-filtration system. For the 29 days of retention and depuration, a peristaltic pump (0.17 liters per min) was used to filter water through a cellulose acetate membrane (393-mm diameter and 1.2-μm pore size) (Millipore Corp., Bedford, MA) to remove larger debris and pseudofeces. To remove virus particles, the water was filtered through a hollow-fiber polysulfone dialysis ultrafilter with a molecular mass cutoff of ~30 kDa (Fresenius F200NR; Fresenius Medical Care, Lexington, MA) and then recirculated back into the tank. Oysters were fed 1 ml/tank of concentrated shellfish diet 1800 once every week, on the days of oyster sampling.

Oysters ($n = 6$) were removed from the tanks at days 1, 4, 8, 15, 22, and 29 for analysis. The exteriors of the oyster shells were rinsed in deionized water, and the oysters were immediately aseptically shucked and the digestive diverticula (DD) dissected. Oyster dissection tools and shucking knives were rinsed and flame sterilized after each oyster dissection. Following dissection, the DD were stored at −80°C until further processing. Individual oyster DD were processed for viral RNA and tested for five viruses by quantitative reverse transcription-PCR (qRT-PCR) as outlined below. The experiment was repeated for *C. virginica* (40 oysters per salinity for a total of 135 oysters), with the exception that *C. virginica* oysters were not sampled on day 4.

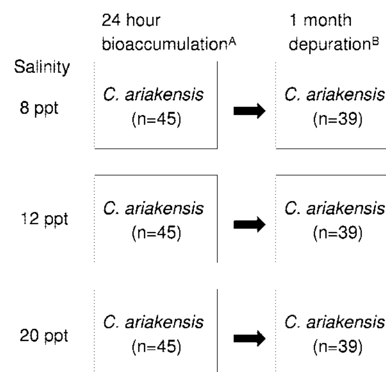

FIG. 1. Illustration of experiment with bioaccumulation, retention, and depuration of viruses by oysters. (A) Bioaccumulation. Oysters were exposed to five virus types (approximately 1,000 PFU/ml, with the exception of NoV at 1,000 PCR units/ml) for 24 h in tanks containing 14 liters of synthetic seawater. Six oysters from each bioaccumulation tanks were sampled on day 1 (after 24 h of bioaccumulation). (B) Depuration. The remaining oysters ($n = 39$) were placed in depuration tank containing approximately 80 liters of sterile synthetic-seawater water for 1 month. The same experimental format was repeated for *C. virginica*.

Viral RNA recovery from oyster DD. Virus recovery from individually dissected DD was done using a modified proteinase K, guanidine, and glassmilk method as described previously (19). Briefly, chopped oyster DD (1.0 to 1.5 g) was placed in a 50-ml copolymer tube, and an equal volume of proteinase K (Amresco, St. Louis, MO) (20 mg/ml) was added to the DD, allowing the release of virions into solution. Following degradation of the DD tissue during a 37°C incubation with shaking at 320 rpm for 1 h, samples were incubated in a 65°C water bath for 15 min to inactivate the proteinase K and centrifuged (3,000 × *g*, 5 min at 4°C). The supernatant was collected and viral nucleic acid further extracted by the addition of 15 μl of a glassmilk, silica bead suspension (Qbiogene, Inc., Carlsbad, CA) and 615 μl 6.6 M guanidium isothiocyanate in buffer (0.07 M Tris-HCl [pH 6.4], 0.03 M EDTA [pH 8.0], 0.17% Triton X-100). Tubes were incubated (20 min at 25°C) in a rotating incubator to facilitate viral RNA binding to the silica beads. Samples were transferred to a microcentrifuge tube and centrifuged (12,000 × *g*, 1 min at 20°C), and the supernatant was discarded in a single pouring motion. The pellet was washed and centrifuged twice (12,000 × *g*, 1 min at 20°C) with 1 ml of 5 M guanidine in buffer (0.1 M Tris-HCl [pH 6.4]); once in 1 ml of ice-cold ethanol; and once in 1 ml of ice-cold acetone, and the final silica pellet air dried for 10 min. Viral RNA attached to the silica beads was eluted with 100 μl of 10 mM Tris-HCl–1 mM EDTA (pH = 8.0) (TE buffer). The samples were incubated in a 56°C water bath for 10 min, allowing the TE buffer to hydrate the RNA and disassociate from the silica bead. RNA precipitation occurred by the addition of 2 volumes of ethanol and 0.1 volumes of 3 M sodium acetate (pH = 5.2) and incubation at −80°C for 1 to 15 h. The final sample was pelleted by centrifugation (20,000 × *g*, 20 min at 4°C) and the supernatant discarded. Samples were air dried, resuspended in 100 μl of molecular-grade water containing 5U/μl GeneAmp RNase inhibitor (Applied Biosystems Inc., Foster City, CA), and stored at −80°C until analysis by qRT-PCR. Total assay time was less than 6 h.

qRT-PCR of viral RNA. Each processed oyster DD sample was tested for five viruses: MNV-1, NoV, HAV, PV, and MS2. For viral RNA amplification, a OneStep RT-PCR kit (Qiagen, Valencia, CA) was used. Briefly, 10 μl of the processed oyster sample was added to 15 μl of the following master mix: 1× Qiagen buffer; 0.4 mM deoxynucleoside triphosphates; 1 μl of a hot-start polymerase enzyme mix; 5 U of GeneAmp RNase inhibitor (Applied Biosystems Inc., Foster City, CA); 0.2 μM downstream primer; 0.2 μM upstream primer; 0.2 μM probe (Table 1); and 6.25 μl diethyl pyrocarbonate-treated water (Quality Biological, Inc., Gaithersburg, MD). All viral RNA samples underwent the following cycling conditions: reverse transcription (32 min at 50°C); denaturation of the reverse transcriptase and activation of the hot-start polymerase (95°C; 15 min); and 60 cycles of template denaturation (94°C, 15 s), primer annealing (55°C, 15 s), and primer extension (72°C, 30 s) in a Smart Cycler qRT-PCR thermocycler (Cepheid, Sunnyville, CA). Positive and negative control samples were

TABLE 1. qRT-PCR primer and gene-specific fluorescent-probe selection

Virus	GenBank accession no. (GenInfo identifier)	Primer or probe	Probe label	Sequence	Product size (bp)	Product region	Reference
NoV	M87661 (gi1061311)	NVKS1		5′-ACAGCATGGGACTCAACACA-3′	190	ORF1, nonstructural polyprotein region	Bae and Schwab (2)
		NVKS2		5′-GGGAAGTACATGGGAATCCA-3′			
		NVKS3	6-FAM[a]	5′-TCACCAGAATTGGCCGAGGTTGT-3′			
MNV-1	AY228235 (gi29150715)	MNVKS1		5′-AGGTCATGCGAGATCAGCTT-3′	159	ORF1, protease, polymerase region	Bae and Schwab (2)
		MNVKS2		5′-CCAAGCTCTCACAAGCCTTC-3′			
		MNVKS3	6-FAM	5′-CAGTCTGCGACGCCATTGAGAA-3′			
HAV HM 175	M14707 (gi:329582)	HAV1-us		5′-CAGCACATCAGAAAGGTGAG-3′	192	VP1 and VP3 junction	Schwab et al. (22)
		HAV2-ds		5′-CTCCAGAATCATCTCCAAC-3′			
		HAV3	6-FAM	5′-TGCTCCTCTTTATCATGCTATG-3′			
PV	AJ293918 (gi9998764)	PanEn1_us		5′-CCTCCGGCCCCTGAATG-3′	197	5′ untranslated region	Schwab et al. (22)
		PanEn2_ds		5′-ACCGGATGGCCAATCCAA-3′			
		PanEn3	6-FAM	5′-TACTTTGGGTGTCCGTGTTTC-3′			
MS2 (ATCC 15597B1)	V00642 (gi15081)	MS2KS1		5′-CTCTCTGGCTACCGATCGTC-3′	235	Replicase gene	Bae and Schwab (2)
		MS2KS2		5′-ACACTCCGTTCCCTCAACG-3′			
		MS2KS3	CAL Red[b]	5′-ACACGCGGTCCGCTATAACGAGT-3′			

[a] The FAM (6-carboxyfluorescein) quencher is BHQ I.
[b] The CAL Red quencher is BHQ II.

analyzed with each thermocycler run to ensure reagent and cycling efficiency and specificity. Serial dilutions of viral RNA from the same virus stocks of NoV, HAV, MNV-1, MS2, and PV employed during oyster bioaccumulation experiments were used as positive controls during qRT-PCR.

Statistical methods. Chi-square tests were used to evaluate oyster mortality between species, and Student t tests compared the presence of multiple viruses between species. Odds ratios were calculated using logistic regression models and Fisher exact tests with the Stata 8 software program (StataCorp LP, College Station, TX).

RESULTS

Crassostrea virginica ($n = 40$) and *C. ariakensis* ($n = 45$) oysters were placed in aerated tanks with 8-, 12-, or 20-ppt salinities, resulting in a total of 255 oysters being evaluated in six tanks. During the sample time period, shellfish mortality was monitored (Table 2). Compared to *C. virginica*, *C. ariakensis* oysters persisted in all experimental water salinity levels. Only one *C. ariakensis* oyster perished during the 29-day test period. *C. virginica* oysters, however, experienced significant mortality (43%) at the lower salinity level of 8 ppt (Table 2). Mortality of *C. virginica* oysters was statistically greater in the 8-ppt-salinity tank than that of *C. ariakensis* oysters ($P < 0.001$).

Bioaccumulation, retention, and depuration. Following the 24-h bioaccumulation (day 1), oysters ($n = 3$) from each tank (for a total of 18 oysters) were evaluated for MNV-1, NoV, HAV, PV, and MS2. The remaining oysters were placed in depuration tanks receiving flowthrough dual filtration; oysters were harvested at days 4 (*C. ariakensis* only), 8, 15, 22, and 29 and tested for the presence of viral RNA. Table 3 indicates the overall number and percentage of oysters that tested positive for each virus type. Because oysters were exposed to viruses only during the 24-h bioaccumulation phase of the experiment and only nine oysters of each species were tested at day 1, reporting results from entire study more accurately (and conservatively) illustrates the individual number of oysters that bioaccumulated viruses. For example, at least 37 (69%) *C. ariakensis* oysters and 18 (40%) *C. virginica* oysters bioaccumulated MNV-1 during the 24-h bioaccumulation (Table 3).

Table 4 indicates the final days of detection for viral pathogens in the two oyster species at the three salinities. Overall, across the three tested salinities, qRT-PCR analyses revealed that *C. ariakensis* oysters efficiently bioaccumulated MNV-1, NoV, and HAV but did not consistently bioaccumulate PV and MS2 (Tables 3 and 4). Similarly, MS2 was also not bioaccumulated by *C. virginica* oysters (Table 3), and overall virus bioaccumulation in *C. virginica* oysters was salinity dependent (Table 4). *C. virginica* oysters bioaccumulated MNV-1, NoV, HAV, and PV at 12 ppt, but only variable bioaccumulation was found at 8-ppt and 20-ppt salinities (Table 4).

C. ariakensis oysters were positive for MNV-1, NoV, and HAV at day 29 in all salinities (Table 4) and positive in a large percentage of the total tested samples (Table 3). MS2 and PV were bioaccumulated to a lesser extent and were present in a lower percentage of *C. ariakensis* tested samples (7 and 2%,

TABLE 2. Shellfish mortality by species during retention and depuration experiments

Oyster species (n[a])	Mortality [no. (%) of dead oysters][b] at salinity of:		
	8 ppt	12 ppt	20 ppt
C. virginica (40)[c]	17 (43)[e]	0 (0)	3 (8)
C. ariakensis (45)[d]	0 (0)	1 (2)	0 (0)

[a] n, no. of oysters per tank.
[b] Tanks were monitored daily, and dead oysters were removed and recorded.
[c] *C. virginica* oysters were evaluated for each salinity level, for a total of 120 oysters.
[d] *C. ariakensis* oysters were evaluated for each salinity level, for a total of 135 oysters.
[e] Mortality of *C. virginica* oysters is statistically greater at a salinity of 8 ppt than that of *C. ariakensis* as determined by a chi-square test ($P < 0.001$).

TABLE 3. Overall numbers of oysters testing positive for each virus type

Oyster species (n[a])	No. (%) of oysters positive for virus type				
	MNV-1	NoV	HAV	MS2	PV
C. virginica (45)	18 (40)	7 (16)	8 (18)	2 (4)	1 (2)
C. ariakensis (54)	37 (69)	30 (56)	27 (50)	1 (2)	4 (7)

[a] n, total no. of oysters analyzed. *C. virginica* oysters ($n = 15$ per tank) and *C. ariakensis* oysters ($n = 18$ per tank) were individually analyzed for each salinity level.

TABLE 4. Endpoint days of detection for viruses in *Crassostrea ariakensis* and *Crassostrea virginica* during depuration experiments at three salinities

Virus	Endpoint day of virus detection for indicated oyster species at salinity (ppt) of[a]:					
	8		12		20	
	CA	CV	CA	CV	CA	CV
MNV-1	29	22	29	22	29	22
NoV	29	ND	29	22	29	25
HAV	29	1	29	8	29	1
MS2	ND[b]	15	15	ND	15	ND
PV	1[c]	ND	ND	1	ND	ND

[a] CA, *C. ariakensis*; CV, *C. virginica*.
[b] ND, not detected.
[c] Day 1 is equivalent to 24 h after oysters were initially exposed to viruses (bioaccumulation).

TABLE 5. Odds of a *Crassostrea ariakensis* oyster harboring MNV-1, NoV, or HAV compared to that for *C. virginica*[a]

Oyster species	Odds of detection of virus type					
	MNV-1		NoV		HAV	
	OR	*P* value	OR	*P* value	OR	*P* value
C. virginica	1		1		1	
C. ariakensis	4.5	0.01	8.4	<0.001	11.4	<0.001

[a] A logistic regression model evaluated the odds of a virus-positive oyster. Each model was adjusted for oyster species, salinity, and depuration time. Each *P* value analyzes the OR for *C. ariakensis* compared to that for *C. virginica*.

respectively) (Table 3). *Crassostrea virginica* oysters were positive for MNV-1 viral RNA until day 22 at all salinities, but their retention of NoV, HAV, and MS2 was variable between salinities (Table 4).

Logistic regression models evaluated the odds of obtaining an oyster positive for viral RNA by oyster species, adjusting for time and salinity (Tables 5 and 6). Models excluded PV and MS2 because of initial poor viral bioaccumulation (Table 3). The odds of *C. ariakensis* harboring viruses was statistically greater than the odds of *C. virginica* harboring the same viruses, adjusting for time and salinity (MNV-1 OR, 4.5; $P = 0.01$; NoV OR, 8.4; $P \leq 0.001$; HAV OR, 11.4; $P \leq 0.001$) (Table 5).

Logistic regression models further evaluated the effects of the variables of salinity and time on the presence of a virus for each oyster species separately (Table 6). Unlike *C. virginica*, salinity did not affect the ability of *C. ariakensis* oysters to bioaccumulate or retain virus ($P > 0.05$) (Table 6). *Crassostrea virginica* was largely salinity dependent (Table 6). The odds of a *C. virginica* oyster testing positive for MNV-1, NoV, or HAV were significantly greater for oysters in tanks of 12-ppt salinity ($P < 0.05$) than in tanks of 8-ppt salinity (Table 6). Similar odds ratios resulted when *C. virginica* oysters in tanks of 12-ppt salinity were compared to *C. virginica* oysters in tanks of 20-ppt (data not shown).

Time was also a significant predictor of a virus-positive oyster (Table 6). For *C. ariakensis* oysters, the odds of an oyster testing positive for MNV-1, NoV, or HAV decreased significantly ($P < 0.01$) with each day of depuration, and there were no statistical differences between the depuration rates of the three viruses (Table 6). For *C. virginica*, the chance of detecting MNV-1 decreased significantly ($P < 0.005$) with each day of depuration (Table 6). NoV and HAV were not bioaccumulated at 8 and 20 ppt by *C. virginica* oysters (Table 4), and thus, depuration time could not be used to predict a virus-positive oyster in logistic regression models (Table 6).

Oysters contain viral RNA from multiple virus types. Each individual processed oyster was evaluated for the presence of five different virus types. At least one virus was detected in 74% of all *C. ariakensis* oysters tested, compared to 42% of all tested *C. virginica* oysters (Table 7). Both species can retain at least four enteric viruses simultaneously, but unlike *C. virginica* oysters, a large portion of *C. ariakensis* oysters (42.6%) tested positive for three viruses ($P < 0.01$) (Table 7). More than one-third of *C. ariakensis* oysters were positive for three virus types, even after 4 weeks of depuration (data not shown).

NoV surrogates. MNV-1, HAV, and MS2 have different capabilities of predicting the presence of NoV viral RNA in experimental oysters (Table 8). Additionally, PV never predicted the presence of NoV with depuration time, oyster species, and salinity in the adjusted regression model and thus is not included in the table. The odds of an oyster testing positive for NoV is 25.5 ($P < 0.001$) times greater when the oyster also tests positive for MNV-1 than when an oyster tests negative for MNV-1, adjusting for oyster species, salinity, and time (Table 8). Similar results were obtained for HAV (OR = 19.4; $P < 0.001$) (Table 8). PV and MS2 were not statistical predictors for the presence of NoV (Table 8).

DISCUSSION

We examined the rates at which two oyster species, *C. ariakensis* and *C. virginica*, bioaccumulated, retained, and depurated five different viral pathogens in three salinity ranges (8, 12, and 20 ppt) that represent conditions of the Chesapeake Bay.

In our study, oysters were fed and oxygenated to optimize viability. However, during the test period, *C. virginica* experienced significant mortality in the 8-ppt-salinity tanks (Table 2). Generally, oysters tolerate salinity fluctuations, but 15 to 18 ppt is the optimum range for *C. virginica* oysters, with a minimum salinity of 5 to 7 ppt necessary for survival (11). *Crassostrea virginica* oysters do exist in low-salinity areas naturally but are usually slow growing and small in size and produce low spatfalls. Oyster populations living in waters with an average salinity of 15 ppt are dense and highly reproductive (11). Environmental factors can affect the physiological state of the oysters and thus in turn alter the ability of an oyster to actively feed, filter, and thus bioaccumulate viruses (25). This is evidenced by the poor viral bioaccumulation seen in our *C. virginica* oysters in the low-salinity water tank.

Only one *C. ariakensis* oyster perished during the entire test period (Table 2), indicating the robustness of the species under the three salinity conditions. *Crassostrea ariakensis* oysters will likely feed, filter, and actively bioaccumulate pathogens under the tested range of salinities in the Chesapeake Bay. The salinity tolerance of *C. ariakensis* oysters was noted previously in a comparative field study of the two oyster species (3). Similar to our findings, the authors reported the mean cumulative mortality of *C. ariakensis* in low salinities (<15 ppt) to be 14%,

TABLE 6. Effect of salinity and depuration time on a virus-positive oyster[a]

Oyster species	Model covariate	Odds of detection of virus type					
		MNV-1		NoV		HAV	
		OR	*P* value	OR	*P* value	OR	*P* value
C. ariakensis	Salinity						
	8 ppt	1		1		1	
	12 ppt	2.18	0.39	1.30	0.72	4.07	0.07
	20 ppt	2.18	0.39	1.69	0.47	1.32	0.71
	Depuration time	0.86	<0.005	0.92	0.01	0.91	<0.005
C. virginica	Salinity						
	8 ppt	1		1		1	
	12 ppt	12.65	0.02	21.21[b]	0.02	16.20[b]	0.04
	20 ppt	1.00	1.00	3.20[b]	1.00	3.20[b]	1.00
	Depuration time	0.86	<0.005				

[a] A logistic regression model evaluated the odds of a virus-positive oyster. Each model was adjusted for salinity and depuration time.
[b] Fisher's exact tests were used for NoV and HAV models with *C. virginica* because time was not a predictor of the presence of a virus in an oyster at 8 and 20 ppt.

compared to a mean cumulative mortality of 81% for *C. virginica* oysters (3).

Logistic regression models evaluated the effect of oyster species, salinity, and depuration time on the presence of viral RNA in an oyster. Analyses revealed that the odds of *C. ariakensis* oysters harboring enteric viruses were statistically greater than the odds of *C. virginica* oysters harboring the same viruses (for MNV-1, OR = 4.5; $P < 0.01$; for NoV, OR = 8.4; $P < 0.001$; for HAV, OR = 11.4; $P < 0.001$) (Table 5). At least one virus was detected in 74% of all *C. ariakensis* oysters but was detected in only 42% of all *C. virginica* oysters (Table 7). Variability of viral bioaccumulation between shellfish species has been previously noted (5). The authors suggested that Pacific coastal shellfish species can bioaccumulate large quantities of viruses quickly and may have a greater capacity for bioaccumulation than *C. virginica* oysters. Similarly, protozoan retention has been shown to vary between *C. ariakensis* and *C. virginica* species (8).

The effects of the covariates, salinity and depuration time, on the prediction of a virus-positive oyster for each oyster species were evaluated. Salinity was not a significant predictor of a virus-positive *C. ariakensis* oyster, since this oyster species bioaccumulated and retained MNV-1, NoV, and HAV similarly across all tested salinity levels (Table 4; Table 6). However, the odds of a *C. virginica* oyster testing positive for MNV-1, NoV, or HAV was statistically significantly greater ($P < 0.04$) in the 12-ppt-water-salinity tanks than for oysters in the 8- or 20-ppt-salinity tanks (Table 6).

A previous study evaluating the effect of salinity on viral bioaccumulation found that water salinity of 7 ppt increased PV binding in shellfish mucus 20% over that in shellfish at 28 ppt (4). It was suggested that virus binding differences occurred because of competition between cations and viral capsids for mucus anions. Our study indicates that salinity largely affects the physiological health of *C. virginica* oysters and subsequently viral bioaccumulation and retention at low salinities. However, *C. ariakensis*, unaffected by salinity, will not only survive the range of salinities found in the Chesapeake Bay but also likely bioaccumulate and retain pathogens while living in contaminated water.

Depuration time was a significant predictor of a virus-positive oyster for both oyster species (Table 6). As previously reported, depuration is ineffective in removal of virus from oysters (23, 25, 30). In our studies, oysters were allowed to depurate in tanks receiving flowthrough water filtration for 29 days. While the odds of a *C. ariakensis* oyster harboring MNV-1, NoV, and HAV decreased significantly ($P < 0.01$) with every additional day of depuration (Table 6), *C. ariakensis* oysters were still positive for MNV-1, NoV, and HAV at day 29 at all salinities (Table 4). A similar effect was seen in our studies for *C. virginica* oysters in tanks of 12 ppt, where oysters were positive for MNV-1 and NoV up to day 22 (Table 4). Our study was the first to evaluate the rate of depuration of viruses

TABLE 7. Positivities of *C. ariakensis* and *C. virginica* oysters for multiple virus types

No. of virus types detected in one oyster	No. (%) of indicated oysters positive for multiple virus types	
	C. ariakensis[a]	*C. virginica*[c]
5	0 (0)	0 (0)
4	2 (3.7)	2 (4.4)
3	23 (42.6)[b]	3 (6.7)
2	6 (11.1)	3 (6.7)
1	9 (16.7)	11 (24.4)
0	14 (25.9)	26 (57.8)

[a] *C. ariakensis* oysters ($n = 18$ per tank) were individually analyzed for each salinity level, for a total of 54 oysters.
[b] Individual *C. ariakensis* oysters were found to contain three viruses more frequently than *C. virginica oysters* (Student's *t* test; $P < 0.01$).
[c] *C. virginica* oysters ($n = 15$ per tank) were individually analyzed for each salinity level, for a total of 45 oysters.

TABLE 8. Odds of predicting an oyster positive for NoV when another virus is present (NoV surrogate)[a]

Virus	Odds of predicting NoV positivity	
	Odds ratio	*P* value
MNV	25.5	<0.001
HAV	19.4	<0.001
MS2	0.6	0.69

[a] Logistic regression models were adjusted for time, oyster species, and salinity. PV never predicted the presence of NoV with time, species, and salinity in an adjusted model and thus was not included in the table.

by *C. ariakensis* oysters, in comparison to *C. virginica*. Rates of depuration do differ by shellfish species, with *C. ariakensis* depurating inefficiently at all salinities (Table 4). Because viruses were detected at all sampled time points (Table 4), conventional depuration may not effectively eliminate viral pathogens from bivalve mollusks, especially *C. ariakensis* oysters.

Regardless of oyster species and salinity, overall, PV and MS2 were bioaccumulated less efficiently than MNV-1, NoV, and HAV (Table 3). Many early viral bioaccumulation and depuration experiments used various PV strains as a surrogate for the fate of enteric viruses in oysters (25). Investigations indicated that PVs are eliminated most quickly, and often entirely, from oysters during the first 24 to 48 h of depuration (9, 26). In our study, only 17% of all oysters tested at day 1 (24 h after bioaccumulation) were positive for poliovirus (data not shown). Given the fast depuration rate of poliovirus, it is likely that any oysters containing poliovirus at day 1 (i.e., the beginning of the depuration experiment) eliminated the virus prior to the first collection times of days 4 or 8. It is also possible that PV RNA was more labile in seawater and the virus and viral RNA were degraded prior to bioaccumulation. For example, Wait and Sobsey illustrated that PV was inactivated by 90% within 1 to 3 days in seawater in both laboratory and field settings at similar water temperatures to those used in our laboratory studies (20°C) (31). Similarly, coliphage MS2 was also not bioaccumulated or retained uniformly within or between oyster species (Table 3). However, MS2 was found in both oyster species after 14 days of depuration (Table 4), indicating the viral RNA can be stable if sequestered by an oyster.

Recent studies report that molecular interactions can occur between individual Norwalk viruses and type A HBGA located within oyster DD (14, 28, 29). While MNV-1 and HAV have not been evaluated for their ability to also bind to specific HBGAs, similar molecular interactions between these viruses and the oyster DD cells may occur. However, because MS2 infects strains of *E. coli*, molecular interactions occurring between HBGAs and MS2 are improbable. MS2 is most likely sequestered by the oyster rather than being attached to a cell surface receptor. Thus, the behavior of MS2 within oysters is not uniform, nor is it easy to predict. Future studies should evaluate the abilities of other enteric viruses to bind to cells present in oyster DD.

Our research found that both oyster species can harbor multiple virus types simultaneously (Table 7). Statistically, individual *C. ariakensis* oysters tested positive for three viruses more frequently than *C. virginica* oysters ($P < 0.05$) (Table 7). Additionally, more than half (57.4%) of the tested *C. ariakensis* oysters harbored between two and four virus types, indicating *C. ariakensis* may serve as a vehicle for multiple infectious agents (Table 4). Mixed norovirus genotypes have been found in the stools of patients characterized in oyster-associated outbreaks (7, 15, 27) and from oysters implicated in an international outbreak (15). One laboratory-based depuration study evaluated two viruses, NoV and feline calicivirus (FCV), simultaneously from individual oysters (30). This study found only one virus type at the last day of detection (day 10) (30). Our study is the first to document that individual oysters can harbor and retain up to three viruses for 4 weeks after the initial exposure to virally contaminated water.

MNV-1 was evaluated as a surrogate for human NoV retention and depuration by oysters (Table 8). Because human NoVs cannot easily be replicated in cell culture, little is known about the fate and transport of human NoVs in the environment and their survivability following depuration and relaying activities (1, 12, 17, 25). To our knowledge, two studies have evaluated the persistence of human NoV in oysters during depuration (23, 30). One of these studies evaluated depuration for only 48 h, with NoV GI being found in individual oysters at that time (23). The second study evaluated bioaccumulation and depuration of NoV GII and FCV by *Crassostrea gigas* oysters. NoV GII was detected after 10 days of depuration, while FCV depurated quickly, within 1 day of depuration (30). Our research indicates that NoV can be retained for 29 days in *C. ariakensis* oysters and for 22 days in *C. virginica* oysters (Table 4).

Of all viruses, MNV-1 most closely matched the behavior of NoV in the two oyster species. Logistic regression models indicate that the odds of an oyster testing positive for NoV is 25.5 times greater ($P < 0.001$) when the oyster also tests positive for MNV-1 than when an oysters tests negative for MNV-1 (Table 8). HAV was also very predictive of the presence of NoV (OR, 19.4; $P < 0.001$) (Table 8). Previously, studies have employed viruses such as PV and coliphage MS2 to model the fate and transport of human NoV (13, 18, 25); however, in our studies, neither virus statistically predicted a NoV-positive oyster (Table 8).

According to our and other recent studies, *C. ariakensis* oysters are more tolerant of wide salinity ranges (3), grow and filter water faster (10), bioaccumulate and retain viral and protozoan pathogens longer (8), and exhibit an inability to depurate some viral pathogens compared to the native *C. virginica*. Prior to the introduction of *C. ariakensis*, the Chesapeake Bay region should consider the public health risks of the consumption of *C. ariakensis* oysters. If *C. ariakensis* oysters are consumed, they should only be served cooked given the greater odds of their harboring a virus under all salinity ranges and time points. This study can also provide information to recommend depuration, relaying, harvesting, disinfection, and possibly cooking standards and to inform public officials as to when sewage-contaminated oyster beds may be reopened.

Conclusions. This study revealed that enteric viruses can be bioaccumulated and retained by both *C. ariakensis* and *C. virginica*. As seen in previous studies, depuration activities were ineffective in removing viral pathogens. Additionally, viral pathogens were retained longer and depurated more slowly by *C. ariakensis.* For MNV-1, NoV, and HAV, *C. ariakensis* had a statistically greater odds of harboring viruses during the test period. Because *C. ariakensis* can retain pathogens for lengthy time periods, the introduction of the nonnative oyster into the Chesapeake Bay may present public health consequences for consumers.

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