

ORIGINAL ARTICLE

Co-localized *Crassostrea virginica* and *Crassostrea ariakensis* Oysters differ in bioaccumulation, retention and depuration of microbial indicators and human enteropathogens

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Abstract**Aims:** To evaluate the bioaccumulation, retention and depuration rates of nine pathogens and surrogates when two oyster species were co-localized in tanks of seawater.**Methods and Results:** *Crassostrea ariakensis* ($n = 52$) and *Crassostrea virginica* ($n = 52$) were exposed to five virus types, two protozoan and two microsporidian species for 24 h. Oysters were then placed in depuration tanks, and subsets were removed and analysed for micro-organisms at weekly intervals. The odds of *C. ariakensis* oysters harbouring mouse norovirus-1 (MNV-1), human norovirus (NoV) or hepatitis A virus (HAV) were significantly greater than the odds of *C. virginica* oysters harbouring the same viruses (MNV-1 OR = 5.05, $P = 0.03$; NoV OR = 6.97, $P = 0.01$; HAV OR = 7.40, $P < 0.001$). Additionally, compared to *C. virginica*, *C. ariakensis* retained significantly higher numbers of transmissible stages of all protozoan and microsporidian species ($P < 0.01$). *Crassostrea ariakensis* oysters are also capable of retaining multiple human pathogens for at least 1 month.**Conclusions:** *Crassostrea ariakensis* oysters were statistically more likely to harbour enteropathogens and microbial indicators, compared to *C. virginica*. Individual *C. ariakensis* were also statistically more likely to retain multiple viruses, protozoa and microsporidia than *C. virginica*, highlighting the role the species may play in the transmission of multiple diseases.**Significance and Impact of the Study:** Nonnative *Crassostrea ariakensis* oysters are under review for their introduction into the Chesapeake Bay. The results of this study suggest that nonnative *C. ariakensis* oysters may present a serious public health threat to people consuming the oysters raw from contaminated sites.**Introduction**

The native population of Chesapeake Bay *Crassostrea virginica* oysters have experienced great declines over the past several decades because of factors such as pollution, over-harvesting and two oyster parasites *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) (NRC, 2005). To help alleviate the oyster industry's economic downturn, the region is evaluating the introduction of a non-native oyster species, *Crassostrea ariakensis*. *Crassostrea*

ariakensis oysters will be introduced as reproductively sterile triploids into the Bay and harvested separately from native *C. virginica* oysters (NRC, 2005). However, because the sterility process is not 100% effective, it is likely the oysters will eventually reproduce in the Bay and cohabitate with the native *Crassostrea virginica* oyster species (NRC, 2005).

The rate at which the two oyster species bioaccumulate, retain and depurate viral, protozoan and microsporidian pathogens has been evaluated when the two species were

placed in separate tanks over three different salinity levels (Graczyk *et al.* 2006; Nappier *et al.* 2008). However, because of the potential for *C. ariakensis* oyster reproduction in the Chesapeake Bay, it is also important to evaluate whether a public health threat exists in the event that the two oyster species co-localize in shellfish harvesting regions.

Consumption of raw oysters frequently causes outbreaks of gastroenteritis (Sobsey and Jaykus 1991; Lees 2000; Koopmans *et al.* 2002; Le Guyader *et al.* 2003). *Cryptosporidium parvum*, *Giardia lamblia* and human-virulent microsporidia, including *Encephalitozoon hellem* and *Enterocytozoon bieneusi* are human enteropathogens that inflict considerable morbidity on healthy people and can cause mortality in immunosuppressed individuals (Wolfe 1992; Weber *et al.* 1994; Graczyk *et al.* 1998). The transmissive stages of these pathogens (i.e., oocysts, cysts and spores) are resistant to environmental stressors and are therefore long lasting and ubiquitous in an aquatic environment (Wolfe 1992; Rose *et al.* 1997; Kucerova-Pospisilova *et al.* 1999). Similarly, human noroviruses (NoVs) and hepatitis A virus (HAV) are often present in faecally contaminated waters, causing many cases of oyster-associated enteric disease (Lees 2000; Parashar and Monroe 2001).

In a study of two oyster species assayed in separate tanks of seawater, the odds of *C. ariakensis* harbouring the enteric viruses NoV and HAV were 8.4 and 11.4 times greater than the odds of *C. virginica* oysters harbouring the same viruses (Nappier *et al.* 2008). Likewise, *C. ariakensis* were found to harbour viable *C. parvum* oocysts five times longer than *C. virginica* oysters (Graczyk *et al.* 1998). Bioaccumulation, retention and depuration rates have not been directly compared between oyster species, but *G. lamblia* and microsporidia can be retained by *C. ariakensis* for up to 14 and 27 days, respectively (Graczyk *et al.* 2006). To date, no known studies have assessed bioaccumulation, retention and depuration of microsporidia in *C. virginica* oysters (Graczyk *et al.* 2006).

To help fully capture the public health impact of the introduction of a nonnative species, it is necessary to evaluate the rate at which the native and nonnative oyster species bioaccumulate, retain and depurate enteric pathogens when co-localized in tanks of seawater. The rate of bioaccumulation can be affected by environmental factors, such as temperature, overcrowding and salinity, as well as oyster physiological factors, such as size and species (Sobsey and Jaykus 1991). Larger oysters have the capacity to filter more water (Hudson *et al.* 2004) and are thus potentially exposed to greater numbers of pathogens in polluted waters (Sobsey and Jaykus 1991). Compared to *C. virginica*, *C. ariakensis* is a larger oyster, tolerable to wider ranges of water salinity (Calvo *et al.*

2001) and able to retain viral, microsporidian and protozoan pathogens longer (Graczyk *et al.* 1998; Nappier *et al.* 2008).

Typically, viruses are bioaccumulated in the digestive diverticula (DD) of the oyster, and thus this organ can be isolated for viral analysis (Schwab *et al.* 1998). Following dissection, viral RNA can be extracted from the DD, using guanidium-based methods (Nappier *et al.* 2008). Viral RNA is subsequently analysed by quantitative reverse transcription-PCR (qRT-PCR) (Nappier *et al.* 2008). Protozoa and microsporidia have been found in oyster haemolymph and gills (Graczyk *et al.* 2006), and the identification and viability of *C. parvum*, *G. lamblia*, *E. hellem* and *E. bieneusi* can be assessed by fluorescent *in situ* hybridization (FISH) (Graczyk *et al.* 2006).

Previous oyster bioaccumulation and depuration studies have predominately evaluated only one oyster species and one group of pathogens. The objectives of this study were to compare bioaccumulation, retention and depuration of nine pathogens or surrogates by two oyster species *C. ariakensis* and *C. virginica* over a 1-month period.

Materials and methods

Pathogen preparation

A diarrhoeal stool sample containing Norwalk virus (NoV) GI-1 (Norwalk/1968/US), commonly denoted substrain 8fIb (kindly provided by Christine Moe, Emory University, Atlanta, GA), was diluted 10-fold in Dulbecco's phosphate-buffered saline (D-PBS; pH 7.4, without calcium chloride or magnesium chloride; Invitrogen, Inc.) and emulsified with an equal volume of Vertrel XF (DuPont, Wilmington, DE, USA) by homogenization. Virus-containing supernatant was recovered by centrifugation (5000 g, 15 min at 4°C).

Mammalian viral stocks including MNV-1 (kindly provided by Herbert Skip Virgin, Washington University, St Louis, MO, USA), HAV HM175 and PV type-1 LSc (PV) were generated by inoculation onto confluent monolayers of appropriate cell lines (Mouse leukaemic monocyte macrophage [RAW 267.4], foetal rhesus monkey kidney FRhK4 and buffalo green monkey kidney [BGMK], respectively) as described previously (Schwab *et al.* 2000; Bae and Schwab 2008). MS2 (ATCC 15597-B1) was propagated with *E. coli* C3000 host cells (Bae and Schwab 2008).

The *C. parvum* and *G. lamblia* (genotype E) stocks originated from an experimental infection of a calf, and the microsporidian spores originated from *in vitro* cell line infections (*E. hellem*) and a human faecal sample (*E. bieneusi*) (Graczyk *et al.* 2004). The oocysts, cysts and

spores were enumerated using a haemocytometer and stored at 4°C for up to 3 weeks prior to experiments.

Oysters

Viable *C. virginica* oysters were obtained from Baltimore, MD. Triploid *C. ariakensis* oysters were shipped overnight from a Virginia Institute of Marine Sciences field trial site (Virginia Beach, VA). Upon arrival to the laboratory, all oysters were rinsed and immediately placed in a tank containing approx. 100 l of 12 ppt salinity sterile Instant Ocean (Marine Enterprises International, Inc., Baltimore, MD). All oysters were market size, approx. 8 cm in length and between 1 and 2 years of age.

Bioaccumulation of micro-organisms by oysters

Aerated 14-l tanks of water and marine salts (Instant Ocean, Marine Enterprises International, Inc.), maintained between 20 and 23°C at a salinity of 12 ppt, were inoculated with five different viruses – MNV-1, NoV, HAV, PV and MS2 – at approx. 1000 PFU ml⁻¹ of each virus, with the exception of NoV (1000 PCR Units (PCRU) ml⁻¹). Additionally, approx. 1.0 × 10⁵ transmissible stages of the protozoan species, *C. parvum* oocysts and *G. lamblia* cysts, and the fungal microsporidia spores, *E. hellem* and *E. bienersi*, were added to the bioaccumulation tanks.

After 1 h of micro-organism mixing in the aerated saline tanks, *C. ariakensis* (*n* = 52) and *C. virginica* oysters (*n* = 52) were added and bioaccumulation commenced with continued aeration. Within the first 2 h of bioaccumulation, oysters were fed 1-ml tank⁻¹ 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 approx. 80 l of sterile Instant Ocean seawater adjusted to 12 ppt salinity and 23°C (Fig. 1). For each depuration tank, water was circulated,

constantly aerated and filtered 5–8 h day⁻¹ using a previously described flow-through dual filtration system (Nappier *et al.* 2008). Filtration was used to remove depurated micro-organisms from the water column and to eliminate secondary microbial uptake (i.e. bioaccumulation) during depuration. Briefly, a peristaltic pump (0.17 l min⁻¹) 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, protozoa, microsporidia and pseudo-faeces. To remove virus particles, the water was filtered through a hollow-fibre polysulfone dialysis ultrafilter with a molecular weight cut-off of ~30 000 Da (30 kDa) (Fresenius F200NR; Fresenius Medical Care, Lexington, MA, USA) and then recirculated back into the tank. Oysters were fed 1-ml tank⁻¹ of concentrated shellfish diet 1800 once every week, on the days of oyster sampling.

Oysters (*n* = 12) were removed at days 1, 4, 8, 15, 22 and 29 for analysis. The exterior of the oyster shells were rinsed in deionized water, and the individual oysters were immediately aseptically shucked and the DD dissected. Oyster dissection tools and shucking knives were rinsed and flame sterilized after each oyster dissection. Following dissection, DD were stored at -80°C until further processing. Individual oyster DD were processed for viral RNA and tested for five virus types by qRT-PCR. Oyster gills and haemolymph were pooled from groups of three oysters and tested for the protozoan and microsporidian species by FISH or direct immunofluorescent antibody (IFA) assays described later.

Viral RNA recovery and detection from oyster DD

Viral RNA recovery from individually harvested DD was carried out using a modified proteinase K, guanidine and glassmilk method, as described previously (Nappier *et al.* 2008). Each processed oyster sample was tested for five types of viruses, MNV-1, NoV, HAV, PV and MS2, and analysed by quantitative reverse transcription-PCR, as previously described (Nappier *et al.* 2008).

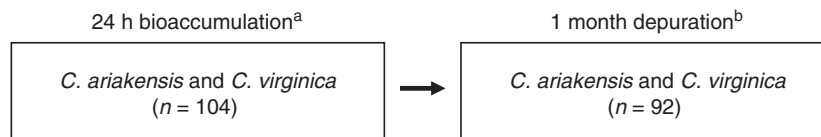


Figure 1 Illustration of experiment with bioaccumulation, retention, and depuration of enteric pathogens or surrogates by oysters. (a) Bioaccumulation. Oysters (*n* = 104) were exposed to nine types of pathogens or surrogates for 24 h in duplicate tanks each containing 52 oysters in 14 l of aerated synthetic seawater (12 ppt; 23°C). A total of six oysters of each oyster species were sampled on day 1 (after 24 h of bioaccumulation) from the bioaccumulation tanks. (b) Depuration. The remaining oysters (*n* = 92) were equally divided into duplicate depuration tanks containing approx. 80 l of sterile synthetic-seawater water for 1 month (12 ppt; 23°C).

Recovery of protozoa and microsporidia from oyster DD

In addition to harvesting DD, haemolymph and gills were also collected from each oyster, as previously described (Fayer *et al.* 1998). The haemolymph and gill washings from three oysters were then pooled. Pooled samples were centrifuged (10 000 g, 10 min at 4°C), the supernatant discarded, and the pellet stored in 75% ethanol at 4°C. Alcohol was washed from the pellets by centrifugation (10 000 g, 10 min) twice in 1 ml of sterile PBS, and the final pellet was suspended in 100 µl PBS, which was portioned into two aliquots. One aliquot was processed for *C. parvum* and *G. lamblia* using a combined FISH and direct IFA method, and the other aliquot was processed for assaying human-infective microsporidia with FISH (Graczyk *et al.* 2006). Briefly, for the protozoa, the walls of the *C. parvum* and *G. lamblia* transmissive stages were allowed to permeabilize, samples were washed and centrifuged (10 000 g for 3 min), and the pellet was resuspended in 100 µl of PBS (Graczyk *et al.* 2006). All combined FISH and direct IFA assay reactions were carried out in Eppendorf tubes in a total volume of 100 µl of hybridization buffer at 48°C (Graczyk *et al.* 2006). The FISH reactions for human-infective microsporidia were carried out in Eppendorf tubes in a total volume of 100 µl of hybridization buffer at 57°C for 3 h (Hester *et al.* 2000; Graczyk *et al.* 2004). All FISH probes are described previously (Hester *et al.* 2000; Graczyk *et al.* 2004, 2006). After hybridization, the tubes were centrifuged twice (2000 g, 5 min at 4°C), and the pellets were resuspended in 100 µl of sterile PBS. Five 20 µl samples were transferred into lysine-coated wells (5 mm diameter) on a Teflon-coated glass slide (Carlson Scientific, Inc., Peotone, IL, USA) and air-dried. The entire area of a well was examined with the aid of an Olympus BH2-RFL epifluorescence microscope with a dry 60× objective and a BP450-490 exciter filter (Graczyk *et al.* 2006). The samples were uncoded prior to analysis of the results, and pathogens were enumerated without knowledge of the samples' identities.

Statistical methods

Statistics were used to assess the null hypothesis that the two oyster species bioaccumulated, retained and depurated pathogens at the same rate. Student's *t*-tests were used to compare presence of multiple viruses between oyster species. Odds ratios were calculated using logistic regression models and fisher exact tests with STATA 8 (StataCorp LP, College Station, TX, USA). Log-linear regression models were created to evaluate the effects of oyster species and depuration time on the number of protozoan and micro-

sporidian transmissive stages found in each oyster with STATA 8 (StataCorp LP).

Results

Bioaccumulation, retention and depuration

Oysters were exposed to nine micro-organisms including, viruses (MNV-1, NoV, HAV, PV, MS2), protozoa (*C. parvum* and *G. lamblia*) and microsporidia (*E. hellem* and *E. bienersi*) for 24 h, and placed in depuration tanks receiving flow-through dual filtration. Subsets of oysters were removed for analysis at days 1 (24 h after bioaccumulation), 4, 8, 15, 22, and 29, with each subset tested for the presence of all nine micro-organisms. Viruses were analysed from individual oyster DD ($n = 48$). Pools of gills ($n = 24$) and haemolymph ($n = 24$) were created from a total of 72 oysters and analysed for protozoa and microsporidia.

The total number (and percent) of oysters positive for each virus type during the 29-day experiment was determined (Table 1). Oysters were only exposed once during the 24-h bioaccumulation; thus, the table illustrates conservative estimates of total viral bioaccumulation. Twenty-one (88%) *C. ariakensis* oysters bioaccumulated MNV-1 (Table 1). Similarly, more than 50% of all individual *C. ariakensis* oysters tested positive for NoV and HAV (Table 1). Overall, *C. virginica* oysters did not test positive for viruses as frequently as *C. ariakensis*. MNV-1, NoV and HAV were detected in 14 (58%), 4 (17%) and 7 (29%) of *C. virginica* oysters, respectively (Table 1). PV and MS2 were minimally bioaccumulated by *C. virginica* and *C. ariakensis* oysters (Table 1).

The number of viable transmissive stages (i.e. oocysts, cysts or spores) found in total pools of oyster gills and haemolymph at each sample day by oyster species is outlined in Fig. 2. On day 1 (i.e. 24 h after bioaccumulation), pools of oyster haemolymph and gills tested positive for all protozoan and microsporidian species (Fig. 2). *Crassostrea ariakensis*, however, harboured three times as many *G. lamblia* and *E. bienersi*, 2.5 times as many *E. hellem* and 1.3 times as many *C. parvum*, compared to *C. virginica* oysters on day 1 (Fig. 2).

Table 2 documents the endpoint day of detection for each type of virus, microsporidia and protozoa. *Crassostrea ariakensis* oysters were positive for MNV-1, NoV or HAV at day 29. Overall, MS2 and PV were bioaccumulated to a lesser extent (Table 1) and detected only up to day 4 (Table 2). Like *C. ariakensis*, *C. virginica* were positive for MNV-1 and HAV at the last sample period, but NoV was detected only up to day 15 (Table 2).

Similarly, the same *C. ariakensis* oysters were positive for *G. lamblia*, *E. hellem* and *E. bienersi* for 22 days and *C. parvum* for 29 days of depuration (Table 2). *Crassos-*

Table 1 Overall number of oysters testing positive for each virus type

| Oyster species (n*) | No. (%) of oysters positive for virus type | | | | |
|---------------------------|--|-----------------|--------------------|--------|-------|
| | Mouse norovirus-1 | Human norovirus | Haepatitis A virus | MS2 | PV |
| <i>C. virginica</i> (24) | 14 (58) | 4 (17) | 7 (29) | 1 (4) | 0 (0) |
| <i>C. ariakensis</i> (24) | 21 (88) | 13 (54) | 18 (75) | 3 (13) | 1 (4) |

*n, total no. of oysters analysed. *C. virginica* oysters (n = 24) and *C. ariakensis* oysters (n = 24) were individually analysed.

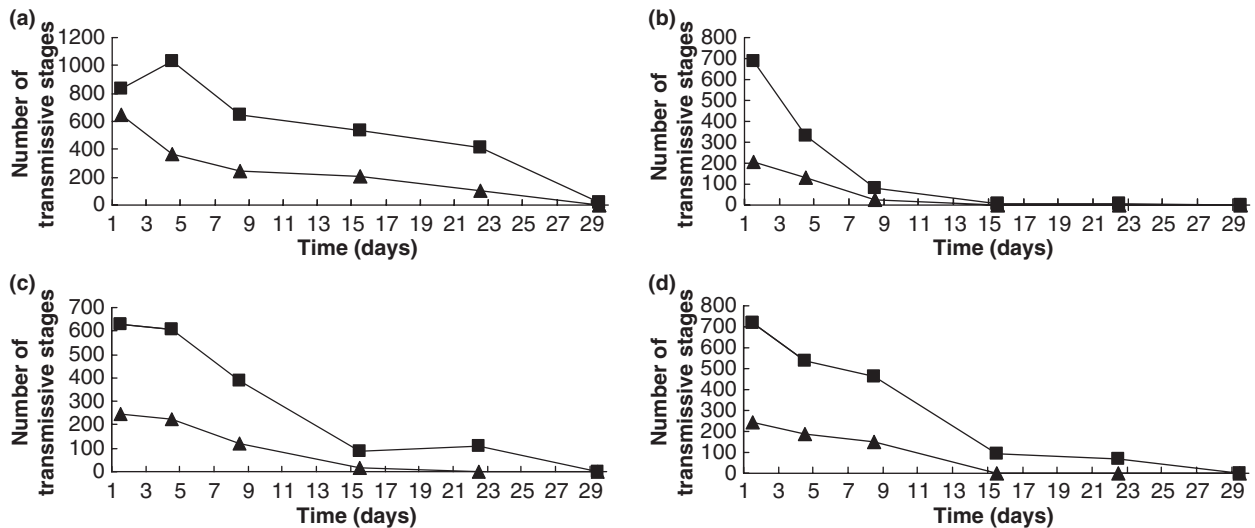


Figure 2 No. of viable transmissible stages (i.e. oocysts, cysts, or spores) found in oysters during 29 day depuration. Gill and hemolymph data were combined from *C. ariakensis* (n = 6) and *C. virginica* (n = 6) oysters at each time point. Day 1 equals 24 h after bioaccumulation. *C. ariakensis* (■); *C. virginica* (▲). (a) *C. parvum*; (b) *G. lamblia*; (c) *E. hellem*; (d) *E. bienersi*.

trrea virginica oysters depurated all protozoan and microsporidian species before the final day of depuration (Table 2). Oyster gills and haemolymph were analysed separately, but protozoan and microsporidian species were found in both organs at any given endpoint day of detection (Table 2).

Further, as evidenced by Fig. 2, protozoan and microsporidian species depurated at different rates. *Crassostrea ariakensis* depurated approximately one log₁₀ of *G. lamblia* during the first 8 days of depuration, one log₁₀ *E. hellem* and *E. bienersi* during 15 days of depuration and one log₁₀ *C. parvum* after 29 days (Fig. 2). As seen in *C. ariakensis* oysters, *C. parvum* persisted longer than any other protozoa or microsporidia in *C. virginica* oysters (day 22), and *G. lamblia* and *E. bienersi* were detected only up until day 8 and *E. hellem* until day 15 (Fig. 2 and Table 2).

Logistic regression models evaluated the odds of obtaining an oyster positive for viral RNA by oyster species, adjusting for time (Table 3). Models excluded PV and MS2 because of limited viral bioaccumulation. The odds of *C. ariakensis* harbouring viruses MNV-1, NoV or HAV were statistically greater than the odds of *C. virginica*

harbouring the same viruses, adjusting for time (MNV-1 OR = 5.05, *P* = 0.03; NoV OR = 6.97, *P* = 0.01; HAV OR = 7.40, *P* ≤ 0.001) (Table 3). Depuration time was not a significant predictor of a virus-positive oyster for HAV and MNV-1 (Table 3). However, time of depuration approached significance for predicting the presence of NoV. The chance of detecting a NoV-positive oyster decreased 7% (*P* = 0.06) with each additional day of depuration (Table 3).

Log-linear regression models were developed to evaluate the effect of oyster species, depuration time and oyster organ (gill vs haemolymph) on the number of detected transmissible stages of protozoa and microsporidia (Table 4). Transmissible stages detected in separate pools of gills and haemolymph from oysters (n = 72) were modelled. The adjusted model indicated that *C. ariakensis* harboured statistically significant higher (*P* < 0.001) transmissible stages of all protozoa or microsporidia, compared to *C. virginica* (Table 4). There was an estimated average log increase of 0.82, 1.13, 1.10 and 1.18 transmissible stages of *C. parvum*, *G. lamblia*, *E. hellem* and *E. bienersi* respectively, in pools of *C. ariakensis* oysters,

Table 2 Endpoint days of detection for enteropathogens in *C. ariakensis* and *C. virginica* oysters during depuration experiment

| Enteropathogen | Endpoint day of enteropathogen detection for indicated oyster species* | |
|--------------------|--|---------------------|
| | <i>C. ariakensis</i> | <i>C. virginica</i> |
| Virus* | | |
| Mouse norovirus-1 | 29 | 29 |
| Human norovirus | 29 | 15 |
| Haepatitis A virus | 29 | 29 |
| MS2 | 4 | ND |
| PV | 4 | 1‡ |
| Protozoa† | | |
| <i>C. parvum</i> | 29 | 22 |
| <i>G. lamblia</i> | 22 | 8 |
| Microsporidia† | | |
| <i>E. hellem</i> | 22 | 15 |
| <i>E. bienuesi</i> | 22 | 8 |

ND, not detected.

*Individual oyster digestive diverticula ($n = 48$) was tested for the presence of all five viruses.

†Gills and haemolymph were pooled separately from three oysters. Each pool ($n = 48$) was tested for the two protozoan and two microsporidian species. All protozoan and microsporidian species were found in both organs (gills and haemolymph) at every endpoint day of detection.

‡Day 1 is equivalent to 24 h after oysters were initially exposed to enteropathogens (bioaccumulation).

Table 3 Odds of a *C. ariakensis* oyster harbouring mouse norovirus-1 (MNV-1), human norovirus (NoV) or haepatitis A virus (HAV) compared to that for *C. virginica**

| Model covariate | Odds of detecting a virus type | | | | | |
|----------------------|--------------------------------|----------------|------|----------------|------|----------------|
| | MNV-1 | | NoV | | HAV | |
| | OR | <i>P</i> value | OR | <i>P</i> value | OR | <i>P</i> value |
| Oyster species | | | | | | |
| <i>C. virginica</i> | 1 | | 1 | | 1 | |
| <i>C. ariakensis</i> | 5.05 | 0.03 | 6.97 | 0.01 | 7.40 | <0.001 |
| Depuration time | 0.98 | 0.60 | 0.93 | 0.06 | 0.98 | 0.57 |

*A logistic regression model evaluated the odds of a virus-positive oyster. Each model was adjusted for oyster species and depuration time. Each *P* value indicates the significance of the odds of *C. ariakensis* compared to that of *C. virginica*.

compared to pools of *C. virginica* oysters. More protozoan and microsporidian were detected in the oyster haemolymph compared to in the oyster gills ($P < 0.05$) (Table 4). Further, time was a significant predictor (<0.001) of the detection of protozoan and microsporidian transmissive stages in all oyster pools (Table 4).

Oysters contain viral RNA from multiple virus types

Individually processed oysters were evaluated for the presence of five different virus types. According to Table 5, at least one virus was detected in 87.5% of all *C. ariakensis* oysters tested, compared to 66.7% of all tested *C. virginica* oysters. Table 5 also indicates that both species can retain at least four enteric viruses simultaneously, but unlike *C. virginica* oysters, a larger portion of *C. ariakensis* oysters harboured three (37.5%) virus types (Student's *t*-test: $P < 0.01$). Additionally, individual *C. ariakensis* oysters were positive for three virus types, even after 4 weeks of depuration (data not shown).

Discussion

We examined the rates at which two oyster species *C. ariakensis* and *C. virginica* bioaccumulated, retained and depurated nine different enteric pathogens or surrogates at 12 ppt salinity. The two oyster species were initially co-exposed to the viruses, protozoa and microsporidia for 24 h (i.e. bioaccumulation) and then allowed to depurate in tanks of clean, circulating 12 ppt seawater for 29 days. A study evaluating the two oyster species at three salinity ranges found significant mortality of *C. virginica* oysters at the lower salinity level of 8 ppt (Nappier *et al.* 2008). Additionally, viral bioaccumulation and retention of was poor for *C. virginica* oysters at the higher salinity levels of 20 ppt (Nappier *et al.* 2008). Thus, this experiment evaluated both oyster species only at the medium salinity level of 12 ppt. During the duration of the experiment, neither species experienced significant mortality (data not shown).

Individual oysters were tested for each of the five viruses, and pools of oyster gills and haemolymph were assessed for the protozoan and microsporidian species. Over 50% of *C. ariakensis* oysters harboured the human enteric viruses NoV and HAV, and 88% harboured the NoV surrogate MNV-1 (Table 1). *Crassostrea virginica* were positive for NoV, HAV and MNV-1 in 17, 29 and 58% of oysters respectively (Table 1). Because the data in Table 1 are based on samples taken during the entire experiment, the table offers conservative estimates of overall bioaccumulation, clearly illustrating the bioaccumulation efficiency of viruses by the *C. ariakensis* oyster species. Further statistical analyses revealed that the odds of *C. ariakensis* oysters harbouring MNV-1, NoV or HAV were significantly greater than the odds of *C. virginica* oysters harbouring the same viruses (MNV-1 OR = 5.05, $P = 0.03$; NoV OR = 6.97, $P = 0.01$; HAV OR = 7.40, $P < 0.001$) (Table 3). Similar odds ratios were calculated in a study evaluating the two oyster species in tanks separately (Nappier *et al.* 2008).

| Model covariate | Log-linear regression coefficients for indicated enteropathogen species | | | | | | | |
|----------------------|---|---------|-------------------|---------|------------------|---------|--------------------|---------|
| | <i>C. parvum</i> | | <i>G. lamblia</i> | | <i>E. hellum</i> | | <i>E. bienuesi</i> | |
| | β | P value | β | P value | β | P value | β | P value |
| Oyster species | | | | | | | | |
| <i>C. virginica</i> | 1 | | 1 | | 1 | | 1 | |
| <i>C. ariakensis</i> | 0.82 | <0.001 | 1.13 | <0.001 | 1.10 | <0.001 | 1.18 | <0.001 |
| Organ | | | | | | | | |
| Gills | 1 | | 1 | | 1 | | 1 | |
| Haemolymph | 0.15 | <0.001 | 0.07 | 0.05 | 0.61 | <0.001 | 0.64 | <0.001 |
| Depuration time | -0.09 | <0.001 | -0.29 | <0.001 | -0.14 | <0.001 | -0.15 | <0.001 |

*TS were detected in separate pools of gills and haemolymph from oysters ($n = 72$). Linear regression models were adjusted for oyster species (*C. ariakensis* vs *C. virginica*), organ (haemolymph vs gills) and depuration time. β , rate coefficient.

Table 5 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 | |
|---|--|-----------------------|
| | <i>C. ariakensis</i> * | <i>C. virginica</i> ‡ |
| 5 | 0 (0) | 0 (0) |
| 4 | 4 (16.7) | 1 (4.2) |
| 3 | 9 (37.5)† | 3 (12.5) |
| 2 | 5 (20.8) | 1 (4.2) |
| 1 | 3 (12.5) | 11 (45.8) |
| 0 | 3 (12.5) | 8 (33.3) |

**C. ariakensis* oysters ($n = 24$) were individually analysed.

†Individual *C. ariakensis* oysters were found to contain three viruses more frequently than *C. virginica* oysters (Student's *t*-test; $\alpha = 0.05$).

‡*C. virginica* oysters ($n = 24$) were individually analysed.

Likewise, Fig. 2 illustrates that *C. ariakensis* bioaccumulated protozoan and microsporidian species with greater efficiency. At day 1, *C. ariakensis* harboured three times as many *G. lamblia* and *E. bienuesi*, 2.5 times as many *E. hellem* and 1.3 times as many *C. parvum*, compared to *C. virginica* oysters (Fig. 2). The adjusted log-linear regression model also indicated that *C. ariakensis* harboured significantly higher transmissible stages of protozoa and microsporidia, compared to *C. virginica* ($P < 0.01$), adjusting for time (Table 4). This is the first study to directly compare pathogen bioaccumulation and retention by two oyster species when in tanks together. When food was placed in the tanks during bioaccumulation, oysters filtered water, competing for available energy resources. Pathogens, also present in the water, were likely adsorbed to the food particles and ingested during feeding. *Crassostrea ariakensis* oysters grow and filter water faster (Hudson *et al.* 2004), thus potentially ingesting more food and exposing themselves to more pathogens. However, it is unclear whether *C. ariakensis* oysters sensed the threat of another species and purposefully fil-

Table 4 Log-linear regression coefficients (β) for the number of protozoan and microsporidian transmissible stages (TS) found in pools of oyster gills and haemolymph from *C. ariakensis* and *C. virginica* oysters during depuration*

tered faster to compete for available food resources. Regardless, the concentration of viral, microsporidian and protozoan human pathogens and surrogates from surrounding waters provides evidence of an increased public health threat from the consumption of *C. ariakensis* oysters.

Further, all oysters were allowed to depurate in clean, circulating seawater for 29 days. Overall, depuration time was not a significant predictor of a virus-positive oyster (Table 3). *Crassostrea ariakensis* and *C. virginica* oysters both retained MNV-1 and HAV for 29 days. NoV was present in *C. ariakensis* oysters at day 29, but was more quickly depurated by *C. virginica*, in which NoV was only detected until day 14 (Table 2). As seen in previous studies, depuration is ineffective in virus removal from oysters (Sobsey and Jaykus 1991; Schwab *et al.* 1998; Koopmans *et al.* 2002). Previous studies of depuration indicated that rates of virus removal can vary by oyster species, virus type, depuration system (static or flowing) and temperature (Sobsey and Jaykus 1991).

Similarly, *C. ariakensis* harboured protozoan and microsporidian species longer than *C. virginica*, with viable *C. parvum* still present at 29 days (the last day of depuration) (Table 2). Viable *G. lamblia* and *E. bienuesi* were present almost three times longer in *C. ariakensis* than in *C. virginica* oysters. The present study supports previous findings that oysters inefficiently depurate *Cryptosporidium* (Gomez-Couso *et al.* 2003; Graczyk *et al.* 2006). Depuration time did significantly affect protozoan and microsporidian loads in oysters (Table 4), and Fig. 2 illustrates progressively decreasing pathogen levels in oyster tissue over time. Regardless, protozoa and microsporidia were still present after 3 weeks in oysters, indicating depuration is not a realistic means of purifying shellfish. Additionally, the protozoan and microsporidian recovery technique is not 100% efficient. A previous study of protozoan recovery indicated that the processing method may only recover protozoa with approx. 50% extraction efficiency (Graczyk *et al.* 1999), indicating that

numbers of reported viable protozoa and microsporidia in the present study are potentially underestimated.

This is the first demonstration of *C. virginica* bioaccumulation and depuration of microsporidia. In our study, *E. hellem* and *E. bienersi* were retained for 22 days by *C. ariakensis*, but only 15 and 8 days by *C. virginica* respectively. Previous findings indicate that *C. ariakensis* may harbour the microsporidia species in their haemolymph for up to 27 days at salinities between 12 and 20 ppt (Graczyk *et al.* 2006). In the present study, statistical analyses revealed that there were significantly greater microsporidia spores in the oyster haemolymph compared to the oyster gills ($P = 0.02$) (Table 4). This information is useful for future studies of microsporidia in oysters, as haemolymph is easier to extract and process for spore detection. The ultimate fate of microsporidia bioaccumulated by oysters is unknown, but it has been postulated that they are digested by haemocytes, present in the haemolymph (Graczyk *et al.* 2006).

To our knowledge, no other studies have looked at the ability for oysters to harbour protozoa, microsporidia and viruses simultaneously. Individual oysters were tested for viruses, and the same oysters' gills and haemolymph were pooled to identify protozoa and microsporidia. Because the same oysters were tested for protozoa and microsporidia, we can infer that some oysters were positive for viruses, microsporidian and protozoan species. Additionally, individual *C. ariakensis* oysters tested positive for three virus types more frequently than *C. virginica* oysters ($P < 0.05$) (Table 5). Over half (54.2%) of the tested *C. ariakensis* oysters harboured between three and four virus types, indicating that *C. ariakensis* may serve as a vehicle for multiple infectious agents. This study highlights the importance of oysters to act as a vehicle for the transmission of human pathogens and provides useful information for the risk assessment of foodborne infections related to the consumption of contaminated *C. ariakensis* oysters.

Further, the human NoV surrogate MNV-1 was bioaccumulated and retained by both oyster species more frequently than any other virus. According to our study, MNV-1 is a promising enteric pathogen surrogate, because it was present when enteric pathogens were present and detected longer or more often than enteric pathogens, an attribute important for conservative public health estimates of pathogen degradation. Before *C. ariakensis* are introduced, MNV-1 should be used to further assess inactivation of enteric pathogens in the presence of other oyster sanitation techniques, such as relaying, cooking or high pressure processing. The purpose of this study was to directly compare detection of viral RNA from five virus types by qRT-PCR in oyster bioaccumulation and depuration trials. Future research should be undertaken

to recover infectious MNV-1 viral particles from oysters and evaluate the ability of the two oyster species to bioaccumulate and retain infectious virus particles.

The present study revealed that multiple enteric pathogens and surrogates can be bioaccumulated and retained by both *C. ariakensis* and *C. virginica*. Overall, however, *C. ariakensis* were statistically more likely to harbour enteric pathogens and surrogates, compared to *C. virginica* oysters. *Crassostrea ariakensis* also bioaccumulated pathogens and surrogates more efficiently and retained them longer than *C. virginica* oysters.

As seen previously (Nappier *et al.* 2008), depuration time did not significantly affect the removal of MNV-1 or the human viruses, NoV and HAV from oysters. While depuration did reduce protozoan and microsporidian loads in oysters, all species were present after 3 weeks of depuration in *C. ariakensis* oysters. Depuration is not an effective oyster sanitation practice, and new methods should be explored.

Bioaccumulation, retention and depuration of microsporidia and protozoa have not been extensively evaluated in oysters. This study revealed that microsporidian and protozoan species were found in statistically greater concentrations in pools of the oyster haemolymph, compared to the oyster gills (Table 4).

While the introduction of the *C. ariakensis* oysters may provide ecological services by efficiently removing infectious human waste from surface waters, they may subsequently increase the public health threat associated with the nonnative oysters, which are intended for human consumption. According to this and other recent work, *C. ariakensis* oysters are more tolerable to salinity ranges (Calvo *et al.* 2001); grow and filter water faster (Hudson *et al.* 2004); bioaccumulate and retain viral, microsporidian and protozoan pathogens longer (Graczyk *et al.* 2006) and exhibit an inability to depurate some pathogens compared to the native *C. virginica*. Prior to the species introduction, the Chesapeake Bay region should fully consider the public health risks of the consumption of *C. ariakensis* oysters. If *C. ariakensis* oysters were introduced, we would recommend routine testing of oysters and their harvesting waters for human pathogens and faecal indicators. 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.

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