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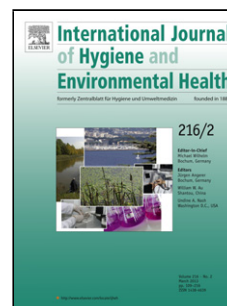
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# Bioaccumulation and persistence of faecal bacterial and viral indicators in *Mytilus edulis* and *Crassostrea gigas*

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## Abstract

This study investigated the response of two shellfish species - mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*) to microbial contamination in order to gain a better understanding of the bioaccumulation and persistence of microorganisms under controlled conditions. *M. edulis* and *C. gigas* were placed in sterile laboratory-prepared artificial seawater and initial tests were carried out to ensure both faecal indicator bacteria (FIB) and bacteriophages were below detection limits. FIB and phages were isolated, purified and dosed into experimental tanks containing the shellfish species. The GB124 phages were bioaccumulated to the highest concentration in *M. edulis* (1.88 log<sub>10</sub>) and *C. gigas* (1.27 log<sub>10</sub>) after 24 hrs. Somatic coliphages were bioaccumulated to the highest concentration in both *M. edulis* (4.84 log<sub>10</sub>) and *C. gigas* (1.73 log<sub>10</sub>) after 48 hrs. F-RNA phages were bioaccumulated to the highest concentration in *M. edulis* (3.51 log<sub>10</sub>) after 6 hrs but were below detection limit in *C. gigas* throughout the exposure period. *E. coli*, faecal coliforms and intestinal enterococci were bioaccumulated to the highest concentrations in *M. edulis* (5.05 log<sub>10</sub>, 5.06 log<sub>10</sub> and 3.98 log<sub>10</sub>, respectively) after 48 hrs. In *C. gigas*, *E. coli* reached a maximum concentration (5.47 log<sub>10</sub>) after 6 hrs, faecal coliforms (5.19 log<sub>10</sub>) after 12 hrs and intestinal enterococci (3.23 log<sub>10</sub>) after 24 hrs. *M. edulis* bioaccumulated phages to a greater extent than the faecal bacteria, and in both shellfish species, faecal bacteria persisted for longer periods over 48 hrs than the phages. This study highlights significant variation in the levels and rate of accumulation and persistence with respect to both shellfish species and the indicators used to assess risk. The results suggest that phage-based indicators could help elucidate risks to human health associated with pathogenic organisms.

## Introduction

Shellfish are filter-feeding aquatic animals that can bioaccumulate pathogens from contaminated water, thereby posing a potential risk of infection to human consumers. Outbreaks of shellfish-related infections have been reported in many parts of the world and most reports have involved oysters, clams, mussels and cockles<sup>17</sup>. The biological property of the shellfish tissues influences the pattern of accumulation of different pathogens observed in different shellfish species<sup>16</sup>. Several studies have shown that bioaccumulation of pathogens in shellfish (either experimentally under controlled conditions, or naturally in their growing waters) is dependent on a number of factors; including physiology of the shellfish and/or pathogen, the rate of metabolism in the shellfish, the duration of exposure of the shellfish to the pathogen or its source, the exposure dose, and other factors, such as salinity and temperature, that can also influence the filtration rate in the shellfish<sup>5,12,13</sup>. There appears to be a differential selection process involved in bioaccumulation, based on the size and shape of particles, which is influenced by the bioaccumulation efficiency of the shellfish species as well as the ability of the labial palp and gills to reject or filter certain particles<sup>3,26</sup>.

Several research studies have been undertaken across a variety of geographic areas in recent years to evaluate the presence of microorganisms within shellfish<sup>1,6,13,20,26</sup>. Enteric viruses have been detected in shellfish harvested from areas classified as ‘Category A’ status according to EU standards<sup>11</sup>, suggesting that the current hygiene classifications for shellfisheries, based on levels of bacterial indicators, may not offer an indication of risk of enteric viral pathogens. Several studies have proposed bacteriophages as alternative indicators of enteric viral contamination<sup>2,7,8,15,18,24</sup>. In this study, the parameters investigated in *C. gigas* and *M. edulis* were *E. coli*, faecal coliforms, intestinal enterococci, somatic

coliphages and F-RNA coliphages. The variables examined included exposure time, salinity and temperature.

The aim of this laboratory-based study was to evaluate the bioaccumulation rate and retention of bacterial and viral indicators (bacteriophages) in shellfish under controlled conditions. The specific objectives of the experiments were first to determine the rate of bioaccumulation in *M. edulis* and *C. gigas* when inoculated with artificially-dosed faecal indicator bacteria and bacteriophages under differing conditions (salinities and temperatures) and, secondly, to examine the persistence of these microorganisms in the shellfish under differing conditions and with respect to time.

The research described here evaluates for the first time the bioaccumulation of human-specific *B. fragilis* phages GB124 in *C. gigas*.

## Materials and methods

Clean polyethylene fish tanks (Fisher Scientific), measuring 310 mm (length) by 210 mm (width) by 230 mm (height), with a working volume of 12 litres, were prepared and placed in the laboratory at room temperature. Stainless test tube racks (Fisher Scientific) were placed at the bottom of each tank to act as a support structure for the shellfish and to avoid recontamination by voided faecal indicator bacteria and bacteriophages.

During the initial trials, *M. edulis* were obtained from the estuary of the estuary River Ouse in southeast England and freshly collected *C. gigas* were obtained from a ready-to-sell outlet in Shoreham-by-Sea, West Sussex, England. All shellfish were transported to the laboratory within one hour and transferred to tanks (A – F) containing laboratory-prepared artificial seawater (17.15 g NaCl, 4.18 g MgSO<sub>4</sub>, 3.37 g MgCl<sub>2</sub>, 0.87 g CaCl<sub>2</sub>, 0.44 g KCl)<sup>27</sup> at room temperature. Bubble stones connected to aquarium airlines and fixed to an air pump (Tetratec<sup>®</sup> APS100) were placed in the experimental tanks for aeration. The air pump is suitable for tank sizes up to 100 litres and provides a continuous flow of air at a rate of 100 litres per hour. The tanks were left for a period of two days to allow the shellfish to acclimatise to their new environment.

### Isolation, purification and propagation of faecal indicator bacteria

Colonies of *E. coli*, faecal coliforms and intestinal enterococci on Tryptone Bile Glucuronide (TBX) agar, Membrane Faecal Coliform ‘m-FC’ agar and Membrane *Enterococcus* ‘m-Ent’ agar (Difco, BDMS, UK) respectively, were subcultured repeatedly in order to obtain pure cultures of each bacterium. Isolates were picked aseptically, using sterile inoculating loops, and introduced into freshly prepared single-strength Modified Mineral Glutamate Broth (ssMMGB) and incubated at 37 °C for a further 24 hrs. The concentration of the bacterial

cultures was determined by serial dilution in ¼ strength Ringer's solution and plated using TBX agar, *m*-FC agar and *m*-Ent agar incubated appropriately to ensure that a cell density of approximately  $1 \times 10^8$  colony-forming units per millilitre (CFU/ml) was achieved. The high inoculum density was required to allow for those bacteria that would be voided when introduced into the tanks.

### **Isolation, purification and propagation of phages**

Clear, distinct plaques (zone of lysis) of somatic coliphages, F-RNA coliphages and bacteriophages infecting *B. fragilis* GB124, on Modified Scholtens' Agar (MSA), Tryptone-Yeast Extract Glucose Agar (TYGA) and *Bacteroides* Phage Recovery Medium Agar (BPRMA) plates, respectively, were picked using sterile glass Pasteur pipettes for isolation, propagation and purification using previously described methods<sup>4,24</sup>. In brief, the agar cores, containing distinct single plaques were suspended in 400 µl of phage buffer (19.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 85.5 mM NaCl, 1 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>) in microcentrifuge tubes (Fisher Scientific, UK). The phage suspensions were incubated at 4 °C for at least four hrs, to allow complete diffusion of phage into the buffer, mixed gently and then filtered through 0.22 µm membrane cellulose nitrate filters (Millipore). The phage suspensions were then diluted serially in phage buffer (up to ten times) and retested using the standardised double agar-layer method (ISO 10705). The process of phage isolation and analysis was repeated three times by selecting phages on plates containing well-spaced zones of lysis in order finally to obtain a high titre (approximately  $1 \times 10^6$  plaque-forming units per millilitre – PFU/ml) purified phage.

To concentrate the purified phages, 5 ml of phage buffer were added to plates showing near complete lysis of the host bacterium, and left for at least one hour at room temperature with

occasional swirling to allow diffusion of phage into the buffer. Thereafter, the top agar-layer and the liquid were scraped into a 50 ml centrifuge tube, mixed using a vortex mixer (Fisherbrand) and left at room temperature for 30 mins. The agar-layer was removed from the suspension by centrifugation at 2000 g for 15 mins. Finally, the supernatant was filtered through a 0.22 µm membrane cellulose nitrate filter (Millipore), labelled as ‘stock’ and stored in light-tight glass tubes in the dark at 4 °C for no longer than 6 months. The phage ‘stock’ was diluted serially in phage buffer and retested using the standardised double agar-layer method (ISO 10705) to determine the titre of the stock and to ensure that a titre of  $1 \times 10^8$  PFU/ml was achieved. Again, the high phage density was required to allow for those that would be voided when introduced into the tanks.

### **Design of bioaccumulation study**

A lower temperature of approximately 8 °C, achieved using a cooled incubator (LMS, UK), an ‘optimal’ (room) temperature of approximately 20 °C and an upper temperature of approximately 24 °C, achieved using 200 W submersible aquarium fish tank heater (XiLong, China), were selected to represent the range of water temperatures commonly observed in temperate river catchments, such as the R. Ouse in East Sussex, UK.

A lower salinity of approximately 5 ppt, an ‘optimal’ salinity of approximately 16 ppt and an upper salinity of approximately 25 ppt were selected to represent the range of water salinities commonly observed at points along temperate river catchments, such as the R. Ouse, UK (Supplementary Table 1). In brief, 47.02 g NaCl, 11.48 g MgSO<sub>4</sub>, 9.10 g MgCl<sub>2</sub>, 2.38 g CaCl<sub>2</sub> and 1.12 g KCl were dissolved in 10 litres of distilled water to achieve approximately 5 ppt artificial seawater; 117.60 g NaCl, 28.70 g MgSO<sub>4</sub>, 22.80 g MgCl<sub>2</sub>, 5.95 g CaCl<sub>2</sub> and 2.80 g KCl were dissolved in 10 litres of distilled water to achieve



approximately 16ppt artificial seawater; and 210 g NaCl, 50 g MgSO<sub>4</sub>, 40 g MgCl<sub>2</sub>, 10 g CaCl<sub>2</sub> and 4 g KCl were dissolved in 10 litres of distilled water to achieve approximately 25 ppt artificial seawater.

The time increments and overall duration of bioaccumulation of faecal indicator bacteria and phages in the shellfish matrices were based in part on previous findings<sup>6</sup>, where variations were observed in the bioaccumulation efficiency of viral pathogens in different shellfish species using a maximal theoretical bioaccumulation (MTB) calculation. These authors reported the highest bioaccumulation efficiencies after 1hr in mussels and, after 24 hrs in oysters and flat oysters. In this study, a slight modification was made in the time selected for analysis, as the shellfish and artificial seawater in tanks were examined prior to commencement of the dosing experiments (zero hours) to ensure the absence of background faecal contamination, which could have affected the results. Background levels of contamination in all shellfish and artificial seawater samples were found to be below the at zero hours. The detection limits for faecal indicator bacteria in shellfish flesh were 20 MPN/100 g and 1 CFU/100 ml for overlying waters; and for phages these were 100 PFU/100 g in shellfish digestive gland and 100 PFU/100 ml in overlying waters). Microbiological analyses were undertaken after 6, 12 and 24 hrs to determine the rate of bioaccumulation of the indicators in the shellfish while analyses after 48 and 96 hrs were undertaken to examine the pattern of persistence of the indicators in the shellfish. Figure 1 presents a schematic diagram of the bioaccumulation study.

Controls – Positive and negative control tanks (E and F) were also run in parallel and both were maintained at an optimum salinity of approximately 16 ppt and temperature of approximately 20 °C. These optimum conditions were based on previous findings<sup>22</sup>, in which

variation was observed in filtration rates in shellfish when the temperature was reduced below an optimum temperature (20 °C) to 10 °C. In fact, *C. gigas*, *O. edulis* and *M. edulis* demonstrated 25 %, 45 % and 25 % reductions in filtration rate, respectively. Similarly, another study<sup>23</sup> observed that increasing salinity above 15 ppt or reducing it below 15 ppt was detrimental to the survival of faecal coliforms in seawater. The positive control tank contained artificial seawater and shellfish dosed with faecal indicator bacteria and phages, whereas the negative control tank contained only artificial seawater and shellfish.

### **Dosing experimental tanks with faecal indicator bacteria and phages**

A dosing volume of 2.5 ml for each of the six microorganisms commonly observed in faecally impacted waters (*E. coli*, faecal coliforms, intestinal enterococci, somatic coliphages, F-RNA coliphages and bacteriophages infecting *B. fragilis* GB124) was added to experimental tanks (volume of water in each tank was 10 litres) containing *C. gigas* (15-18 live oysters) or *M. edulis* (30 live mussels).

### **Enumeration of dosed faecal indicator bacteria and phages in shellfish**

On each occasion, three *C. gigas* and five *M. edulis* were removed from the experimental tanks at set intervals (6, 12, 24, 48, 96 hrs) and processed. The flesh and the intravalvular fluids were assessed for *E. coli*, faecal coliforms and intestinal enterococci using the MPN methods mentioned above, while the digestive glands were assessed for somatic coliphages, F-RNA coliphages and bacteriophages infecting *B. fragilis* GB124 using a standardised double agar-layer method (ISO 10705).

**Mortality rate of *M. edulis* and *C. gigas***

The experimental tanks were examined at regular intervals. Those *M. edulis* and *C. gigas* with shells that did not close tightly when tapped or agitated, with gills retracted, were removed from the experimental tanks and recorded.

**Statistical analysis**

The mean values of faecal indicator bacteria and bacteriophages observed in shellfish and overlying waters were subjected to descriptive statistics using SPSS Statistics Version 20.0. The relationship between temperature, salinity and mean values of bioaccumulated microbial indicators in mussels and oysters in the laboratory-based bioaccumulation experiment were analysed using a Pearson's correlation matrix.

## Results

After three hours of bioaccumulation, *M. edulis* and *C. gigas* in tanks B (high salinity, high temperature), D (high salinity, low temperature), E (positive control) and F (negative control) were observed to be open (with all their gills out) and were filtering more water than those in tanks A (low salinity, high temperature) and C (low salinity, low temperature) (i.e., the filtering activities exhibited by shellfish in higher salinity artificial seawater were greater compared to those in lower salinity artificial seawater). This is likely to account in part for the variation observed in the bioaccumulation efficiency of the shellfish.

After 48 hrs, the artificial seawater in experimental tanks containing *C. gigas* at higher temperatures (A and B), including positive control tank (E) turned turbid, with the oysters displaying visible signs of poor health (i.e., the valves were not tightly closed and there was an observed reduction in filtration rate due to non-active gills), whereas the artificial seawater in experimental tanks C and D (at low temperatures) remained clear. Conversely, artificial seawater in experimental tanks at low temperatures (C and D) and high temperatures (A and B) remained clear and the *M. edulis* appeared to be in good health (i.e., valves were tightly closed) throughout the duration of the experiment.

## Bioaccumulation and persistence of faecal bacteria

### *E. coli*

*E. coli* were bioaccumulated to the highest maximum and mean concentrations ( $6.26$  and  $4.92 \pm 2.51 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ , respectively) in *M. edulis* 48 hrs from time zero in tank D (high salinity, low temperature) containing artificial seawater at 25 ppt salinity and bioaccumulated to the lowest level ( $2.69 \pm 1.59 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ ) in the positive control tank E (Supplementary Figure 1).

*E. coli* were bioaccumulated to the highest maximum and mean concentrations ( $6.26$  and  $4.52 \pm 2.34 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ , respectively) in *C. gigas* 6 hrs from time zero in 100 g) in tank D (high salinity, low temperature) (Table 2), which contained artificial seawater at 25ppt salinity and were bioaccumulated to the lowest level ( $3.92 \pm 1.98 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ ) in tank C (low salinity, low temperature) and positive control tank E. The pattern of persistence of *E. coli* in *M. edulis* between 48 and 96 hrs showed a mean reduction from  $5.05$  to  $3.96 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ , but increased in *C. gigas* from  $4.42$  to  $4.65 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$  (Supplementary Figure 1). *C. gigas* bioaccumulated *E. coli* to slightly higher densities compared with *M. edulis* (Figure 2).

### **Faecal coliforms**

Faecal coliforms were bioaccumulated to the highest maximum concentration ( $6.26 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ ) in tanks C and D in *M. edulis* 48hrs from time zero (Table 1), but the highest mean concentration ( $4.92 \pm 2.51 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ ) was observed in tank D (high salinity, low temperature) containing artificial seawater at 25 ppt and the lowest mean ( $2.81 \pm 1.63 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ ) was observed in the positive control tank E (Supplementary Figure 2).

Faecal coliforms were bioaccumulated to the highest concentration ( $5.96 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ ) in tanks B (high salinity, high temperature) and D (high salinity and low temperature) in *C. gigas* 12 hrs from time zero (Table 2). The highest mean concentration ( $4.35 \pm 2.19 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ ) was observed in tank D (high salinity, low temperature) and the lowest mean concentration ( $3.84 \pm 1.89 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ ) was observed in the positive control tank E. The pattern of persistence of faecal coliforms in *M. edulis* between 48 and 96hrs showed a mean reduction from  $5.06$  to  $4.09 \text{ Log}_{10} \text{ CFU}/100 \text{ g}$ , but increased in *C. gigas* from  $4.53$  to

4.83 Log<sub>10</sub> CFU/100 g. *C. gigas* bioaccumulated faecal coliforms to slightly higher densities compared with *M. edulis* (Figure 2).

### **Intestinal enterococci**

Intestinal enterococci were bioaccumulated to the highest maximum and mean concentration (4.36 and  $3.46 \pm 1.70$  Log<sub>10</sub> CFU/100 g, respectively) in *M. edulis* 48 hrs from time zero in tank D (high salinity, low temperature) (Table 1), and were bioaccumulated to the lowest concentration ( $2.29 \pm 1.33$  Log<sub>10</sub> CFU/100 g) in tank B (high salinity, low temperature) (Supplementary Figure 3).

Intestinal enterococci were bioaccumulated to the highest maximum and mean concentration (3.60 and  $2.55 \pm 1.32$  Log<sub>10</sub> CFU/100 g, respectively) in *C. gigas* 24 hrs from time zero in tank C (low salinity, low temperature) (Table 2) and were bioaccumulated to the lowest concentration ( $2.14 \pm 1.07$  Log<sub>10</sub> CFU/100 g) in the positive control tank E. The pattern of persistence of intestinal enterococci in *M. edulis* between 48 and 96 hrs showed a mean reduction from 3.98 to 3.29 Log<sub>10</sub> CFU/100 g, and in *C. gigas* showed a mean reduction from 3.00 to 2.91 Log<sub>10</sub> CFU/100 g. *M. edulis* bioaccumulated intestinal enterococci to slightly higher densities compared with *C. gigas* (Figure 2).

## **Bioaccumulation and persistence of bacteriophages**

### **Bacteriophages infecting *Bacteroides fragilis* GB124**

Human-specific *B. fragilis* GB124 phages were bioaccumulated to the highest concentration in *M. edulis* 24 hrs from time zero (Table 1), but the highest mean concentration ( $2.45 \pm 1.21$  Log<sub>10</sub> PFU/100 g) was observed in the positive control tank containing artificial seawater at 16ppt salinity and the lowest concentration ( $1.93 \pm 1.50$  Log<sub>10</sub> PFU/100 g) was observed in

tank B (high salinity, high temperature). However, GB124 phages were below their detection limit in *M. edulis* in tanks C and D throughout the experiment. The maximum concentration of GB124 phages in *M. edulis* ( $3.26 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ ) was observed in tank A (low salinity, high temperature). This suggests that lower salinities may be favourable to the bioaccumulation of human-specific GB124 *B. fragilis* phages in *M. edulis*. After 96 hrs, the human-specific *B. fragilis* GB124 were no longer isolated from *M. edulis* in tanks A and B, whereas those in tanks E still contained the phages (Supplementary Figure 4).

In *C. gigas*, GB124 phages were also bioaccumulated to their highest concentration after 24 hrs (Table 2). However, this time the highest mean concentration of  $0.54 \pm 1.33$  was observed in tank B (high salinity, high temperature) and the lowest concentration ( $0.46 \pm 1.14$ ) was observed in tank D (high salinity, low temperature). GB124 phages were below their detection limit in *C. gigas* in tanks C and E throughout the experiment. The maximum concentration of GB124 phages in *C. gigas* ( $3.26 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ ) was also observed in tank B (high salinity, high temperature). The pattern of persistence of GB124 phages in *M. edulis* between 48 and 96 hrs showed a mean reduction from 1.73 to  $0.56 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ , and was below their detection limit in *C. gigas* during this period. *M. edulis* bioaccumulated GB124 phages to higher densities compared with *C. gigas* (Figure 2).

### **Somatic coliphages**

Somatic coliphages were bioaccumulated to the highest concentration in *M. edulis* after 48 hrs of contamination (Table 1), but the highest mean concentration ( $4.03 \pm 1.98 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ ) was observed in tank C (low salinity, low temperature) and the lowest concentration ( $3.68 \pm 1.81 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ ) was observed in tank A (low salinity, high temperature). The maximum concentration of somatic coliphages ( $5.03 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ )

was also observed in mussels from tank C (low salinity, low temperature) (Supplementary Figure 5).

In *C. gigas*, somatic coliphages were bioaccumulated to their highest concentration after 48 hrs (Table 2). The highest mean concentration ( $1.39 \pm 1.52 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ ) was observed in tank C (low salinity, low temperature) and the lowest mean concentration ( $0.51 \pm 1.26 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ ) was observed in tank D (high salinity, low temperature). The maximum concentration of somatic coliphages in *C. gigas* was observed in tank B (high salinity, high temperature). Concentrations of somatic coliphages in *M. edulis* between 48 and 96 hrs showed a mean reduction from 4.84 to 4.61  $\text{Log}_{10} \text{ PFU}/100 \text{ g}$ , and concentrations in *C. gigas* showed a mean reduction from 1.73 to 1.21  $\text{Log}_{10} \text{ PFU}/100 \text{ g}$ . *M. edulis* bioaccumulated somatic coliphages to higher densities compared with *C. gigas* (Figure 2).

### **F-RNA coliphages**

F-RNA coliphages were bioaccumulated to their highest concentration in *M. edulis* after 6 hrs (Table 1), but the highest mean concentration ( $2.99 \pm 1.52 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ ) was observed in the positive control tank E containing artificial seawater at 16 ppt salinity and 20 °C and the lowest mean concentration ( $0.98 \pm 1.52 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ ) was observed in tank C (low salinity, low temperature). The maximum concentration of F-RNA coliphages ( $3.82 \text{ Log}_{10} \text{ PFU}/100 \text{ g}$ ) was again observed in tank E. Concentrations of F-RNA coliphages in *M. edulis* between 48 and 96 hrs showed a mean reduction from 3.47 to 1.73  $\text{Log}_{10} \text{ PFU}/100 \text{ g}$  (Supplementary Figure 6).



F-RNA coliphages were below their detection limit in *C. gigas* in tanks A, B, C, D and E throughout the entire experiment. *M. edulis* bioaccumulated F-RNA coliphages to higher densities compared with *C. gigas* (Figure 2).

### **Mortality rate of *M. edulis* and *C. gigas***

In experimental tanks containing *M. edulis*, the total numbers of organisms that died after 96 hrs of exposure were 12 and 4 (representing percentage mortality rates of 40 % and 13 %) in tanks A (low salinity, high temperature) and B (high salinity, high temperature), respectively (Supplementary Figure 7). A common feature of tanks A and B was their high water temperature (approx. 24 °C), and this may be one of the factors responsible for the relatively high mortality rate recorded in these tanks. No shellfish mortality was recorded in tanks C and D, nor in the positive and negative control tanks. In experimental tanks containing *C. gigas*, the total number of organism deaths recorded after 96 hrs of exposure were 5, 5 and 5 (representing percentage mortality rates of 33 % each) in tanks A, B and E (Supplementary Figure 7). Similarly, a common feature of tanks A and B was their relatively high temperature (approx. 24 °C), and again this may be one of the factors responsible for the relatively high high mortality rates recorded in the tanks. No mortality was recorded in tanks C and D, nor in the negative control tank. In general, *M. edulis* and *C. gigas* in experimental tanks at the lower temperature (approx. 8 °C) survived better than those that experienced the higher temperature (approx. 24 °C).

## Discussion and conclusions

The rate of bioaccumulation and persistence of three faecal indicator bacteria (*E. coli*, faecal coliforms and intestinal enterococci) and three bacteriophages (somatic coliphage, F-RNA coliphages and bacteriophages infecting *B. fragilis* GB124) were examined in two shellfish species, namely oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*), in artificial seawater at low and high salinities (approx. 5 and 25 ppt) and low and high temperatures (approx. 8 and 24 °C) over a 96 hour exposure period. Previous shellfish bioaccumulation studies have predominantly evaluated bacteria<sup>14,20</sup>, protozoa<sup>26</sup>, enteric viral pathogens<sup>6,12</sup> and bacteriophages<sup>1,13</sup> in clams<sup>6</sup>, mussels<sup>6,14,20</sup> and oysters<sup>1,6,13,26</sup>. This study represents the first known attempt to investigate bioaccumulation of human-specific bacteriophages infecting *B. fragilis* GB124 in controlled laboratory experiments involving oysters. During the experiment, mussels and oysters demonstrated approximately 8 % and 13 % mortality rates, respectively. A common feature of experimental tanks in which mortality occurred was the elevated temperature (approx. 24 °C), which may be one of the factors responsible for elevated observed mortality rate. This may also be partly due to the fact that the shellfish species used in the experiment grew and were harvested from a temperate climate in which ambient temperatures rarely reach 24 °C, except occasionally during the summer periods.

All the mussels bioaccumulated *E. coli*, faecal coliforms, intestinal enterococci, somatic coliphages and F-RNA coliphages in all experimental tanks, including the positive control tank. The recorded levels of GB124 phages were below their detection limit in mussels in two experimental tanks at low and high salinities and both at low temperatures (approx. 8 °C). Although phages infecting *B. fragilis* have been demonstrated to be resistant to unfavourable conditions<sup>8</sup>, it may be that the low filtration activity of mussels at low temperature (approx. 8 °C) prevented the phages from being accumulated to detectable levels in this study. This

observation may also be due to changes in the physiology<sup>16</sup> of mussels, caused by the low temperature. This observation is contrary to the findings of previous studies that focused on the bioaccumulation of phages in mussels under natural conditions, in which GB124 phages were bioaccumulated to high levels during the spring and winter<sup>24</sup>. All oysters bioaccumulated *E. coli*, faecal coliforms, intestinal enterococci and somatic coliphages in all experimental tanks, including the positive control tank (in which salinity was approx. 16 ppt and temperature was approximate 20 °C). F-RNA coliphages were undetected in any oysters during this study. This differential selection process observed in oysters may likely be due to the ability of the labial palp and gills to reject certain microorganisms<sup>3</sup>. Again, GB124 phages were not detected in oysters from the tank containing artificial seawater at low salinity (approx. 5 ppt) and low temperature (approx. 8 °C). Changes in temperature and salinity, as well as other environmental factors, can affect the physiological state of oysters, altering their ability to feed, filter and bioaccumulate viruses<sup>12,21</sup>. In this study, statistical analysis revealed that bioaccumulated levels of somatic coliphages, F-RNA coliphages and GB124 phages were significantly greater in mussels than in oysters. Intestinal enterococci also followed this same pattern of bioaccumulation; in that they were observed to be higher in mussels than in oysters. However, *E. coli* and faecal coliforms exhibited a different pattern of bioaccumulation compared with the phages and intestinal enterococci, in that they were recorded at significantly greater levels in oysters than in mussels.

Many authors have highlighted the effect of temperature and salinity on physiological indicators (such as clearance and absorption efficiency) in shellfish species<sup>10,23</sup>. Another study observed that the clearance rate and absorption efficiency in mussels (*Perna perna*) exhibited inhibition at extreme salinities of 15 and 40 ppt<sup>19</sup>. In this study, the clearance rate of *E. coli*, faecal coliforms, intestinal enterococci, somatic coliphage, F-RNA coliphages and

bacteriophages infecting *B. fragilis* GB124 in mussels and oysters after 96 hrs exposure period varied. In addition, the maximum concentration of F-RNA coliphages was observed in tank E, which suggests that optimum salinities may be more favourable for the bioaccumulation of F-RNA coliphages in *M. edulis*.

The application of Pearson's correlation matrix revealed that, under laboratory-controlled conditions, temperature significantly influenced the bioaccumulation of all microbial indicators in mussels – *E. coli* (R = 0.895), faecal coliforms (R = 0.919), intestinal enterococci (R = 0.884), somatic coliphages (R = 0.924), F-RNA coliphages (R = 0.938), and GB124 phages (R = 0.936). In contrast, temperature was observed to have a minimal effect on the bioaccumulation of the microbial indicators in oysters – *E. coli* (R = 0.012), faecal coliforms (R = 0.086), intestinal enterococci (R = 0.389), somatic coliphages (R = 0.018), and GB124 phages (R = 0.117). These bioaccumulation results clearly demonstrate variation between shellfish species under identical laboratory conditions and support the findings of other authors<sup>1,6,13,14,26</sup>. The effect of salinity on the bioaccumulation of all microbial indicators in mussels and oysters under controlled conditions in the laboratory was minimal compared with the effect observed in studies into the bioaccumulation of faecal indicator bacteria and phages in shellfish under natural conditions<sup>24</sup>. This suggests that salinity does not drive the bioaccumulation process under laboratory conditions, but in the environment it likely to inversely relate to rainfall and the pollution inputs from land-runoff into surface waters.

The results from this laboratory-based study revealed that the rate of bioaccumulation and persistence of microbial indicators in shellfish under controlled conditions varies between the shellfish species, and also varies from what has been observed in the natural environment,

where shellfish species may be contaminated naturally during filter-feeding in contaminated estuarine or coastal waters. This work has elucidated the rate of bioaccumulation and persistence of faecal indicator bacteria and bacteriophages in mussels and oysters under controlled conditions and has demonstrated differing accumulation patterns of faecal indicator bacteria and bacteriophages two widely consumed shellfish species. The study provides the shellfisheries industry with new information that will inform future shellfish harvesting purification processes in order to reduce the incidence of shellfish-related infections. The significant variations in the levels and rates of bioaccumulation and persistence observed in this study, with respect to both indicator organisms and shellfish species, suggests that a ‘toolbox’ of approaches is required to assess potential risk to shellfish consumers more effectively. The findings support the suggestion that enumeration of bacterial indicators (such as *E. coli*) as prescribed widely by legislation, may not offer the best available indication of risk to human health from shellfish-borne infectious disease and that phage-based indicators may help to elucidate these risks.

## **Acknowledgment**

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## Figure Legends

Figure 1. Schematic diagram of bioaccumulation experiment

Figure 2. Boxplot of bioaccumulation of faecal indicator bacteria and bacteriophages in *M. edulis* and *C. gigas*. The median value is represented by a line inside the box, 95 % confidence intervals (bars).

Supplementary Figure 1. Bioaccumulation of *E. coli* in *M. edulis* (a) and *C. gigas* (b) in artificial seawater with respect to temperatures and salinity.

Supplementary Figure 2. Bioaccumulation of faecal coliforms in *M. edulis* (a) and *C. gigas* (b) in artificial seawater with respect to temperatures and salinity.

Supplementary Figure 3. Bioaccumulation of intestinal enterococci in *M. edulis* (a) and *C. gigas* (b) in artificial seawater with respect to temperatures and salinity.

Supplementary Figure 4. Bioaccumulation of bacteriophages infecting *Bacteroides fragilis* GB124 in *M. edulis* (a) and *C. gigas* (b) in artificial seawater with respect to temperatures and salinity.

Supplementary Figure 5. Bioaccumulation of somatic coliphages in *M. edulis* (a) and *C. gigas* (b) in artificial seawater with respect to temperatures and salinity.

Supplementary Figure 6. Bioaccumulation of F-RNA coliphages in *M. edulis* (a) and *C. gigas* (b) in artificial seawater with respect to temperatures and salinity.

Supplementary Figure 7. Mortality rate of *M. edulis* (a) and *C. gigas* (b) in artificial seawater with respect to temperatures and salinity.

Table 1. Summary of bioaccumulation and persistence of indicator organisms in mussels (*M. edulis*) with respect to temperature and salinity. Mean concentration after each exposure period  $\pm$  standard deviation  $\text{Log}_{10}$  PFU/100 g of shellfish digestive gland or  $\text{Log}_{10}$  CFU/100 g of shellfish intravalvular fluid and flesh; ND – Non detects (i.e.  $\leq$  detection limit); Figures in bold denote the highest mean concentration over exposure period and letters in parenthesis represent the tank in which highest values were recorded.

Table 2. Summary of bioaccumulation and persistence of indicator organisms in oysters (*C. gigas*) with respect to temperature and salinity. Mean concentration after each exposure period  $\pm$  standard deviation  $\text{Log}_{10}$  PFU/100 g of shellfish digestive gland or  $\text{Log}_{10}$  CFU/100 g of shellfish intravalvular fluid and flesh; ND – Non detects (i.e.  $\leq$  detection limit); Figures in bold denote the highest mean concentration over exposure period and letters in parenthesis represent the tank in which highest values were recorded.

Supplementary Table 1. Experimental design of bioaccumulation study. ppt – part per thousand.

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**Table 1: Summary of uptake, bioaccumulation and persistence of indicator organisms in mussels (*M. edulis*) with respect to temperature and salinity**

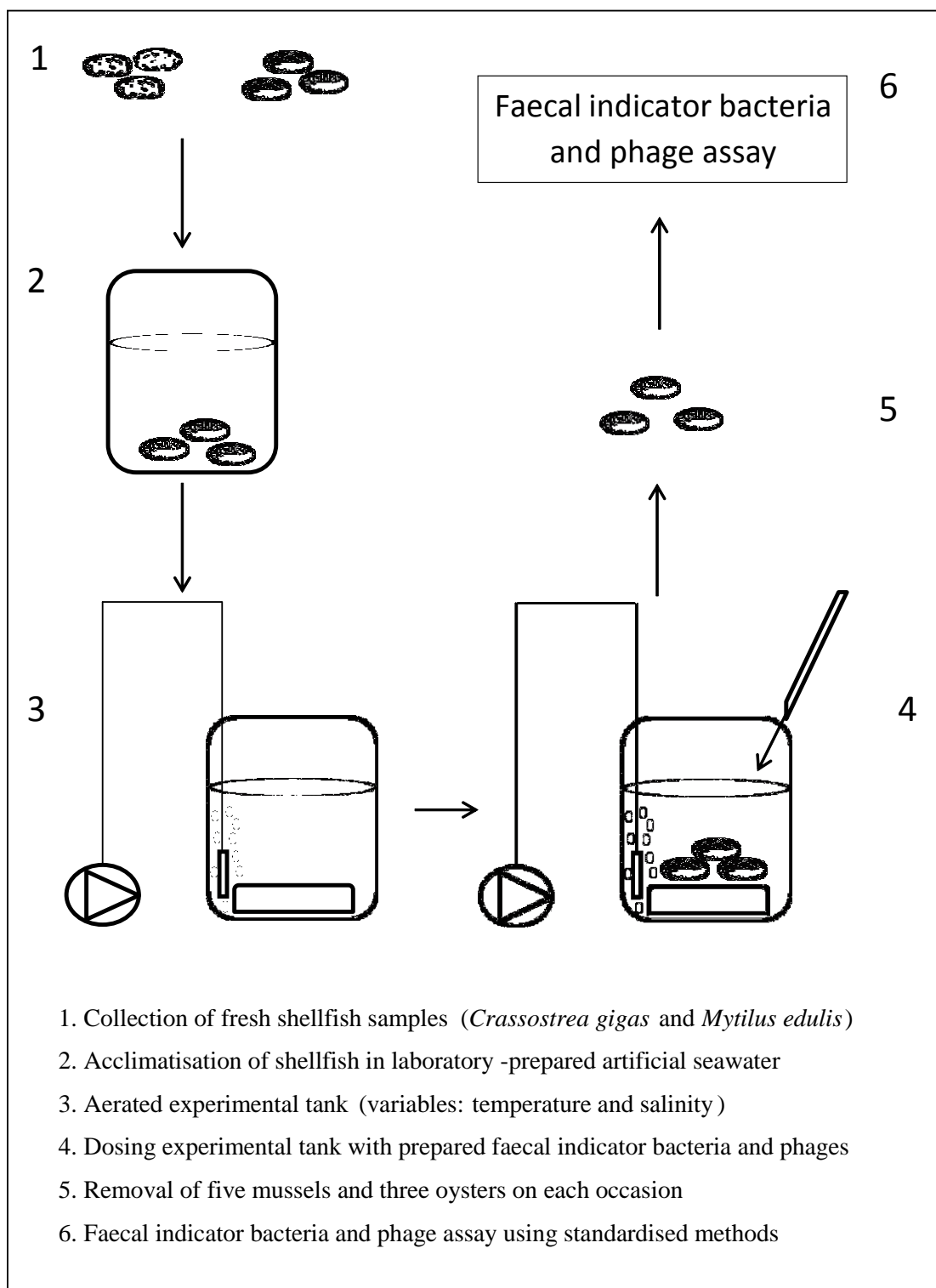
Indicator	Mean concentration Log <sub>10</sub> /PFUCFU/100g ± SD				
	Exposure period – hours (Tank)				
	6	12	24	48	96
GB124 phages	1.73±1.58	1.82±1.66	<b>1.88±1.72 (E)</b>	1.73±1.58	0.56±1.24
Somatic coliphages	4.61±0.10	4.57±0.16	4.55±0.27	<b>4.84±0.12 (C)</b>	4.61±0.26
FRNA phages	<b>3.51±0.42 (E)</b>	2.75±1.58	2.09±1.92	3.47±0.29	1.73±1.58
<i>E. coli</i>	4.17±1.30	4.58±1.25	4.66±1.39	<b>5.05±1.08 (D)</b>	3.96±0.57
Faecal coliforms	4.24±1.26	4.76±1.40	4.77±1.34	<b>5.06±1.10 (C, D)</b>	4.09±0.57
Intestinal enterococci	3.51±0.42	3.39±0.60	3.22±0.94	<b>3.98±0.54 (D)</b>	3.29±0.57

**Key:** Mean concentration after each exposure period ± standard deviation Log<sub>10</sub> PFU/100g of shellfish digestive gland or Log<sub>10</sub> CFU/100g of shellfish intravalvular fluid and flesh; ND – Non detects (i.e. ≤ detection limit); Figures in **bold** denote the highest mean concentration over exposure period and letters in parenthesis represent the tank in which highest values were recorded.

**Table 2: Summary of uptake, bioaccumulation and persistence of indicator organisms in oysters (*C. gigas*) with respect to temperature and salinity**

Indicator	Mean concentration Log <sub>10</sub> /PFUCFU/100g ± SD				
	Exposure period – hours (Tank)				
	6	12	24	48	96
GB124 phages	0.56±1.24	ND	<b>1.27±1.74 (B)</b>	ND	ND
Somatic coliphages	1.73±1.58	ND	1.23±1.69	<b>1.73±1.58 (C)</b>	1.21±1.66
FRNA phages	ND	ND	ND	ND	ND
<i>E. coli</i>	<b>5.47±0.62 (D)</b>	5.39±0.39	5.29±0.70	4.42±0.47	4.65±0.35
Faecal coliforms	4.99±0.66	<b>5.19±0.94 (B, D)</b>	5.18±0.61	4.53±0.39	4.83±0.33
Intestinal enterococci	2.30±0.62	2.79±0.22	<b>3.23±0.41 (C)</b>	3.00±0.31	2.91±0.20

**Key:** Mean concentration after each exposure period ± standard deviation Log<sub>10</sub> PFU/100g of shellfish digestive gland or Log<sub>10</sub> CFU/100g of shellfish intravalvular fluid and flesh; ND – Non detects (i.e. ≤ detection limit); Figures in **bold** denote the highest mean concentration over exposure period and letters in parenthesis represent the tank in which highest values were recorded.



**Figure 1: Schematic diagram of uptake and bioaccumulation experiment**

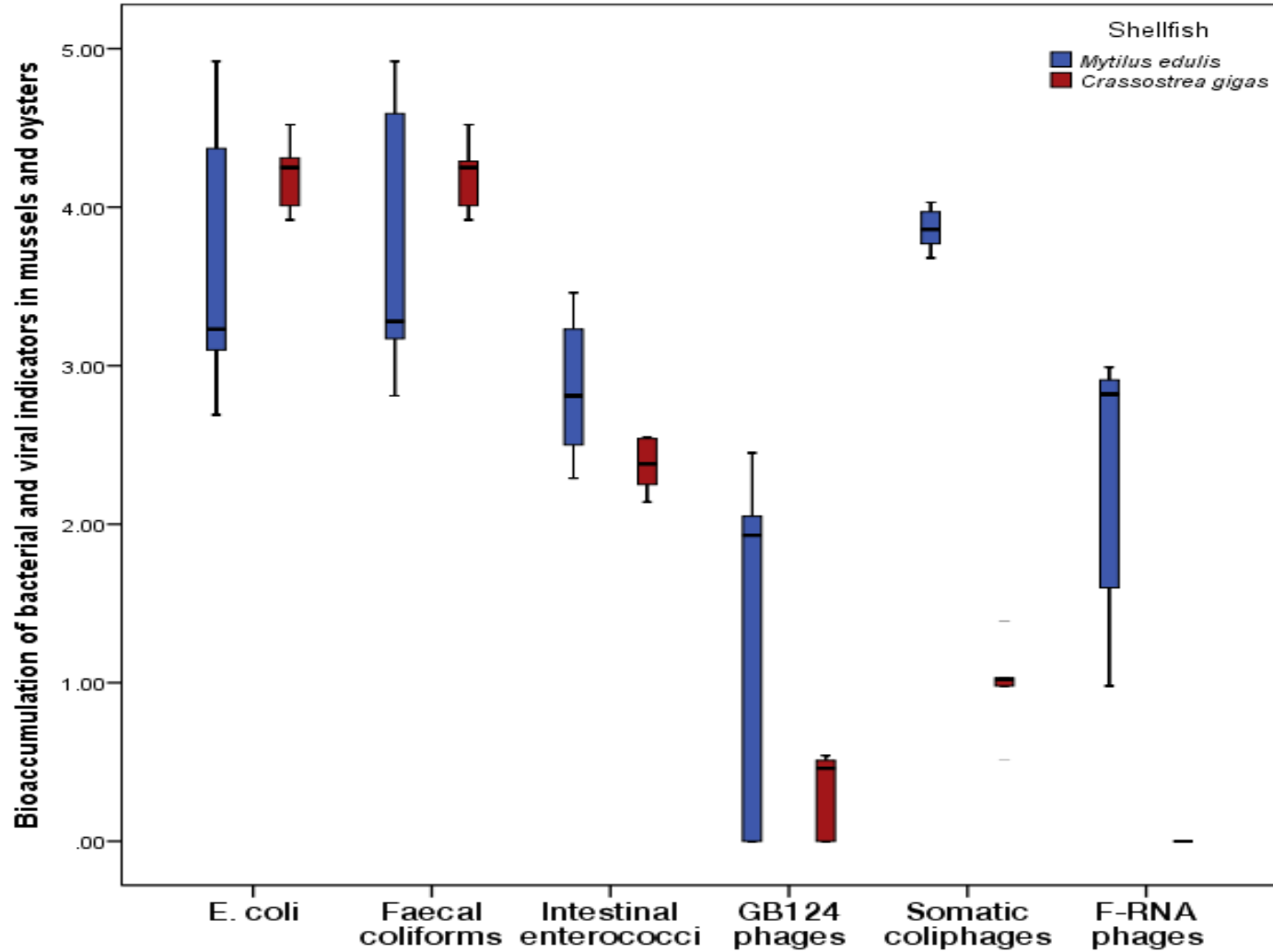


Figure 2: Boxplot of bioaccumulation of faecal indicator bacteria and bacteriophages in *M. edulis* and *C. gigas*. The median value is represented by a line inside the box, 95% confidence intervals (bars).