Environ Sci Pollut Res (2017) 24:28268–28276
https://doi.org/10.1007/s11356-017-0736-1

SHORT RESEARCH AND DISCUSSION ARTICLE

Comparison of bioaccumulation and elimination of *Escherichia coli* and male-specific bacteriophages by ascidians and bivalves

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Received: 20 July 2017 / Accepted: 9 November 2017 / Published online: 20 November 2017

Abstract Levels of *Escherichia coli* and male-specific bacteriophages (MSBs) were determined in the filter feeders obtained from retail markets, commercial farms, and wild beds in Korea. The accumulation and elimination of *E. coli* and MSBs were compared between ascidians and bivalves (oysters and mussels) during relaying and depuration. *E. coli* concentrations in ascidians from retail markets ranged between < 20 and 460 most probable number/100 g while MSBs were not detected. *E. coli* levels in bivalves from commercial farms and wild beds were not significantly different but bacterial levels in ascidians were consistently lower. Ascidians exhibited much lower ability than bivalves to accumulate *E. coli* and MSBs during relaying in a polluted coastal area. This study also shows that an equilibrium was developed between levels of microbes in water and ascidians and shellfish during relaying. *E. coli* and MSBs in ascidians decreased quickly during depuration in a clean seawater tank. However, after 1 day, *E. coli* in bivalves decreased by only 1.1–1.6 logs, and the elimination of MSBs was negligible. Therefore, depuration is an effective means to reduce the health risk of contaminated ascidians.

Responsible editor: Philippe Garrigues

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Keywords Ascidian · Bivalve · Filter feeder · Male-specific bacteriophage · Bioaccumulation · Elimination · Korea

Introduction

The edible ascidian *Halocynthia roretzi* is a commercially important seafood species in Korea, Japan, and other Asian countries. Ascidians are cultured extensively in coastal regions of eastern and southern Korea and northeastern Japan (Mizuta et al. 2002). Similar to other tunicates, ascidians are suspension feeders that filter phytoplankton and other suspended particulate matter from the surrounding water (Bone et al. 2003; Petersen 2007; Riisgård and Larsen 2010). Besides ingesting phytoplankton and other food particles, filter-feeding animals (e.g., tunicates and bivalves) may also concentrate pathogenic bacteria and viruses in their guts, so that consumption of filter feeders from polluted waters presents a risk to human health.

For many decades, the consumption of raw or undercooked bivalves, such as oysters and clams, has been implicated in numerous food-poisoning outbreaks caused by pathogenic microorganisms (Rippey 1994; Potasman et al. 2002; Lees et al. 2010; Sair et al. 2002; Iwamoto et al. 2010; Yang et al. 2017). Fecal coliforms, including *Escherichia coli*, are used as fecal contamination indicators to assess the quality of bivalves and to determine whether bivalves are safe for raw consumption (Mok et al. 2016a). Many countries have established regulatory limits using fecal indicators for bivalves and their growing areas (European Commission (EC) 2005; New Zealand Food Safety Authority (NZFSA) 2006; US Food and Drug Administration (US FDA) 2015; Korea Ministry of Food and Drug Safety (KMFDS) 2016). Male-specific bacteriophages (MSBs) have been also used for tracking pollution in marine estuaries and determining the risk of illness

associated with the consumption of raw bivalves (Burkhardt et al. 1992b). Furthermore, in Korea, peeled whole ascidians are commonly consumed raw, including the hepatopancreas. For this reason, as for bivalves, careful attention must be paid to the food safety of ascidians.

Several experiments have been conducted to determine the kinetics of uptake and elimination of fecal indicator bacteria and pathogens by shellfish. The accumulation and elimination kinetics of enteric bacteria and viruses vary among bivalve species (Cabelli and Heffernan 1970) and depend on the type of microorganism (Burkhardt et al. 1992a; Burkhardt and Calci 2000; Mok et al. 2016b) and environmental conditions (Burkhardt et al. 1992a). Although ascidians and bivalves are both filter feeders, they differ in size and in their particle capture mechanisms (Riisgård and Larsen 2010). It is well established that the filtration rates of ascidians vary with the species and environmental conditions (Randløv and Riisgård 1979; Ribes et al. 1998; Petersen and Svane 2002). Feed particle size and capture mechanisms have been studied extensively in ascidians (Bone et al. 2003; Petersen 2007; Riisgård and Larsen 2010). However, few studies have been conducted to evaluate the microbiological quality of live ascidians destined for raw consumption. Although bacterial contamination of ascidians can occur at any step from pre-harvest through to final preparation, prevention of initial contamination at the harvesting area is important because, as noted, the animal is commonly consumed raw in Asian countries.

In the present study, microbiological quality was evaluated based on the levels of indicators of fecal contamination, including total coliforms, fecal coliforms, *E. coli*, and MSBs in live bivalves (oysters and mussels) and ascidians collected from retail markets or fields. The characteristics of bioaccumulation and elimination of *E. coli* and MSBs by *H. roretzi* and by oysters and mussels were compared by relaying them at a polluted area and then depuration in seawater tanks to investigate the importance of ascidians as vectors in the transmission of pathogenic microorganisms. While levels of these microorganisms in bivalves (oysters and mussels) at retail markets have been studied as well as bioaccumulation and depuration, this is the first work that we are aware of that evaluates the levels of fecal indicators within ascidians as a vector in the transmission of pathogenic microorganisms.

Materials and methods

Sample collection

Live ascidians (*H. roretzi*) were obtained from local retail markets in Tongyeong and Busan, Korea, during 2009–2010. Samples of *H. roretzi*, oysters (*Crassostrea gigas*), and mussels (*Mytilus galloprovincialis*) were also collected from a commercial aquaculture farm and from wild beds. The commercial farm was located in an area designated for shellfish growing for export that met water quality standards of the National Shellfish Sanitation Program (NSSP) for approved areas (US FDA 2015). The wild bed was located in a shallow water coastal region near Busan City, which is susceptible to land-based pollutants. Samples of animals were collected monthly from the commercial farm and from the wild bed. All collected samples for microbiological analysis were maintained below 10 °C during transport to the laboratory. Ascidians, oysters, and mussels for accumulation and elimination experiments were taken from the designated shellfish-growing area.

Analysis of fecal indicator bacteria

The levels of total coliforms, fecal coliforms, and *E. coli* in the samples were enumerated by the most probable number (MPN) method. The MPN method used was a five-tube test using three tenfold serial dilutions; referring the numbers of tubes yielding positive results to published standard tables gave the concentrations of bacteria in the samples. The recommended procedures for the examination of seawater and shellfish according to the American Public Health Association (APHA) were applied for total coliform and fecal coliform enumeration (APHA 1970). The presumptive test was performed using lauryl tryptose broth (Difco, Detroit, MI, USA) at 35 ± 0.5 °C. The confirmative test used brilliant green-lactose broth (Difco) at 35 ± 0.5 °C for total coliforms, and EC medium (Difco) at 44.5 ± 0.2 °C for fecal coliforms. In addition, the ISO/TS 16649-3 method (ISO 2015) was used for *E. coli* enumeration. Tubes of Minerals-modified Glutamate Broth (Oxoid, Basingstoke, UK) were incubated at 37 ± 1 °C for 24 ± 2 h for the presumptive test. Each positive culture tube showing yellow color was subsequently confirmed by subculture onto a Tryptone Bile X-glucuronide Agar (Oxoid) plate at 44 ± 1 °C for 22 ± 2 h. Results are expressed as MPN/100 mL for seawater and MPN/100 g for ascidian and bivalve meat samples. The limits of detection (LODs) of these methods were the following: 18 MPN/100 g of meat and 1.8 MPN/100 mL of seawater for total and fecal coliforms, and 20 MPN/100 g of meat and 2.0 MPN/100 mL of seawater for *E. coli*.

MSB analysis

The MSB levels in ascidians and bivalves meat were assayed using the double-layer agar method described by Burkhardt et al. (1992b). The *E. coli* culture HS[pFamp]R (ATCC 700891) was obtained from the American Type Culture Collection through their Korean distributor and used as the bacterial host strain for the MSBs. The bacterial host culture

was prepared as described in the US EPA method 1601 (US EPA 2001). The assays used the antibiotics, streptomycin sulfate, and ampicillin to prevent competing bacterial growth. The MSBs were quantified by plaque formation on the host *E. coli* on the agar medium. The results are expressed as the numbers of plaque-forming units (PFU) per 100 g or per 100 mL of sample. The LODs were 17 PFU/100 g of meat and 10 PFU/100 mL of seawater.

Bioaccumulation experiments

Live ascidians, oysters, and mussels collected from the designated shellfish-growing area were transferred to water near the outflow of a sewage treatment plant to compare the accumulation rates and final levels of *E. coli* and MSBs by ascidians and bivalves. Ascidians, oysters, and mussels harvested from the designated shellfish-growing area were used for relaying, after confirming that they were free from *E. coli* and MSBs. After scrubbing with a stiff brush to remove adhering detritus and fouling organisms, 37 animals (7 ascidians, 15 oysters, and 15 mussels) were placed together into each of mesh bags (mesh size 10 mm). The bags were hung from buoys and allowed to sink approximately 50 cm below the surface at the relaying site. Duplicate samples of five ascidians and 12 mussels and oysters were taken from each bag and assayed for the levels of *E. coli* and MSB.

Depuration experiments

The depuration tank (100 × 100 × 70 cm high) had a working volume of 400 L. Sand-filtered (Negatron Water Filter, Busan, Korea), UV-irradiated (four 39-W lamps, Sungchang, Anyang, Korea) seawater was used for the depuration experiments. Prior to the depuration experiments, no detection of indicators in the seawater was confirmed. The water temperature was maintained at 12 ± 8 °C. Contaminated ascidians and bivalves relayed for 5 days were washed thoroughly, and damaged and moribund individuals were rejected. The contaminated animals were loaded into baskets without overlapping and the baskets were placed on the bottom of the depuration tank at a depth of approximately 8 cm. Seawater was supplied to the tank at a rate of 10 L/min. Duplicate samples of five ascidians, 10 oysters, or 10 mussels were retrieved for microbiological analysis.

Water purification by filter feeders

Twenty similarly sized ascidians, oysters, or mussels were shucked (or peeled) and weighed for calculation of average net weight. Sewage containing bacteria and MSBs was filtered with a paper pre-filter (Advantec No. 5A, Toyo Roshi, Japan) for the removal of particles. Filtered sewage (2 L) was added to sand-filtered seawater (120 L), the mixture was stirred with an electronic stirrer for 1 h, and the mixture was then subdivided into 30-L volumes. Prior to subdividing, the levels of *E. coli* and MSBs in the mixture were enumerated. Groups of 22 ascidians, 25 oysters, or 74 mussels providing similar total net meat weights (ca. 400 g) were each placed in 30 L of the seawater mixture and aerated (air filtered with a cotton plug). Seawater was sampled daily for analysis of *E. coli* and MSB concentration to investigate the water purification activities of the ascidians and bivalves. Water temperature and salinity were measured using an YSI 556 Multiprobe system (YSI Inc., Yellow Springs, OH).

Results and discussion

Levels of fecal indicator bacteria and MSBs in ascidians from retail markets

Levels of total coliforms, fecal coliforms, *E. coli*, and MSBs in live ascidians obtained from retail markets in Busan (Gijang market) and Tongyeong (Seoho market and Jungang market), Korea, are summarized in Table 1.

Levels of fecal indicator bacteria in ascidians differed among the markets and sample batches. The maximum levels recorded among the 30 samples taken from the three retail markets were 1100 MPN/100 g total coliforms, 230 MPN/100 g fecal coliforms, and 460 MPN/100 g *E. coli*. The estimated geometric means were 24 MPN/100 g for fecal coliforms and 22 MPN/100 g for *E. coli*. The MSBs were not detected in any of the retailed ascidians.

The levels of *E. coli* in most of the ascidians ($n = 30$) purchased from the three markets complied with the European standard for live and raw seafood (bivalves, echinoderms, tunicates, and gastropods) for human consumption (fewer than 230 *E. coli* per 100 g) (EC 2005), and no MSBs were found. However, the level of *E. coli* (460 MPN/100 g) in one sample obtained from Seoho market, Tongyeong, exceeded the European standard. The geometric mean value of *E. coli* concentration in ascidians from the 30 retail samples (24 MPN/100 g) was higher than that in retailed oysters from the North Atlantic and Pacific coasts but similar to that of Gulf and Mid-Atlantic coast oysters in the USA (DePaola et al. 2010). Even in the ascidian sample that exceeded the European *E. coli* standard of 230 MPN/100 g, the MSB concentration was below the detection limit of 17 PFU/100 mL. Although the ratios of MSBs and *E. coli* change during the sewage treatment process, *E. coli* are generally present in greater concentrations than MSBs in raw sewage (Doré et al. 2003). The sample that exceeded the European standard may have been contaminated by untreated sewage at any stage of growing, harvesting, and handling.

Table 1 Levels of fecal pollution indicator bacteria and male-specific bacteriophages (MSB) in ascidians (*Halocynthia roretzi*) collected from three retail markets in Korea during 2009–2010

Collected market	Total coliform bacteria (MPN/100 g)			Fecal coliform bacteria (MPN/100 g)			*E. coli* (MPN/100 g)			No. of samples exceeded 230 *E. coli* MPN/100 g	MSB (PFU/100 g)			No. of sample batches
	Min	Max	GM	Min	Max	GM	Min	Max	GM		Min	Max	Mean	
Total	< 18	1100	90	< 18	230	24	< 20	460	22	1	< 17	< 17	< 17	30
Gijang retail market, Busan	< 18	790	101	< 18	78	25	< 20	80	22	0	< 17	< 17	< 17	9
Seoho retail market, Tongyeong	< 18	1100	123	< 18	230	30	< 20	460	24	1	< 17	< 17	< 17	12
Jungang retail market, Tongyeong	< 18	340	57	< 18	20	< 18	< 20	40	< 20	0	< 17	< 17	< 17	9

GM, geometric mean; *MPN*, most probable number; *PFU*, plaque-forming unit

Levels of fecal indicator bacteria and MSBs in ascidians and bivalves collected from commercial farms and wild beds

The levels of fecal indicator bacteria and MSBs in the seawater, ascidians, oysters, and mussels collected from commercial farms and wild beds are summarized in Table 2.

Levels of fecal indicator bacteria in the seawater and in the three filter feeders were significantly different among the sample collection sites. Marked differences in bacterial levels were also observed among the ascidians, oysters, and mussels at the same sampling site. Maximum and geometric mean concentrations of total coliforms in ascidians from the commercial farm were lowest among the three filter feeders. However, there were no significant differences in fecal coliforms and *E. coli* levels among ascidians, oysters, and mussels because the levels of fecal indicator bacteria in most of the samples were below the detection limit. In the samples from wild beds, the maximum and geometric mean concentrations of three categories of indicative bacteria in ascidians were lower than those in oysters and mussels. The maximum concentrations of total coliforms in ascidians, oysters, and mussels from wild beds were 230, 4900, and 2400 MPN/100 g, respectively, and the geometric mean concentrations were 43, 221, and 102 MPN/100 g, respectively. The MSBs were not detected in any of the samples. No significant differences were observed in the levels of the indicative bacteria between oysters and mussels. The levels of the indicator bacteria in ascidians were consistently lower than those observed in bivalves. The levels of MSBs could not be compared among filter feeders because the MSBs were not detected in any of the tested samples.

Korea is the world's fourth largest producer of bivalves, contributing almost 2.8% of the global harvest (Pawiro 2010). Some of the shellfish-growing areas meet the approved area criteria of the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish (US FDA 2015) for which the median, or geometric mean, of fecal coliforms in seawater should not exceed 14 MPN/100 mL, and the estimated 90th percentile should not exceed 43 MPN/100 mL. Other shellfish-growing areas located close to pollution sources are affected by the land-based fecal contaminants during wet weather (Ha et al. 2011).

Most bivalve and ascidian farms are located in shallow coastal waters, which may be affected by the land pollution

Table 2 Levels of fecal pollution indicator bacteria and male-specific bacteriophages (MSB) in ascidians (*Halocynthia roretzi*), oysters (*Crassostrea gigas*), and mussels (*Mytilus galloprovincialis*) collected from commercial shellfish-growing farms and wild beds in 2010

Sampling sites	Samples	Total coliform (MPN/100 mL or g)			Fecal coliform (MPN/100 mL or g)			*E. coli* (MPN/100 mL or g)			No. of samples exceeded 230 *E. coli* MPN/100 g	MSB (PFU/100 mL or g)			No. of sample batches
		Min	Max	GM	Min	Max	GM	Min	Max	GM		Min	Max	GM	
Commercial farm	Seawater	< 1.8	22	2.5	< 1.8	4.0	1.8	-	-	-	-	< 10	< 10	< 10	12
	Ascidians	< 18	45	19	< 18	20	< 18	< 20	20	< 20	0	< 17	< 17	< 17	10
	Oysters	< 18	220	27	< 18	45	19	< 20	50	21	0	< 17	< 17	< 17	10
	Mussels	< 18	490	27	< 18	20	< 18	< 20	< 20	< 20	0	< 17	< 17	< 17	12
Wild bed	Seawater	< 1.8	220	17	< 1.8	79	4.1	< 2.0	13	2.3	-	< 10	< 10	< 10	12
	Ascidians	< 18	230	43	< 18	45	19	< 20	20	< 20	0	< 17	< 17	< 17	12
	Oysters	< 18	4900	221	< 18	950	49	< 20	640	28	1	< 17	< 17	< 17	12
	Mussels	< 18	2400	102	< 18	330	29	< 20	130	22	0	< 17	< 17	< 17	12

GM, geometric mean; *MPN*, most probable number; *PFU*, plaque forming unit; -, not tested

sources. Our data showed that bivalves concentrated fecal indicator bacteria to a slightly higher degree than ascidians did. Unfortunately, levels of MSBs in ascidians could not be compared with those in bivalves. Nevertheless, ascidians produced from coastal waters were apparently much safer for raw consumption than oysters harvested from the same areas. The bioaccumulation and elimination characteristics of these filter feeders for *E. coli* and MSBs were compared in relaying and depuration experiments to investigate possible reasons for the differences in the levels of indicator bacteria in filter feeders from the same growing water.

Bioaccumulation and elimination of *E. coli* and MSBs by filter feeders

Bioaccumulation of *E. coli* and MSBs in filter feeders that were relayed from designated shellfish-growing areas to an area contaminated by sewage is shown in Fig. 1. *E. coli* was selected as the only test indicator because this bacterium was recommended as the best biological indicator for contamination of bivalve flesh by fecal pollutants for public health protection (Edberg et al. 2000). Levels of *E. coli* and MSBs in relaying waters fluctuated during the experiment in the ranges 33–170 MPN/100 mL and 10–110 PFU/100 mL, respectively. The concentrations of *E. coli* in the filter feeders reached maximum levels after 1 or 2 days of relaying and fluctuated within 920–4900 MPN/100 g in mussels, 630–1400 MPN/100 g in oysters, and 140–490 MPN/100 g in ascidians. Mussels were consistently contaminated with higher levels of *E. coli* than were oysters collected at the same time, and concentrations of the bacteria in ascidians were much lower than those in oysters. In contrast to the relatively rapid accumulation of *E. coli* in filter feeders (1–2 days), MSB concentrations reached the highest levels in each filter feeder after 4 days of relaying. Figure 1 also shows that an equilibrium was developed between levels of microbes in water and ascidians and shellfish within 4 days for oysters and mussels.

Patterns of MSB accumulation differed among the three species. MSBs were accumulated faster and to higher levels in oysters than in mussels or ascidians. A high level of MSBs accumulated in both bivalves but low levels were observed in ascidians. The MSBs in bivalve meat reached ca. 10^3 PFU/100 g within 2 days after relaying in oysters, and within 4 days in mussels, and remained at 10^3 PFU/100 g. In contrast, the highest level of MSBs recorded in ascidians was only 120 PFU/100 g. Thus, ascidians showed much poorer ability to accumulate *E. coli* and MSBs compared with both bivalves. In most cases, the MSB concentration in the flesh of ascidians was lower than that in seawater.

The accumulation levels of bacteria and MSBs varied among the three species. Accumulation factors for bacteria and MSBs in the ascidians, oysters, and mussels were calculated from the geometric mean concentrations of *E. coli* and MSBs and are presented in Fig. 1. The accumulation factors for *E. coli* were 3.3 for ascidians, 11.4 for oysters, and 23.6 for mussels. Accumulation factors for MSBs were 0.7, 60.9, and 11.2, respectively. *E. coli* were most efficiently accumulated in mussels and MSBs were most efficiently accumulated in oysters. The lowest accumulation factors for both *E. coli* and MSBs were observed in ascidians, especially for MSBs where the concentration was lower than in the surrounding water.

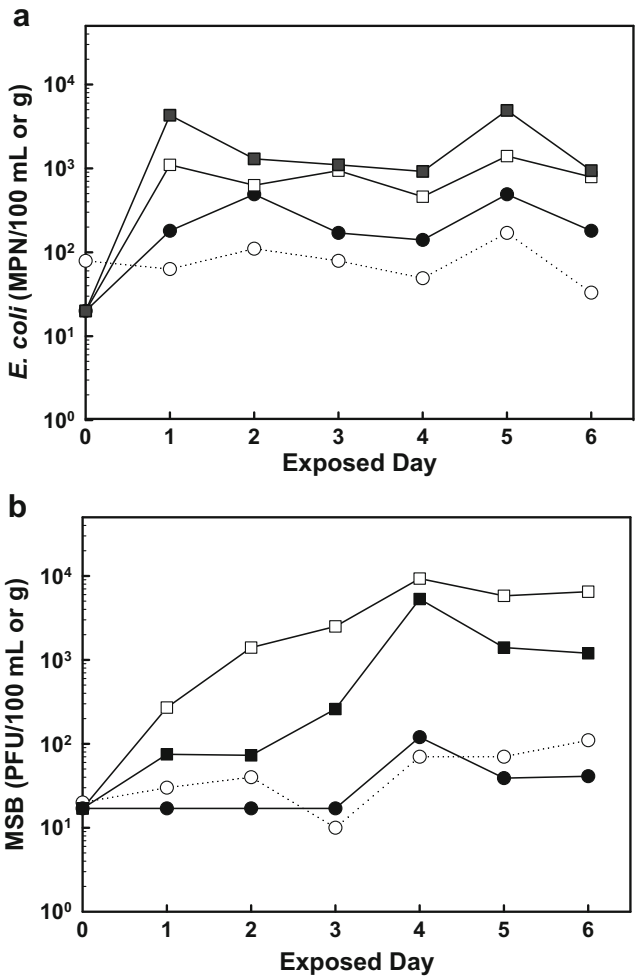

Fig. 1 Bioaccumulation of *Escherichia coli* (A) and male-specific bacteriophages (MSBs) (B) in ascidians (●), oysters (□) and mussels (■) during relaying in a polluted coastal area. Concentrations of *E. coli* and MSBs in the surrounding water are represented as open circles (○). Environmental conditions during relaying were: water temperature, 9.2–10.7 °C; salinity, 31.8–32.6

Many studies have been carried out on the accumulation of enteric bacteria and viruses in various bivalve species (Bedford et al. 1978; Timoney and Abston 1984; Doré and Lees 1995; Burkhardt and Calci 2000). Although bacteria and viruses are usually accumulated in bivalve tissue to levels much higher than in the surrounding water, interspecific differences among filter feeders in their ability to accumulate

microorganisms have been reported, with accumulation factors varying from a fewfold to more than a hundredfold (Kershaw et al. 2013; Burkhardt and Calci 2000). These differences are probably associated with the morphology of the gills in bivalves and the branchial basket in ascidians (Kryger and Riisgard 1988; Doré and Lees 1995; Galbraith et al. 2009; Petersen and Svane 2002). Other biological factors may be involved; for example, norovirus binds specifically to oyster digestive tissues (Le Guyader et al. 2006), and Burkhardt and Calci (2000) showed that F-specific coliphage was selectively accumulated up to 99-fold in oysters (*C. virginica*). A number of studies have shown that mussels accumulate enteric bacteria, such as *E. coli*, faster and to higher levels than in oysters (*C. gigas*). The average *E. coli* accumulation ratios in mussels (*Mytilus* spp.) were from 1.3 to 3.4 times greater than those in oysters (*C. gigas*) (Berry and Younger 2009; Younger and Reese 2013; Kershaw et al. 2013). In the present study, the geometric mean accumulation factor of *E. coli* in mussels was 2.1 times higher than that in oyster, which is consistent with previous reports. However, accumulation factors of *E. coli* and MSBs in tissues of the ascidian *H. roretzi* were lower than those in mussels or oysters.

To investigate the cause of the lower accumulation factors in ascidians, change patterns of *E. coli* and MSBs in a mixture of filtered seawater and sewage for the three filter feeders were assessed (Fig. 2). Species and animal size, water temperature, and the size and concentration of particles are all known to affect filtration activity by filter feeders (Vaughn and Hakenkamp 2001; Petersen 2007). Thus, in the present study, change patterns of *E. coli* and MSBs in ascidians and bivalves were compared under the same environmental conditions. Species-specific differences were observed in decrease rates of *E. coli* and MSBs. The level of *E. coli* in the water holding the ascidians declined more rapidly than that in the water holding oysters and mussels. In contrast, MSBs were most slowly removed from the water holding ascidians. Although *E. coli* was rapidly removed from the water by the ascidians, the bacterium was accumulated to a lower concentration in ascidian tissues than in bivalves (Fig. 1). However, MSBs in ascidians in the surrounding water decreased slower than those in bivalves (Fig. 2) and accumulation of MSBs in ascidians was also lower than that in bivalves (Fig. 1). Bacteria are an important food source for bivalves and tunicates. Stuart and Klumpp (1984) observed that ascidians retained all particles larger than 0.6 μm with ca. 100% efficiency but the retention efficiency of the same-sized particles was only 20% in bivalves. Thus, it was suggested that the bacteria were more easily digested or inactivated by ascidians than by bivalves. It was unclear whether MSBs were passed through the branchial basket of ascidians or were not inactivated in hepatopancreas after uptake by animals.

Other experiments on the elimination of *E. coli* and MSBs during depuration in a clean seawater tank from the contaminated filter feeders are shown in Fig. 3. Different microbial reduction patterns were observed between ascidians and bivalves. In the ascidians, the levels of microorganisms declined quickly from high starting concentrations of *E. coli* (2200 MPN/100 g) and MSBs (310 PFU/100 g) to undetectable levels (< 20 MPN/100 g for *E. coli*, < 17 PFU/100 g for MSB) within 1 day of depuration. However, *E. coli* levels in bivalves, oysters, and mussels decreased by only 1.1 logs and 1.6 logs, respectively, and the elimination of MSBs was negligible after 1 day of depuration. Bivalves were of class C (4600 to 46,000 *E. coli* MPN/100 g flesh) according to microbiological standards of EU legislation (EC 2005) before depuration and improved to better than class Al (< 230 *E. coli* MPN/100 g flesh) for raw consumption within 2 days

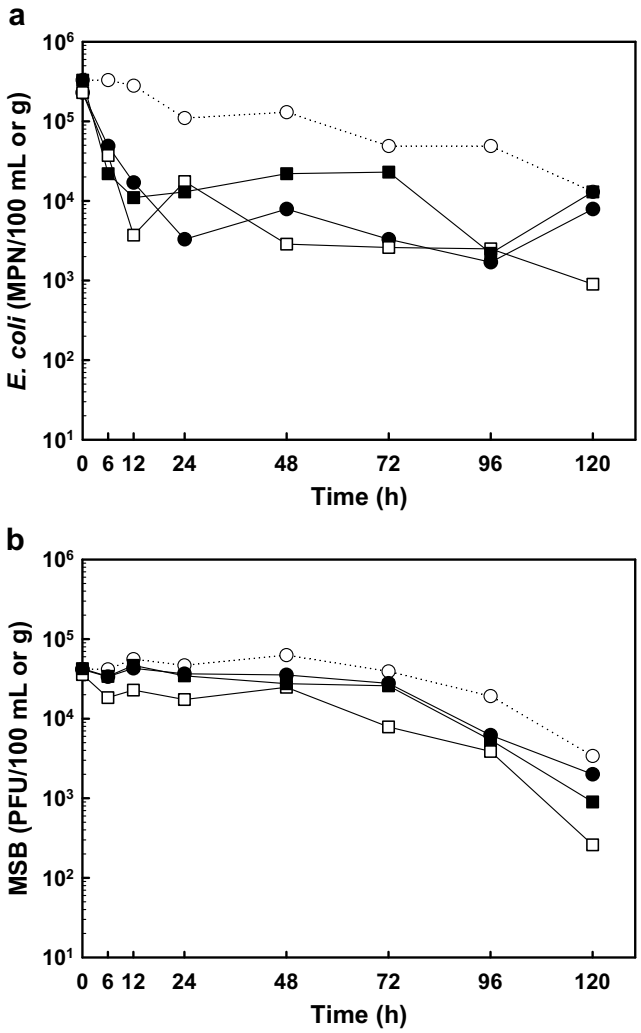

Fig. 2 Change patterns of *Escherichia coli* (**a**) and male-specific bacteriophages (MSBs) **(b)** in a mixture of filtered seawater and sewage for ascidians (●), oysters (□), and mussels (■). Concentrations of *E. coli* and MSBs in control seawater are represented as open circles (○). Approximately 400 g net meat weight of filter feeders was placed in 30 L of seawater. Environmental conditions during examination were: water temperature, 8.8–10.5 °C; salinity, 32.1–32.7

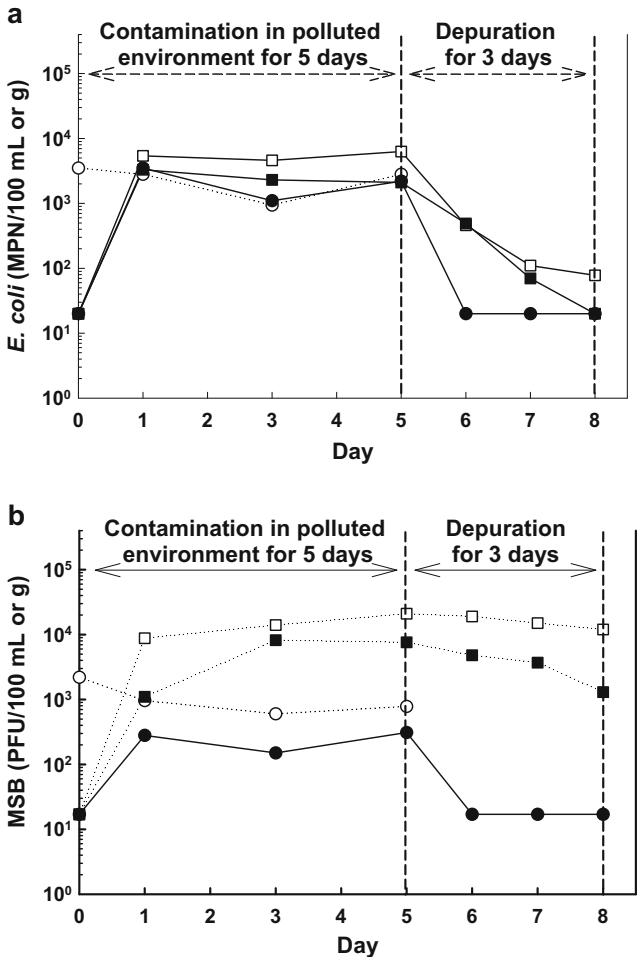

Fig. 3 Bioaccumulation during relaying in a polluted area and depuration in a clean seawater tank of *Escherichia coli* (**a**) and male-specific bacteriophages (**b**) by ascidians (●), oysters (□), and mussels (■). Concentrations of *E. coli* and MSBs in the surrounding water during contamination are represented as open circles (○). Environmental conditions of the relaying water: temperature, 10.5–12.4 °C; salinity, 30.8–32.2. Environmental conditions of the depuration water: temperature, 11.2–12.8 °C; salinity, 32.3–33.4

of treatment. In contrast, the MSB concentrations in bivalves dropped by only 0.14–0.31 logs in the same period.

Several studies have revealed that viruses are retained by bivalves for significantly longer periods than indicator bacteria such as *E. coli* and fecal coliforms (Power and Collins 1989; Doré and Lees 1995; Burkhardt and Calci 2000; Oliveira et al. 2011). In particular, human norovirus has been shown to persist longer than feline calicivirus or poliovirus in oysters under depuration (Ueki et al. 2007; Mcleod et al. 2009). Thus, although depuration is an efficient purification procedure for bacterial contaminants in bivalves, the process has a limited effect on reducing viral levels. Our observations are consistent with these earlier studies. Many studies on accumulation and elimination of enteric bacteria and bacteriophages for disease prevention have focused on bivalves for economic reasons. Unlike bivalves, there have been few reports on the kinetics of accumulation and elimination of bacteria and viruses by ascidians. In this study, we compared microbiological quality and bioaccumulation and elimination properties between ascidians and bivalves. We conclude that the depuration process is an effective way to reduce the health risk of contaminated ascidians but, similar to bivalves, ascidians can be a vector of human disease when they are grown in polluted coastal areas for raw consumption.

Conclusions

In the present study, microbiological quality was evaluated on the basis of bacterial fecal contamination indicators and MSBs in live ascidians collected from the growing areas and the retail markets. In addition, the accumulation and elimination characteristics of *E. coli* and MSBs by ascidian and bivalves were compared during bioaccumulation by relaying them at a polluted area and depuration in seawater tanks. The *E. coli* concentrations of ascidians purchased from three retail markets ranged from < 20 MPN/100 g to 460 MPN/100 g, meeting the European standard for live and raw seafood, with exception of one sample, and MSBs were not detected. No significant differences were observed in the indicator levels for bacteria in oysters and mussels; the indicator levels in ascidians were consistently lower than those in bivalves collected from commercial farms or wild beds.

E. coli was quickly accumulated in the filter feeders but MSB accumulation was delayed during relaying in a polluted coastal area. Mussels were consistently contaminated with higher levels of *E. coli* than were oysters collected at the same time, and the bacterial levels in ascidians were even lower than those in oysters. High levels of MSBs were accumulated in bivalves but low levels of MSBs were found in ascidians. These observations suggest that ability of ascidians to accumulate *E. coli* and MSBs is lower than that of mussels and oysters.

Species-specific differences were also observed in the elimination rates of *E. coli* and MSBs by these filter feeders during depuration in a clean seawater tank. The levels of microorganisms in ascidians decreased quickly from starting concentrations of 2200 MPN/100 g of *E. coli* and 310 PFU/100 g of MSBs to undetectable levels (< 20 MPN/100 g for *E. coli*, < 17 PFU/100 g for MSB) within 1 day of depuration. However, *E. coli* levels in oysters and mussels decreased by only 1.1 logs and 1.6 logs, respectively, and elimination of MSBs was negligible after 1 day of depuration. Therefore, we conclude that depuration is an effective means of reducing the health risk of contaminated ascidians.

Funding information This work was supported by a grant from the National Institute of Fisheries Science in Korea (R2017057).

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