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Efficacy of a Closed-Water Depuration System in Reducing Bacterial Load in Bivalve Shellfish

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Abstract This study evaluated the effectiveness of a closed water depuration system equipped with a sponge filter for reducing bacterial contamination in black clams (*Villorita cyprinoides*) harvested from the Varapuzha region of the Cochin Estuary, Kerala, India. Initial bacteriological analysis revealed high levels of contamination in the clams, indicating potential food safety risks for consumers. The depuration experiment was conducted in a recirculating tank system fitted with a sponge filter, in which clams were maintained in clean water and sampled at regular time intervals up to 72 hours. Bacterial indicator reduction, including Total Coliforms (TC), Faecal Coliforms (FC), Total Heterotrophic Bacteria (THB), and *Vibrio* species were monitored to evaluate the purification efficiency of the system. Results demonstrated a marked reduction in bacterial load during depuration, with the most pronounced decrease occurring within the first 48 hours. Total coliforms and faecal coliforms were reduced by 98.54% and 98.45%, respectively, reaching substantially lower faecal indicator levels, although compliance with specific regulatory standards requires validation using the prescribed indicator organisms and units. Total heterotrophic bacteria also showed a substantial decline, while *Vibrio* species were reduced to a lesser extent. Nevertheless, a gradual increase in bacterial counts was observed on the inner walls of the depuration tank due to biofilm formation, indicating the possibility of recontamination if the system is not properly cleaned. Overall, the sponge filter-based closed water depuration system proved to be a simple, cost-effective, and water-efficient method for improving the microbiological quality of clams. This approach is suitable for small-scale depuration practices and could help to improve shellfish safety and protect public health. This study may provide practical baseline information for optimising low-cost shellfish depuration systems under tropical estuarine conditions.

Keywords Depuration; Black clam (*Villorita cyprinoides)*; Sponge filter; Bacterial reduction; Biofilm; Closed water depuration system

1 Introduction

Seafood constitutes a vital component of the global diet, particularly in developing countries where it serves as a major source of affordable animal protein. Among the various seafood categories, bivalve molluscs such as clams, mussels, oysters, and scallops are especially important due to their high nutritional value, ecological significance, and economic potential (FAO (Food and Agriculture Organization), 2022). In India, bivalves play a significant role in coastal fisheries, contributing substantially to local livelihoods and the seafood export industry (Laxmilatha, 2018; Anil et al., 2024).

Bivalves are filter-feeding organisms that obtain their nutrition by filtering large volumes of the surrounding water. This feeding mechanism allows them to accumulate suspended particulate matter, including phytoplankton, organic detritus, bacteria, and potentially harmful pathogens (Min et al., 2024; Ochoa-Esteso et al., 2024). While this ecological function contributes to water purification and nutrient cycling, it also makes bivalves susceptible to the bioaccumulation of contaminants, particularly when they inhabit or are harvested from polluted aquatic environments (Martínez-Albores et al., 2020). Consequently, bivalves can act as vectors for foodborne illnesses caused by bacterial, viral, and protozoan pathogens, especially when consumed raw or insufficiently processed (Desdouits et al., 2023).

The black clam (*Villorita cyprinoides*) is a commercially important bivalve species in India, accounting for more than 64% of clam landings in Kerala. The major harvesting grounds are located in the Vembanad and Ashtamudi lakes (Suja and Mohamed, 2010). However, important shellfish harvesting areas such as the Cochin Estuary, which forms part of the Vembanad wetland system and is recognised as a Ramsar site, are increasingly affected by anthropogenic pollution, including domestic sewage discharge, municipal runoff, and industrial effluents (Chinnadurai et al., 2020; Chinnadurai et al., 2023; Nandakrishnan and Prasad, 2024).

Several studies have reported microbial contamination in estuarine waters and shellfish from the Cochin region, raising serious public health concerns. Earlier research documented the presence of diarrheagenic strains of *Escherichia coli* and *Salmonella* spp. in the Cochin Estuary, highlighting the risks associated with untreated sewage discharge (Peralta and Andalecio, 2011). Similarly, the bacteriological quality of green mussels (*Perna viridis*) from the same estuary has revealed elevated levels of faecal indicator bacteria and *Vibrio* species (Padua et al., 2023). Clams from the adjacent Vembanad Lake have also been reported to contain faecal indicator bacteria, *Vibrio* species, and *Aeromonas spp.*, posing significant health risks to consumers and seafood handlers (Vaiyapuri et al., 2021; Silvester et al., 2022).

Apart from microbial contamination, the Cochin Estuary is increasingly affected by nutrient enrichment, chemical pollution, habitat degradation, overfishing, and unregulated coastal development, all of which have contributed to the deterioration of water quality (Thasneem et al., 2018). These environmental stressors further increase microbiological risks associated with shellfish consumption, thereby emphasising the need for stringent food safety regulations and effective sanitary measures (European Food Safety Authority [EFSA], 2010).

Globally, regulatory frameworks in regions such as the European Union, the United States, and Australia mandate routine monitoring of microbiological water quality in shellfish-growing areas to safeguard public health and ensure the safety of bivalve molluscs for consumption (European Commission, 2019; U.S. Food and Drug Administration [FDA], 2023). In the European Union, legislation requires competent authorities to classify production and relaying areas for live bivalve molluscs and to routinely monitor faecal contamination using established standards for *E. coli* levels. Harvesting is temporarily suspended when these standards are not met, until corrective actions such as relaying or depuration are carried out (Ciccarelli et al., 2022; Pinn and Le Vay, 2023).

Depuration is a widely adopted post-harvest process in which live bivalves are maintained under controlled conditions in tanks containing clean, treated water, allowing them to naturally purge accumulated contaminants (Martínez-Albores et al., 2020). The efficiency of depuration depends on several factors, including water quality, system design (flow-through or recirculating systems), duration of depuration, and the use of disinfection methods such as ultraviolet irradiation or biofiltration (Oliveira et al., 2011; Küniļi, 2024).

Among the various depuration methods, closed water depuration systems have emerged as a promising alternative to conventional open-flow systems. These systems utilise recirculating water coupled with biological filtration to remove microorganisms while conserving water and allowing greater control over environmental conditions (Campbell et al., 2022; Chinnadurai et al., 2023). Such systems are particularly beneficial in areas where access to clean water is limited or unreliable. During depuration, clams expel ingested microorganisms and contaminants in mucus-coated faecal pellets, which may lead to recontamination if not effectively removed from the system. Therefore, an efficient biofilter is essential for trapping and reducing expelled microorganisms in closed-water depuration systems.

Despite these advantages, closed water depuration systems face challenges such as the formation of biofilms on tank surfaces and filtration units. Biofilms consist of complex microbial communities embedded within an extracellular polymeric substance (EPS) matrix, which provides protection against environmental stressors such as ultraviolet radiation, salinity fluctuations, and antimicrobial agents (Flemming et al., 2016). Biofilm formation begins with bacterial attachment to submerged surfaces, followed by colonisation, EPS secretion, and maturation.

Once established, biofilms can harbour pathogenic and antibiotic-resistant bacteria, potentially turning depuration systems into sources of recontamination if not properly maintained (Azeredo et al., 2017). In aquaculture systems, such biofilms may contribute to water quality deterioration and increased microbial loads. Therefore, regular sanitisation of depuration tanks after each cycle is essential to maintain system efficiency and biosecurity (Canadian Food Inspection Agency (CFIA)., 2017).

The present study aimed to evaluate the effectiveness of a sponge filter-based closed-water depuration system in reducing bacterial contamination in black clams collected from the Varapuzha region of the Cochin Estuary. Changes in THB, TC, FC, and *Vibrio* spp. during depuration were monitored, and the microbial load on tank-wall biofilms was furthermore assessed to evaluate the potential of recontamination.

2 Results

2.1 Bacterial load in initial shellfish samples

The initial bacteriological load of *Villorita cyprinoides* (black clam) was determined prior to depuration. The bacterial parameters assessed included Total Coliforms (TC), Faecal Coliforms (FC), Total Heterotrophic Bacteria (THB), and *Vibrio* spp. (Table 1).

Table 1 Initial bacteriological load in raw shellfish samples

Bacteriological Parameter	Load
Total Heterotrophic Bacteria (THB)	1.68×10^7 CFU/g
Total Coliforms (TC)	2.4×10^4 MPN/100 g tissue
Faecal Coliforms (FC)	1.5×10^4 MPN/100 g tissue
Vibrio spp.	5.1×10^5 CFU/g

2.2 Bacteriological changes in clams during depuration

Bacterial load in the *Villorita cyprinoides* (black clam) samples at different depuration time intervals (0 h, 6 h, 12 h, 24 h, 48h and 72 h) was recorded for all parameters (Table 2).

Table 2 Bacteriological load in shellfish samples at different depuration time intervals

Sample No	Depuration time intervals (Hours)	Total Heterotrophic Bacteria load (CFU/g)	Total Coliforms load (MPN index/100mL)	Faecal Coliforms load (MPN index/100mL)	*Vibrio* spp. load (CFU/g)
1	0.00	1.68×10^7	2.4×10^4	1.5×10^4	5.1×10^5
2	6.00	1.29×10^7	1.5×10^4	1.1×10^4	4.4×10^5
3	12.00	9×10^6	1.1×10^4	4.6×10^3	3×10^5
4	24.00	1.54×10^6	4.6×10^3	2.1×10^3	1.5×10^5
5	48.00	1.03×10^6	3.5×10^2	2.3×10^2	1.2×10^5
6	72.00	1.22×10^6	3.8×10^2	2.3×10^2	1.4×10^5

2.2.1 Reduction in THB (Total Heterotrophic Bacteria) count in *Villorita cyprinoides*

The initial THB count was found to be 1.68×10^7 cfu/g. A 1.21 logs reduction was obtained within the first 48 hours of depuration to a THB count of 1.03×10^6 cfu/g. After 48 hours of depuration around 93.86% (1.21 logs) of THB reduction was observed (Table 2 and Figure 1). However, complete depuration of total heterotrophic bacteria (THB) was not achieved. Total Heterotrophic Bacteria showed a moderate but statistically significant reduction ($F = 18.6$, $p < 0.05$).

2.2.2 Reduction in Total Coliform (TC) Count in *Villorita cyprinoides*

The initial TC count was found to be 2.4×10^4 MPN/100 mL (Table 2). Reduction of 0.72 log was obtained within the 24 hours of depuration, and a further 1.84 logs (98.54%) reduction was observed after 48 hours of depuration to a final TC count of 2.54 logs (Figure 2). Thus, during the entire 72 h depuration process using a sponge filter, a total reduction of nearly 1.8 logs to a final count of 3.8×10^2 MPN/100 mL could be accomplished. However, complete depuration of TC could not be attained, even after 72 h of depuration. TC load of 2.57 logs remained in shellfish.

The reductions in Total Coliform counts were highly significant across depuration intervals ($F = 27.3$, $p < 0.01$), with the most pronounced effects observed within the first 48 hours.

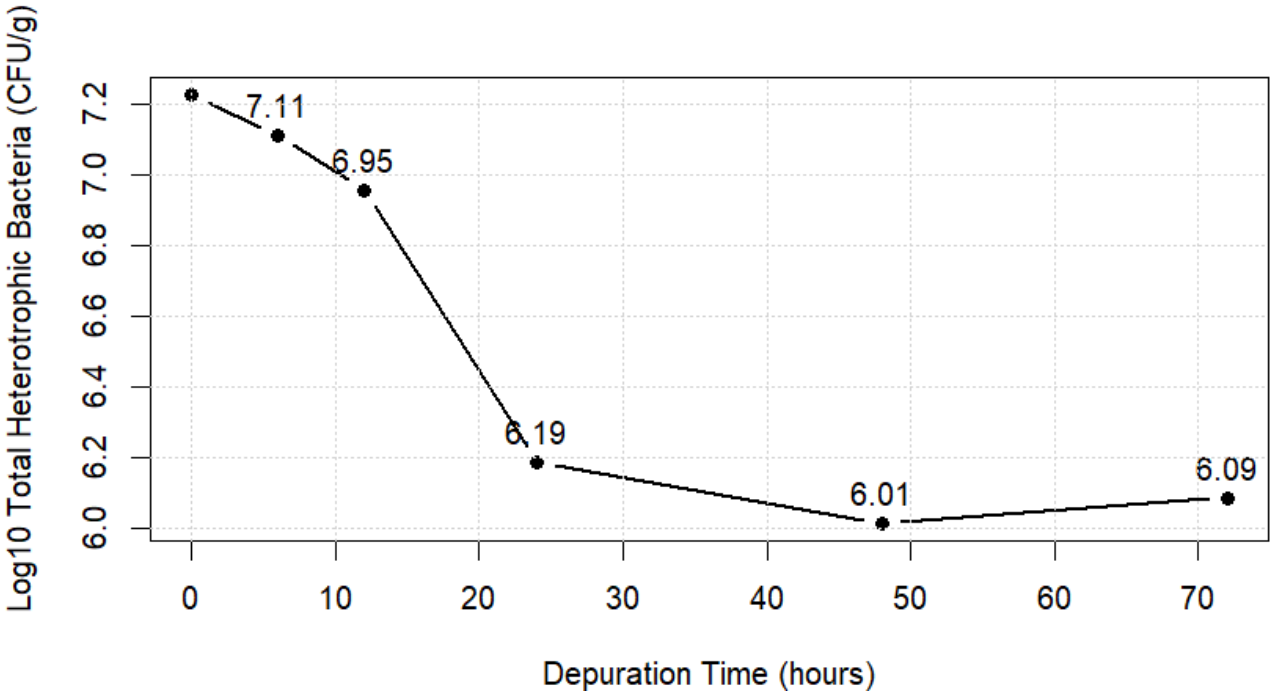

Figure 1 THB reduction in *Villorita cyprinoides* during closed water depuration with a sponge filter

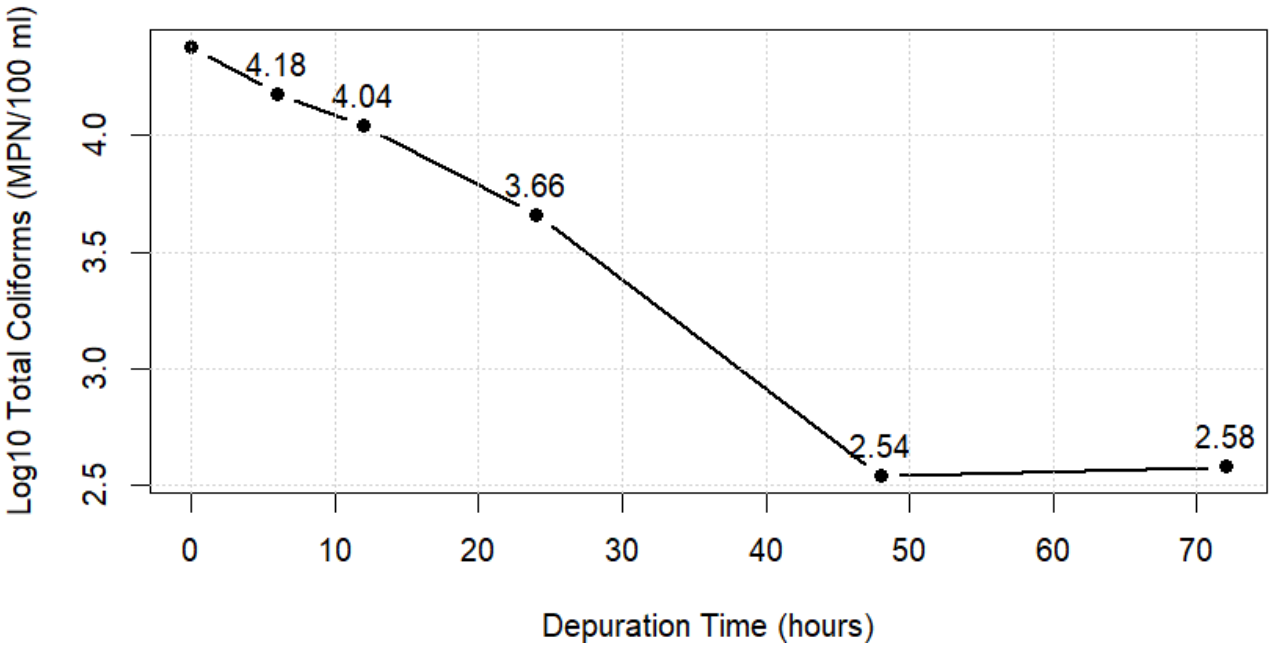

Figure 2 TC reduction in *V. cyprinoides* during closed water depuration with a sponge filter

2.2.3 Reduction in Faecal Coliform (FC) count in *Villorita cyprinoides*

The initial Faecal Coliform (FC) concentration in naturally contaminated *Villorita cyprinoides* from the Cochin Estuary was recorded at 1.5×10^4 MPN/100 mL (4.17 logs), aligning only with Class C shellfish growing area standards under EU regulations. After 48 hours, FC levels decreased to 230 MPN/100 mL (2.36 logs), and by 72 hours, a total removal rate of 98.45% (1.82 logs) was attained, which falls in the acceptable FC regulatory limits of

depurated shellfish (Table 2 and Figure 3). The reductions in Faecal Coliform counts were highly significant across depuration intervals ($F = 25.8$, $p < 0.01$), with the most pronounced effects observed within the first 48 hours.

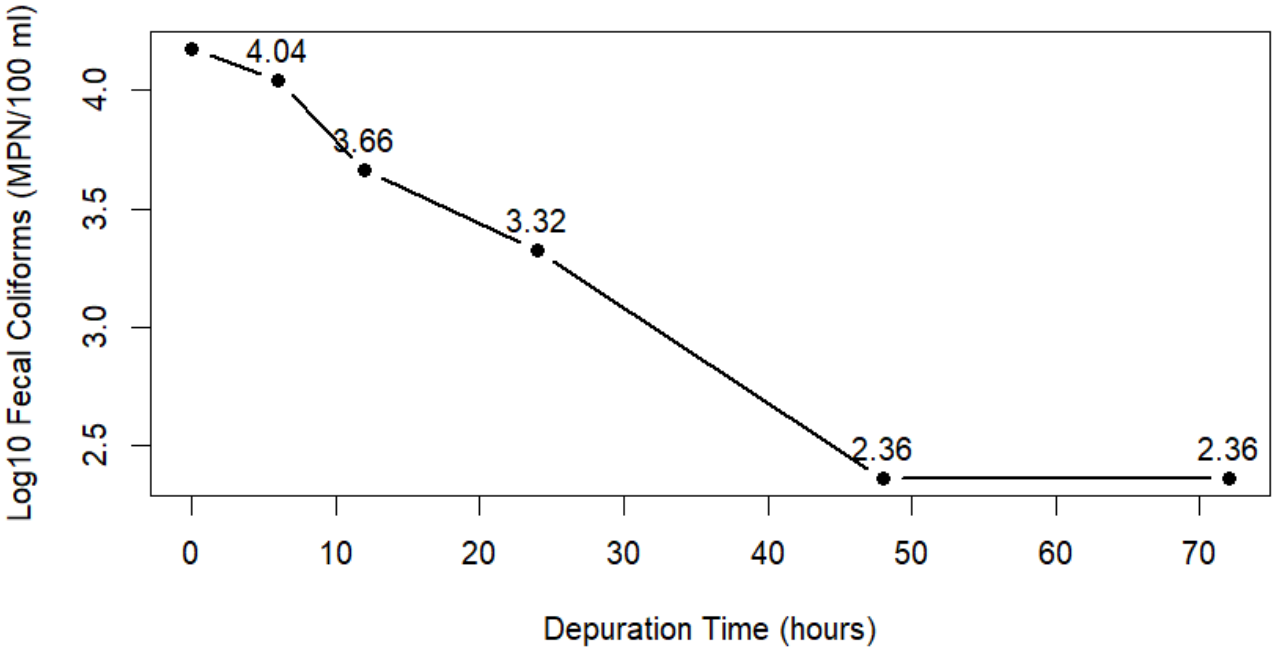

Figure 3 FC reduction in *V. cyprinoides* during closed water depuration with a sponge filter

2.2.4 Reduction in *Vibrio* spp. count in the black clam *Villorita cyprinoides*

The initial *Vibrio* spp. count was found to be $5.1x10^5$ cfu/g. After 48 hours of depuration the *Vibrio* spp. count becomes $1.2x10^5$ cfu/g (reduction around 76.47 %) (Table 2 and Figure 4). Complete depuration of *Vibrio* spp was not achieved within the 72 hours of depuration.

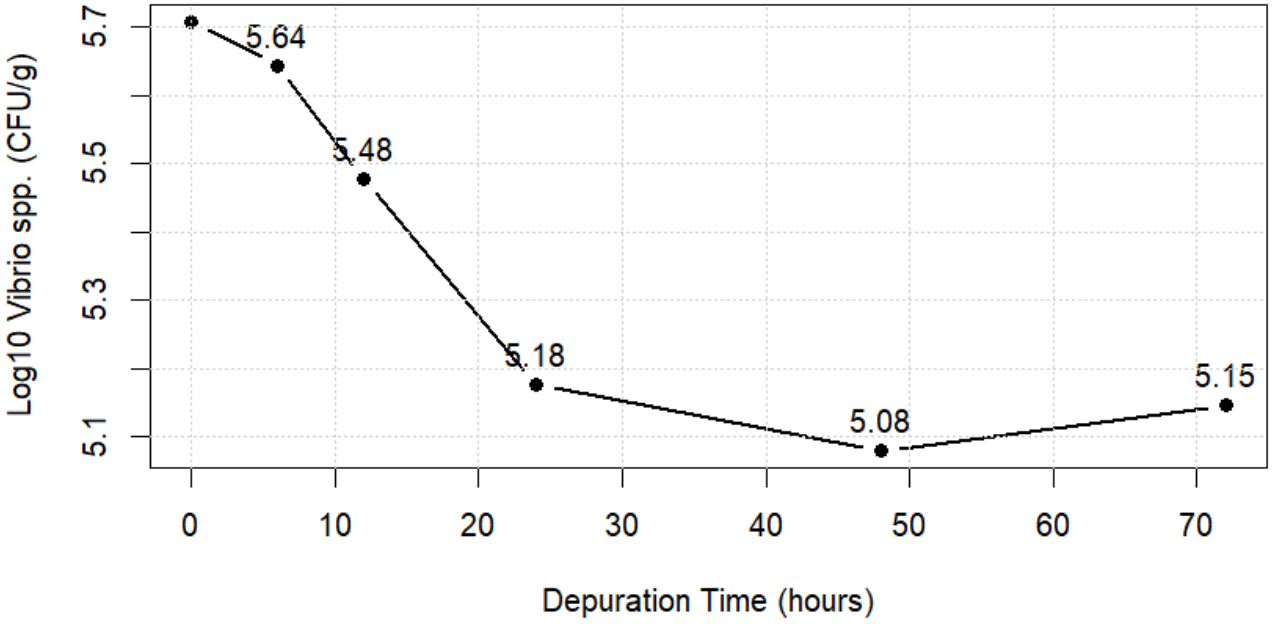

Figure 4 *Vibrio* spp. reduction in *V. cyprinoides* during closed water depuration with a sponge filter

2.3 Bacteriological changes in biofilm-associated microbes on the depuration tank walls during depuration

Bacterial load in the biofilm samples of the depuration tank wall at different depuration time intervals (0 h, 24 h, 48h, 72 h) was recorded for all parameters, including TC, FC, THB and *Vibrio* spp. Count (Table 3). The results showed

a progressive increase in microbial load on the tank walls over time, indicating the formation and accumulation of biofilm-associated bacteria during depuration.

Table 3 Microbial load in biofilm on depuration tank walls at different time intervals

Sample No	Depuration time intervals (Hours)	Total Heterotrophic Bacteria (CFU/g)	Total Coliforms load (MPN index/100mL)	Faecal Coliforms load (MPN index/100mL)	*Vibrio* spp. Load (CFU/g)
1	0.00	1.4×10^3	<3	<3	0
2	24.00	7.5×10^3	21	210	2.9×10^4
3	48.00	1.36×10^5	1100	>1100	2.48×10^5
4	72.00	2.83×10^5	>1100	>1100	2.85×10^6

3 Discussion

The high levels of faecal indicator bacteria detected in raw black clams collected from the Cochin Estuary indicate poor sanitary conditions in the harvesting environment and suggest a potential risk of enteric pathogen contamination. This is consistent with the filter-feeding habit of bivalves, which enables them to accumulate microorganisms from the surrounding water at levels often higher than those in the environment. Similar observations have been reported for shellfish harvested from polluted estuarine systems, where bacterial contamination has been associated with untreated sewage discharge, surface runoff, and other anthropogenic inputs. In the present study, the high initial counts of total coliforms, faecal coliforms, total heterotrophic bacteria, and *Vibrio* spp. confirm that clams collected from the study area may pose a food safety risk if consumed without adequate post-harvest treatment.

The results of the depuration trial demonstrated that the closed-water depuration system equipped with a sponge filter was effective in reducing bacterial contamination in *Villorita cyprinoides.* The most pronounced reduction occurred during the first 48 h of depuration, during which total coliforms, faecal coliforms, and total heterotrophic bacteria declined substantially. This pattern suggests that the early phase of depuration is the most efficient period for bacterial elimination under the present system conditions. Similar findings have been reported in previous studies, where the majority of bacterial reduction in bivalves occurred within the first 24~48 h of depuration. The marked decline observed in the present study indicates that the recirculating sponge filter system provided suitable conditions for effective microbial purging while maintaining clam survival.

Among the microbial indicators examined, faecal coliforms showed one of the highest rates of reduction and reached levels close to accepted regulatory limits after 48 h, further supporting the effectiveness of the system for improving the sanitary quality of the shellfish. In contrast, *Vibrio* spp. showed comparatively lower removal efficiency and persisted throughout the depuration period. This lower reduction may reflect the greater environmental resilience of *Vibrio* spp. and their ability to survive under depuration conditions more effectively than faecal indicator bacteria. The persistence of *Vibrio* spp. observed in this study is important from a food safety perspective, as it indicates that while the system is effective for reducing indicator bacteria, it may be less efficient in eliminating more robust or potentially pathogenic bacterial groups. Therefore, depuration alone may not always be sufficient to completely remove microbiological hazards from shellfish harvested from contaminated environments.

An additional practical finding of this study was the progressive increase in bacterial load on the inner walls of the depuration tank during the later stages of depuration. This trend suggests the formation of biofilms, which may act as reservoirs of microorganisms and contribute to secondary contamination of the system. The slight increase in bacterial counts observed after prolonged depuration may therefore be related to biofilm-associated recontamination. This finding highlights an important operational limitation of closed-water depuration systems: although they are simple, water-efficient, and suitable for small-scale use, their effectiveness depends strongly on proper cleaning and maintenance between depuration cycles. Overall, the present study demonstrates that a sponge filter-based closed-water depuration system can significantly improve the microbiological quality of black clams within a

relatively short period, particularly within the first 48 h. However, regular sanitation and system management are essential to minimize biofilm formation and maintain long-term depuration efficiency.

4 Materials and Methods

4.1 Study area

Live black clams (*Villorita cyprinoides*) were collected from Varapuzha region (10°04′34.85″ N, 76°16′00.33″ E) of Ernakulam District, Kerala, India (Figure 5). This site is an oligohaline zone of the Cochin backwater estuary with salinity recorded at 0.03‰ during the wet season and 0.05‰ during the dry season (Abhilash et al., 2012).

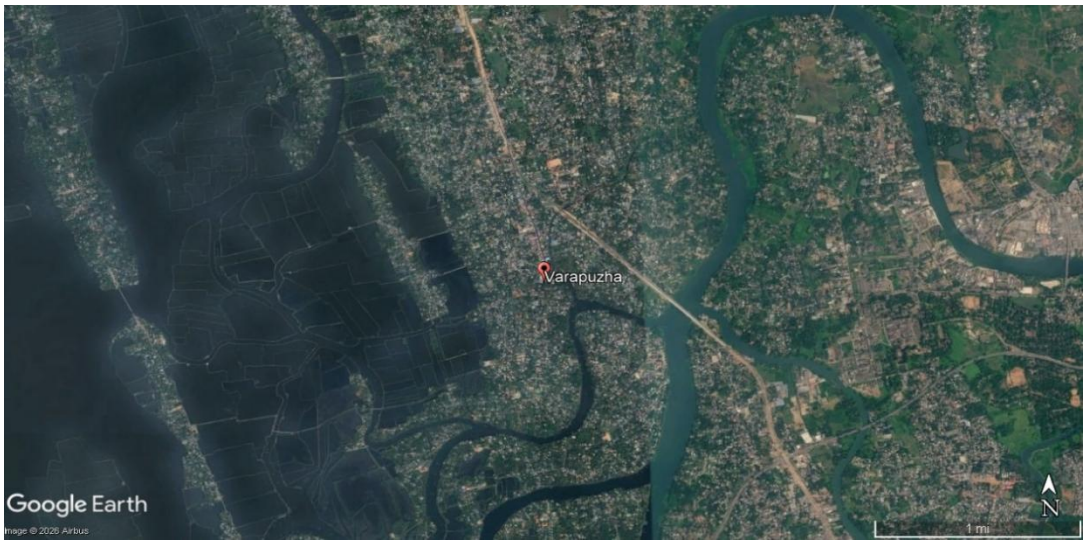

Figure 5 Map showing the sampling site at Varapuzha, Kerala, India

4.2 Collection of shellfish samples

The samples were used for bacteriological analysis and depuration experiments.

Bacteriological analyses were performed on shellfish samples to determine the initial microbial load and to monitor changes during the depuration process. Samples were collected at different depuration intervals (0, 6, 12, 24, 48, and 72 h). Total Coliforms (TC) and Faecal Coliforms (FC) were enumerated using the Most Probable Number (MPN) method, while Total Heterotrophic Bacteria (THB) and *Vibrio* spp. were determined using the total plate count method following standard microbiological procedures (Cappuccino and Sherman, 2014; APHA, 2017). Biofilm samples were also collected from the inner walls of the depuration tank at 0, 24, 48, and 72 h using sterile swabs and analyzed for TC, FC, THB, and *Vibrio* spp. using the same procedures.

4.3.1 Enumeration of Total Coliforms (TC)

Most Probable Number (MPN) method using lactose broth as the medium was used to enumerate total coliform load in the shellfish sample. Ten grams of bivalve tissue is aseptically weighed and homogenized in 90mL of sterile distilled water using tissue homogenizer (Masticator, Spain). From this homogenate 3×10 mL samples were transferred to 10 mL double strength lactose broth. 3×1 mL samples were transferred to 9 mL single strength lactose broth and 3×0.1 mL into 9.9 single strength lactose broth. Tubes were incubated at 37 ºC and observed for growth and gas production in the Durham tubes kept inverted in the test tubes. Gas production after 24 to 48 hours is considered positive for the presence of coliforms. The number of positive tubes in each dilution (10 mL, 1 mL, and 0.1 mL) was recorded and referred to *McCardy's* MPN table to estimate the total coliform load.

4.3.2 Enumeration of Faecal Coliforms (FC)

The tissue was processed as mentioned in the above section 4.3.1, and incubated at 44.5 °C to enumerate faecal coliform in the sample. After the incubation (growth and gas production) positive tubes in each dilution were recorded and referred to McCardy's MPN table to estimate the faecal coliform load.

4.3.3 Enumeration of Total Heterotrophic Bacteria (THB)

Ten grams of tissue was aseptically weighed and transferred into a tissue homogeniser (Masticator, Spain) and homogenised in 90 mL sterile distilled water for one minute. Serial dilutions were made from this homogenised sample by transferring 1 mL to 9 mL sterile distilled normal saline blank. Serial dilutions were made up to 10^{-5}. For enumeration of THB, 0.1 mL of appropriate dilutions (10^{-3} to 10^{-5}) were spread plated on sterile nutrient agar plates. The plates were then incubated at room temperature for 24 to 48 hours. Plates with colony number ranging from 25 - 250 were taken for counting and THB load is expressed as the number of colony forming units per gram of bivalve tissue (cfu/g).

4.3.4 Enumeration of *Vibrio* spp.

For enumeration of *Vibrio* spp., tissue samples were processed as described for THB analysis. Appropriate serial dilutions (10^{-3}~10^{-5}) were spread-plated onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates. The plates were incubated at room temperature for 24~48 h, and plates containing 25~250 colonies were counted. The results were expressed as CFU g^{-1} of bivalve tissue.

4.3.5 Biofilm sample analysis

Biofilm samples were collected from the inner walls of the depuration tank at 0, 24, 48, and 72 h using sterile cotton swabs. The swabs were transferred into sterile conical flasks containing 90 mL sterile distilled water and vortexed to ensure uniform suspension of biofilm-associated bacteria. The resulting suspension was analyzed for TC, FC, THB, and *Vibrio* spp. using the same procedures, media, and incubation conditions described for shellfish samples (Sections 4.3.1~4.3.4).

4.4 Depuration experiment

Prior to the experiment, the collected clams (*Villorita cyprinoides*) were examined to ensure that they were alive and actively filtering. The sampling site, characterized by fine silty-clay substrate rich in suspended particulate organic matter, provided favorable conditions for active siphonal filtration, which was further confirmed by visual observation of siphon extension, valve gaping, and rapid valve closure upon tactile stimulation. Clams showing no response, persistent shell opening, or foul odour were discarded prior to analysis.

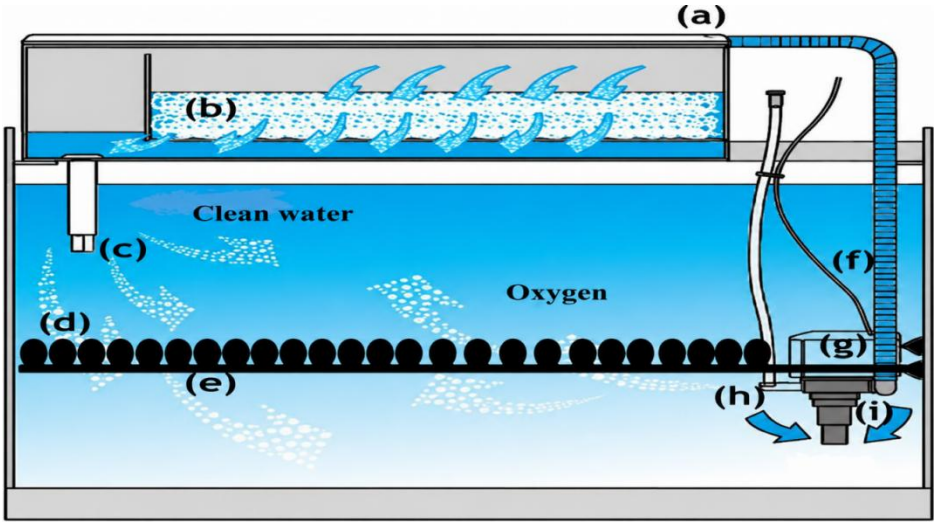

Figure 6 Schematic representation of the depuration tank

Figure caption: (a) Water outlet to bio filter (b) Sponge layer (c) Water outlet to tank after passing through the biofilter (d) Clam (e) Wire mesh (f) Water transporting pipe (g) Water pump (h) Air inlet (i) Water inlet

4.4.1 Design of depuration tank and depuration process

The depuration system consisted of a closed water holding glass tank with a capacity of 55 litres and dimensions 70 x 30 x 30 cm. A wall hung immersion water pump (Dophin P-708, China), placed 10 cm above the bottom of the tank, re-circulated (15 litres/min) the water in the depuration tank which was then passed through sponge filter held

within a holder placed above the tank. For depuration, 45 litres of tap water were used. The experiment was carried out at ambient temperature (29℃~30℃). Approximately, 100 medium sized clams were arranged in monolayer on a plastic mesh rack which was suspended 15 cm above, from the tank bottom to prevent re-contamination from the faecal material settled at the bottom. About 10~15 shellfish were taken out at various intervals of 0 h, 6 h, 12 h, 24 h, 48 h and 72 h using a sterile spatula. Then total coliforms, faecal coliforms, total heterotrophic bacteria and *Vibrio* spp count were enumerated as described above Sections 4.3.1 to 4.3.4. Clams survived well throughout the experiment, however, any dead ones if found were removed from the system immediately. Clams were not fed during the entire period of depuration process.

Author's contributions

Mohamed Hatha conceptualised the work plan, designed the depuration system, reviewed the results and reviewed the manuscript. Kavya, Raj and Suresh carried out the lab work. Kavya also involved in developing the manuscript.

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