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Use of Tilapia Green Water to Eliminate *Vibrio parahaemolyticus* and *Vibrio cholerae* in Cultured Green Mussel, *Perna viridis* (Linnaeus 1758)

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Abstract

Vibrio parahaemolyticus and *Vibrio cholerae* are two widely known pathogens associated with bivalves that cause food-borne diseases including gastroenteritis and cholera. Aquaculture of the green mussel *Perna viridis* (Linnaeus 1758) is an important source of income for coastal and island communities in the Philippines, but issues regarding *Vibrio* bacterial load in this bivalve limits its market potential. Various depuration methods have been tested with ultraviolet light (UVL) method found to be highly efficient, widely practised but reported to be ineffective in removing pathogenic *Vibrio* species. The present study evaluates the potential of a biological approach using tilapia green water (TGW) in eliminating the pathogenic *Vibrio* species associated with the green mussel. Results show that the TGW can significantly decrease the level of *V. parahaemolyticus* and *V. cholerae* down to the safe level at 10^4 CFU.g^{-1} tissue after 76 h of exposure. Depuration of pathogenic *Vibrios* with TGW was found comparable to the treatment using the standard UVL depuration method. Overall, the present findings provide evidence that depuration using TGW can effectively decrease bacterial load of *V. parahaemolyticus* and *V. cholerae* in the green shell *P. viridis.*

Keywords: *Vibrio parahaemolyticus, Vibrio cholerae, Perna viridis,* tilapia green water, depuration, mussel

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Introduction

Perna viridis (Linnaeus 1758) is one of the top farmed commodities in the Philippines. According to the 2016 Philippines Fisheries Statistics, the total mussel production is estimated at 18,774.55 t that contributes USD 51,216,138.00 to the total aquaculture production value (Philippines Statistics Authority 2016).However, shellfish consumption has been linked to food-borne illnesses associated with *Vibrio* species (Aagesen et al. 2013; Agnes et al. 2015). Filter feeders such as molluscan bivalves are known to accumulate microorganisms for these bivalves are farmed in shallow or near-shore estuarine waters, where organic loads are relatively high. Therefore, these bivalves are likely to contain high bacterial loads including human pathogenic *Vibrio* species specifically *Vibrio parahaemolyticus* and *Vibrio cholerae* that are known to be abundant in coastal environments (Food and Environmental Hygiene Department, FEDH 2005). *Vibrio* spp. are Gram-negative, facultative, anaerobic rod-shaped bacteria with twelve identified species that causes food-borne illnesses (Food and Agriculture Organization, FAO 2011). Among these species, *V. parahaemolyticus and V. cholerae* are the most problematic due to their widespread prevalence and pathogenicity (Stauder et al. 2012; Aagesen et al. 2013). *Vibrio parahaemolyticus* is known to cause three distinct medical conditions: gastroenteritis, wound infections, and septicemia, while *V. cholerae* is known to cause cholera. Reports on *V. parahaemolyticus and V. cholerae* occurrence in mussels and disease outbreaks associated with eating these bivalves have been well documented (Rippey 1994; Croci et al. 2002; Lee et al. 2008; Nakaguchi 2013).

Various depuration processes were developed to eliminate pathogenic *Vibrios*. The United States Food and Drug Administration (USFDA) prefers the use of ultraviolet light (UVL) disinfection over other depuration methods such as ozone and chlorine treatment as it has lower residuals that either affects the bivalve's marketability or impose a potential threat to the consumers (Lee et al. 2008). However, Lee et al. (2008) reported that the existing depuration method can only effectively remove bacterial indicators such as *Escherichia coli* and *Salmonella*, but cannot significantly decrease *Vibrio* species. Ultraviolet (UV) depuration cannot entirely stop bacterial replication process because of the photoreactivation, a phenomenon in bacterial cells that enable them to counteract damaging activities caused by natural UV radiation (Lee et al. 2008). Additionally, the *V. parahaemolyticus* and *V. cholerae* were documented to adhere to bivalve cells aided with their N-acetyl glucosamine-binding protein A, type I and type IV pilus proteins (Stauder et al. 2012; Aagesen et al. 2013). These adhesion molecules ensure that the bacteria are well attached inside the gut, preventing its release to the water and eventual exposure to UV radiation during the depuration process. The failure of UVL depuration to eliminate *V. parahaemolyticus* and *V. cholerae* in mussel, *Mytilus galloprovincialis* (Lamarck 1819), is well documented (Croci et al. 2002). The use of biocontrol, specifically the use of tilapia green water (TGW) system to prevent and suppress the growth of pathogenic *Vibrios* in shrimp culture has been well documented (Defoirdt et al. 2007; Dash et al. 2017). Evidence suggests that TGW water supports a complex species of bacteria and fungi that produce low molecular weight metabolites that are inhibitory to the growth of several *Vibrio* species (Tendencia et al. 2003; Lio-po et al. 2005).

Further, it has been shown that the *Vibrio*-inhibitory activity of TGW has been attributed to the mucus produced by tilapia that exhibits potent antibacterial activity against *Vibrio* species (Wibowo et al. 2015). Antibacterial metabolites of green microalgae associated with the TGW culture system have also been reported as the major factor in the inhibition of *Vibrio* growth (Taniguchi et al. 2011; Cadiz et al. 2016). All of these earlier reports indicate the high potential of TGW to suppress and prevent the growth of aquatic *Vibrio* species. However, this system has not been reported in the elimination of *Vibrio* species associated with bivalve mollusc. The present study is the first to investigate the possibility of the biological approach using TGW in eliminating pathogenic *Vibrio* species in cultured mussels.

Materials and Method

Experimental animals

The green mussels were obtained from local growers in the Northern Region of Panay (Capiz, province) Island, Philippines and transported to UPV-Multi-Species hatchery laboratory. The mussels were carefully packed in sealed containers with ice packs to maintain a temperature of 20 ^{o}C to minimise transport stress and avoid mortality. Upon arrival, the mussels were cleaned by removing barnacles and other fouling organisms. Mussels with intact byssus thread were selected, washed three times with seawater and transferred to the holding tank (500 L capacity fibreglass tank) and supplied with ample aeration. *Chaetoceros calcitrans* was given *ad libitum* (maintained at a cell density of 1×10^5 cells.mL^{-1}) daily for 3 days to allow full recovery. Other maintenance routine included daily 100 % water change and removal of dead mussels and other unwanted organisms. After acclimatisation, the mussels with a length of 3–4 cm were selected and carefully transferred to the experimental tanks.

Preparation of tilapia green water

Before the depuration experiment, TGW was prepared in 5 tonne fibreglass tank filled with filtered seawater at a salinity of 24 ppt and provided with continuous aeration. The tank was covered with a corrugated plastic sheet to allow normal light penetration and promote algal photosynthesis. Saline tilapia hybrid *Tilapia mossambica* (Peters 1852) × *Tilapia nilotica* (Linnaeus 1758) maintained at biomass of 500 g.m^{-3} as suggested by Corre et al. (2015) was used in the experiment. The fish was given maintenance diet at 1 % of biomass twice a day at 8:00 a.m. and 5:00 p.m. Water quality was monitored daily and maintained to the optimum requirement of tilapia throughout the experimental period. Following the 7 days of tilapia culture, the natural bloom of green algae, comprising *Chlorella* sp. and *Nannochloropsi*s sp. occurred in the tank reaching a cell density of 1×10^5–1×10^6 cells.mL^{-1}. This algal cell density was maintained in the tank and during the peak of algal bloom new water was added to attain the desired cell densities. In maintaining the TGW, no fertiliser was used since the natural ammonia produced by the fish serves as natural fertiliser for the green algae. Half of the water was changed every 5 days to prevent organic load accumulation in the TGW tank. The change of water was also to ensure continuous bloom of the green algae in the tank.

Depuration method using biological approach

The TGW depuration tank is shown in Figure 1. Each tank is equipped with an elevated purported plastic tray, serving as a false bottom that separates the mussels from the tank bottom were biological wastes could accumulate. Below the depuration tank is another reservoir tank of 5 tonne capacity filled with 24 ppt filtered seawater, used to hold the tilapia and provides the green water for the depuration. A 100-watt water pump (BNH, Philippines) is used to supply the green water to the depuration tank. TGW was supplied at a rate of 300 % tank-volume a day. A similar setup was used in the negative treatment group but instead of green water filtered seawater was filled in the reservoir tank and for the positive treatment group, the seawater supply was passed through a 30 W UV light (BNH, Philippines) going into the depuration tank.

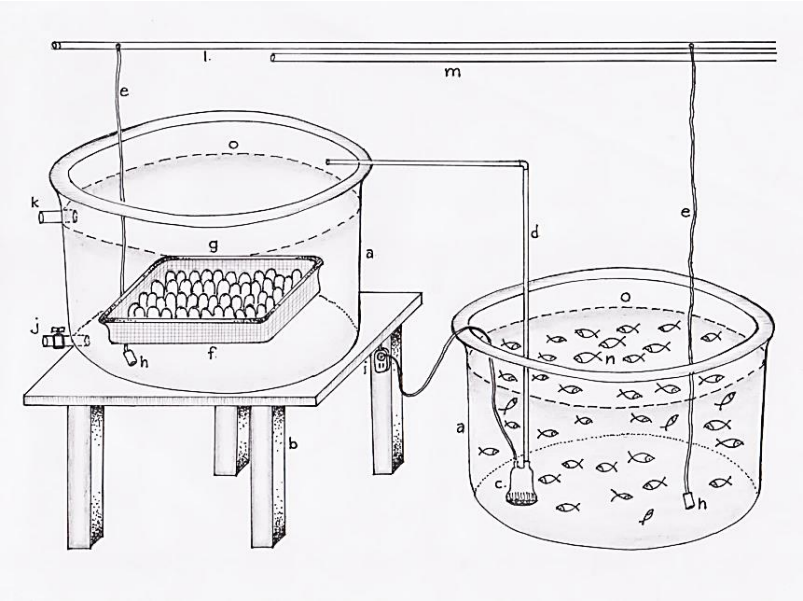

Fig. 1. The depuration setup used in the experiment in treatment with tilapia green water. Where: a, are the fiberglass tanks where tilapia is stocked and the depuration tank where the mussels are submerged; b, is the table holding the depuration tank with the mussel; c, is the submersible pump; d, is the supply pipe of green water from the reservoir going to the depuration tank; e, are aeration hose supplying air to the depuration tank and the tilapia reservoir tank; f, is the tray holding the mussels in the holding tank; g, refers to the mussels being depurated; h, are air stones; i, is the electrical outlet; j, is the depuration tank drain pipe setup; k, is the water level overflow pipe of the depuration tank; l, is the aeration tube and m is the seawater supply tube.

Depuration experiment was conducted following random completely blocked design (RCBD) designed with three treatments run in triplicate. Four hundred fifty individuals of healthy mussels were selected and distributed equally to nine 75 L depuration tanks comprising the three treatment groups consisting of the negative control (NC; filtered seawater), the positive control (PC; UVL depuration), and the TGW depuration treatment group. Tanks were arranged in random and the salinity of seawater in each treatment was maintained at 24 ppt.

Each treatment group was supplied with proper aeration and experimental animals were not fed during the entire experiment. Water temperature was maintained at 25–28 °C to prevent mussel mortality. The experiment was conducted for 76 h and sampling for bacterial counting done every 24 h.

Quantification of Vibrio

Mussel samples from each replicate treatment tanks were collected and processed for bacteriological examination. An initial bacterial count was analysed from 10 mussels prior to the distribution of these animals in their respective treatment tanks. During every sampling, three mussels were collected from each corresponding replicate tanks. The animal for analysis was subjected to cold temperature to anaesthetise and dissected using sterile scissors. The whole mussel meat was homogeniszed using a sterile micro-homogeniser. From the homogenate, a sample of 0.1 g was collected and subjected to complete homogenization using a sterile tissue homogeniser. The prepared tissue homogenates were subjected to a series of ten-fold dilutions in an Eppendorf tube and aliquot of 100 µL were plated in a *Vibrio* chromogenic agar (Pronadisa, Spain). Inoculated Plates were incubated at room temperature for 18–24 h. Colonies with solid blue-green colours were counted as *V. parahaemolyticus* and light purple coloured colonies were counted as *V. cholerae* (as per manufacturer's instructions).

CFU.g^{-1} was calculated using the formula:

$$D = \frac{C}{Vi \times DR} \times \frac{Vh}{W}$$

where: *D*, bacterial density in the tissues (colony forming units (CFU.g^{-1} tissue); *C*, colony numbers on the agar plates; *Vh*, volume of the tissue homogenates (mL); *Vi*, volume of the homogenate diluents inoculated onto the agar plates (mL); *W*, weight of the tissue homogenates (g); *DR*, dilution rates of the homogenates.

Vibrio parahaemolyticus and *V. cholerae* in the depuration water of each treatment were also quantified using the *Vibrio* chromogenic agar. Water from the depuration tank was collected, brought to the laboratory and analysed. A series of serial dilutions were made and 0.1 mL aliquot was prepared and plated in *Vibrio* chromogenic agar as described above.

Statistical analysis

One-Way ANOVA was used to determine the statistical differences between the treatment means at $P < 0.05$. If significant differences were observed, the data was subjected to a Tukey post hoc analysis test to determine the significant differences between the treatment means. All statistical analyses were conducted using SPSS version 16.

Results

Depuration method comparing the biological approach to the standard physical depuration method using UVL was tested to eliminate the pathogenic *V. parahaemolyticus* and *V. cholerae* associated with the green mussel. No mortalities of mussels were observed in all the treatments during the depuration process. Following the 24 h depuration process, a decreasing trend in the level of *V. parahaemolyticus* was observed in all treatment groups except the NC group where the *V. parahaemolyticus* count remained high (10^8 and 10^7 CFU.g^{-1} tissue) until the end of the depuration period (Fig. 2). Both the PC and the TGW group exhibited drastic decline in *V. parahaemolyticus* counts that are significantly lower than that of the NC group at the 24 h of depuration. During the 48 h of depuration the *V. parahaemolyticus* count in the PC group remained at a steady level (10^4 CFU.g^{-1} tissue) that prevails until the 76 h of the depuration. In contrast, the TGW group exhibited a logarithmic reduction in *V. parahaemolyticus* count until reaching the lowest count at 10^4 CFU.g^{-1} tissue on the 76 h of depuration. Analysis of the culture water where the experimental animals are maintained during the depuration indicates no presence of *V. parahaemolyticus* and *V. cholerae* in the UVL group. *Vibrio cholerae* was present at around $<10^1$ CFU.mL^{-1} with *V. parahaemolyticus* count at around 10^1 CFU.mL^{-1} in NC. None of these *Vibrios* were found in the TGW group.

In contrast to the trend observed in *V. parahaemolyticus* counts, all the treatment groups exhibited a significant decline in *V. cholerae* counts after the 24 h depuration period. However, at 24 h of depuration, both the PC and the TGW group exhibited an almost similar magnitude of response lowering the *V. cholerae* to about 10^5 CFU.g^{-1} tissue that is significantly lower than the NC with *V. cholerae* count of 10^7 CFU.g^{-1} tissue. As the depuration progresses, *V. cholerae* count in the NC group remained steady at 10^7 CFU.g^{-1} tissue until the 76 h of the depuration process. *Vibrio cholerae* count in PC group during the 48 h of depuration followed a linear trend exhibiting no change in the bacterial count (10^5 CFU.g^{-1} tissue) that remained steady until the 76 h of depuration. In contrast, a significant declining trend in *V. cholerae* count is imminent from 24 h to 48 h at the TGW group. After reaching the lowest bacterial count at $\approx 10^4$ CFU.g^{-1} tissue on the 48 h, the bacterial count (*V. cholerae)* remained similar until the 76 h of depuration (Fig. 3). Collectively these results suggest that depuration by TGW is comparable to the depuration by UVL in lowering the *V. cholerae and V. parahaemolyticus* tissue content of green mussel to the level that is acceptable (10^4 CFU.g^{-1} tissue) as safe for human consumption (Center for Food Safety and Applied Nutrition-Food and Drug Administration, CFSAN, 2005).

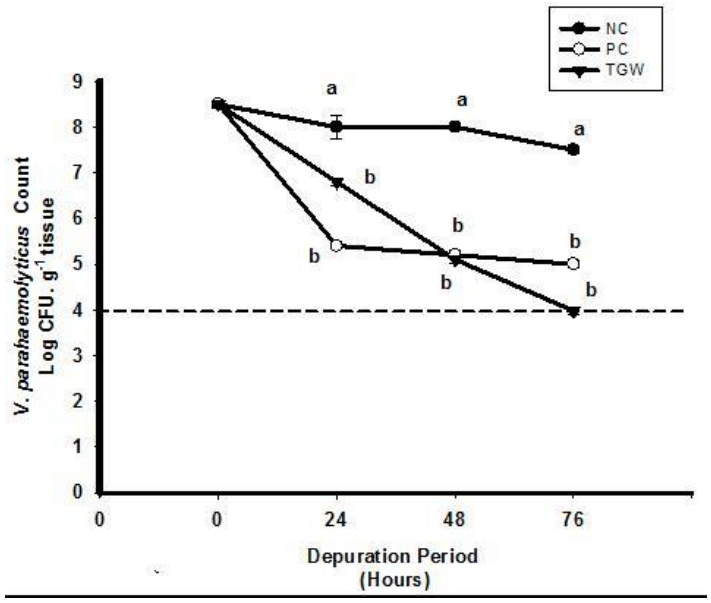

Fig. 2. *Vibrio parahaemolyticus* counts during each sampling period. Black Circle, (NC) Negative Control (Filtered Seawater); White Circle (PC), Positive Control (UV treated seawater); Black Triangle (TGW), Tilapia Green Water. Values in each sampling point having different superscript letters are significantly different ($P < 0.05$). The broken line represents the baseline of the safe level of *V. parahaemolyticus* in mollusc ($1x10^4$ CFU.g^{-1} tissue).

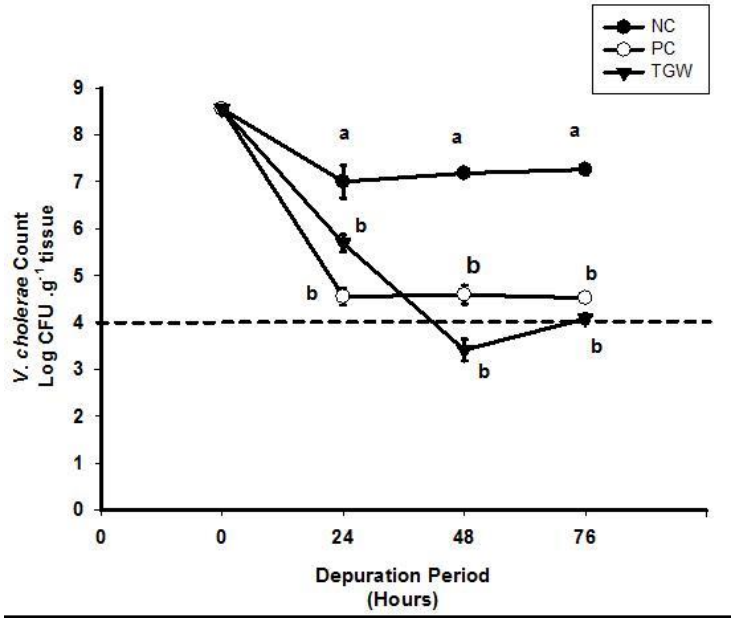

Fig. 3. *Vibrio cholerae* counts during each sampling period. Black Circle, (NC) Negative Control (Filtered Seawater); White Circle (PC), Positive Control (UV treated seawater); Black Triangle (TGW), Tilapia Green Water. Values in each sampling point having different superscript letters are significantly different ($P < 0.05$). The broken line represents the baseline of the safe level of *V. parahaemolyticus* in mollusc ($1x10^4$ CFU.g^{-1} tissue).

Discussion

Foodborne illnesses, caused by pathogenic *Vibrio* species, associated with eating raw and partially cooked bivalve mollusc have become a serious problem of global concern (Broberg et al. 2011). As consumption of mollusc increases coupled with the current increase in sea surface temperature, promoting the dominance of autochthonous bacteria in coastal waters, the risk of foodborne *Vibrio* infection is expected to rise (Rippey 1994). Various depuration techniques have been developed in attempts to address this concern. However, the existing techniques are considered inadequate when it comes to the removal of pathogenic *Vibrio* species associated with bivalve mollusc (Croci et al. 2002). In an attempt to provide solutions to the pressing issues regarding bivalve mollusc natural ability to harbour entero-pathogenic *Vibrio* species, this study was designed to evaluate biological approach in depurating the green mussel, *P. viridis* to lower the load of *V. parahaemolyticus* and *V. cholerae*.

In this study, it was observed that *V. parahaemolyticus* tend to decrease in all groups as the depuration proceeds. The decreasing trend of *V. parahaemolyticus* and *V. cholerae* in the negative control (NC; filtered seawater), though not statically different at every sampling period, might be attributed to the water exchange rate. *Perna viridis* actively pumps water and particles by taking it in through its inhalant siphon, passing it through the gills for filtration and discharging it out through the exhalant siphon. This process is known to partially remove bacterial cells associated with the mussel (Soon and Ransangan 2014). However, the NC treatment was not effective in lowering the pathogenic *Vibrio* count to the safe level of 10^4 CFU.g^{-1} tissue (Center for Food Safety and Applied Nutrition-Food and Drug Administration, CFSAN 2005). Thus water-based depuration is not effective as the remaining bacterial cells in the tissue may multiply to compensate for the cells removed during the exchange of new seawater in the system. In contrast, the positive control (PC; UVL treatment) was able to lower levels of *V. parahaemolyticus* in green mussel to a level that is 3 Log lower (from 10^8–10^5) than the initial count. However, the PC method seems inadequate since the treatment was not able to eliminate the *V. parahaemolyticus*. According to Lee et al. (2008) the existing depuration method can only effectively remove bacterial indicators such as *E. coli* and *Salmonella*, but cannot significantly decrease *Vibrio* species. This is due to the photoreactivation phenomenon by which bacterial cells can counteract damaging activities caused by natural UV radiation (Lee et al. 2008).

Photolyases, an enzyme responsible for the photoreactivation, has been reported in *V. parahaemolyticus* (Zehong et al. 2015). Starke-Griessler et al. (2013) stated that photoreactivation showed recovery after UV irradiation up to 100 % for a UV fluence of 50 J.M^{-2} and below. Furthermore, the results of this present work is comparable with the result of by Lopez-Joven et al. (2011), wherein both sucrose-fermenting and non-sucrose fermenting *Vibrio* species were observed to persist in the clam even on the 216 h of combined UV and filtration depuration processes. In addition, Lopez-Joven et al. (2011) reported that non-sucrose fermenting *Vibrio* (involved in food poisoning) could thrive even after 9 days (216 h) of UVL depuration in warmer conditions.

In contrast, the findings of Loredana et al. (2009) reported that UV treatment in combination with the lower water temperature at 15 °C was able to eliminate *V. parahaemolyticus* in experimentally contaminated temperate bivalve *M. galloprovincialis* depurated for 72 h. *Vibrio parahaemolyticus* and *V. cholerae* are not accustomed to low temperature as their optimum requirement to achieved maximum growth at 37 °C (Kim et al. 2012). The low temperature in the study of Loredana et al. (2009) may have limited the reproductive capacity and mobility of *Vibrio* rendering it susceptible to the UV irradiation. Conversely, no interventions in the culture temperature were performed in the present study since *P. viridis* is a tropical bivalve and were observed moribund if exposed to a temperature lower than 23 ^{o}C for at a period of 76 h.

The TGW treatment was able to lower the pathogenic *Vibrio* species associated with *P. viridis* to about $\approx 10^4$ CFU.g^{-1} tissue after 76 h of depuration that is almost 5 Log lower than the initial count and was found not significantly different with the PC group. The significant decrease in both *V. parahaemolyticus* and *V. cholerae* in the TGW treatment is highly apparent when compared to the NC group that exhibited 3 Log higher bacterial counts. The TGW treatment indicates the significant *Vibrio*-inhibitory influence in green mussel, *P. viridis.* Further, in the present study, TGW depuration was documented comparable to the standard method of UVL depuration in the removal of *V. parahaemolyticus* and *V. cholerae* in *P. viridis* as observed in the PC treatment group. Although studies on the inhibitory effect of TGW on *V. parahaemolyticus* are scarce, especially in bivalve species, the result of the study can be attributed to the tilapia mucus. Wibowo et al. (2015) reported that tilapia mucus ethanol extract exhibited potent inhibitory activity against *V. harveyi*.

Similar observations were recorded by Tendencia et al. (2003) in a laboratory experiment wherein groups with *Tilapia hornorum* (Trewavas 1966) inhibited the growth of luminescent *Vibrio* in tank culture. This inhibitory activity was found associated with *Chlorella* algae growing in the TGW tank. The result of the present study is also comparable to the recent results reported by Cadiz et al. (2016) wherein the TGW exhibited a lower *V. parahaemolyticus* counts in an intensive *P. vannamei* culture. This *Vibrio* inhibition was found highly correlated with the density of *Chlorella* and *Nannochloropsis* growing in the tank with tilapia. Low molecular weight compounds associated with algae in TGW was also reported to inhibit *Vibrio* growth (Lio-po et al. 2005).

Further several species of bacteria and yeast associated with TGW were also documented to produce metabolites exhibiting anti-*Vibrio* activities (Lio-Po et al. 2005). Also Taniguchi et al. (2011) have elucidated that the green algae *Nannochloropsis oculata* have natural inhibitory activity against *V. parahaemolyticus*. *Vibrio parahaemolyticus* counts decreased during the peak of algae photosynthesis. The decrease of *V. parahaemolyticus* is believed to be due to the algal low molecular weight photosynthetic by-products including short chain fatty acids that are responsible for the inhibitory activity (Lio-Po et al. 2005; Taniguchi et al. 2011). All of these earlier reports support the findings of the present study that TGW could effectively inhibit the growth of pathogenic *Vibrio* species including *V. cholerae* and *V. parahaemolyticus* that are associated with the green mussel *P. viridis*.

Though the mechanisms of inhibition were not elucidated in the present study and these aspects warrants intensive investigation in the future to fully realise the potential of TGW in suppressing the growth of *Vibrio* species in aquatic products.

Conclusion

This study concludes that depuration using TGW is comparable to that of using UV irradiation to reduce the levels of *V. parahaemolyticus* and *V. cholerae* in *P. viridis* to safe levels. The present depuration method using TGW could be easily adopted and highly applicable in small island communities where mussel farming is practiced but product depuration is considered challenging due to erratic or absence of electricity.

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