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Effect of three innovative culture systems on water quality and whitespot syndrome virus (WSSV) viral load in WSSV-fed *Penaeus monodon* cultured in indoor tanks

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ABSTRACT

White spot syndrome virus is the most important among the shrimp diseases. It has been devastating the shrimp industry for more than 3 decades. Previous studies reported that greater percentage of yellow colonies on thiosulfate citrate bile salt sucrose agar (yellow vibrios) in the rearing water, abundant supply of natural food such as *Chlorella*, and the use of the greenwater technology (GW) are some ecological ways of preventing WSSV outbreak. The aim of this study was to investigate the efficiency of the 3 systems against WSSV.

Shrimp, experimentally infected with WSSV by feeding with WSSV positive shrimp carcass, was cultured in tanks using three treatments: with tilapia to simulate the GW, seeded with *Chlorella*, and with molasses added to enhance growth of yellow vibrios. Shrimp cultured in seawater served as the control. Survival was recorded and shrimp were analyzed for WSSV quantification using qPCR upon termination.

Analysis showed no significant differences in shrimp survival at 120 h post infection in all treatments and the control. However, from the original viral load of 1.40×10^{1} WSSV/mg sample, WSSV decreased and was significantly lowest in shrimp cultured using GW (7.0×10^{0}), compared to the control (4.82×10^{5}) and the other treatments (3.66×10^{5} for molasses added and 4.64×10^{5} for Chlorella seeded) in which viral load increased 4–5 times. Shrimp survival was highest in *Chlorella* seeded treatment and lowest in GW. Nitrogenous waste concentrations were lowest in molasses added water and highest in GW.

Results suggest that the GW culture technology provides protection against WSSV while addition of molasses lowers nitrogenous waste concentration. The use of GW in combination with the addition of molasses for shrimp culture is suggested.

1. Introduction

Among the viral diseases of shrimp, white spot syndrome virus (WSSV) is the most pathogenic and the longest existing virus with no known proven prevention or control measure (Flegel, 2009). The disease has caused great losses to the shrimp industry and has prompted local shrimp farmers in the Philippines to try innovative techniques of culturing *Penaeus monodon* that prevent WSSV outbreaks. Some of these techniques are the use of greenwater technology (GW) and the enhancement of the growth of yellow colonies on thiosulfate citrate bile salt sucrose agar (yellow vibrios), a *Vibrio* selective medium, by adding molasses into the culture pond water. A cross sectional study done on shrimp farming practices in the Philippines identified feeding with natural food, i.e. algae such as *Chlorella*, as a WSSV protective factor (Tendencia et al., 2011).

In the greenwater technology (GW), shrimp are cultured in water collected from a pond where tilapia or other fish species are grown. Two GW are used by shrimp farmers: 1) shrimp and finfish are cultured in two separate ponds; water used to culture shrimp comes from the finfish pond; 2) the finfish are polycultured with shrimp by stocking in isolated nets or happas inside the shrimp culture pond (Tendencia et al., 2004). Different species can be used to bring the greenwater effect but tilapia is the best candidate species (Tendencia et al., 2006). The greenwater culture system has been reported to be effective against luminous bacteria (Corre et al., 2005) caused by *Vibrio harveyi* (Lavilla-Pitogo et al., 1990) at a tilapia biomass not lower than 300 g/m^3 to every 80 g/m^3 shrimp biomass (Tendencia et al., 2004). The effectiveness of the GW against luminous bacteria has been attributed to the presence of anti-*V. harveyi* factors in the associated bacterial, fungal, and phytoplankton microbiota, the skin mucus and feces of tilapia and bacteria from the skin mucus and feces of tilapia (Lio-Po et al., 2005; Tendencia and Dela Peña, 2010).

Shrimp farmers in Negros Island, Philippines added molasses (5 ppm) into the pond water to enhance the growth of the yellow vibrios. Most of the pathogenic vibrios, like *V. harveyi*, *V. parahaemolyticus* and *V. anguillarum*, produce green colonies on TCBS (green vibrios; Diggles et al., 2000; Junpeng et al., 2007; Travers et al., 2009). Vibrios

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that form yellow colonies (yellow vibrios), like *V. alginolyticus* and *V. gazogenes*, usually have probiotic effect (Austin et al., 1995; Thompson et al., 2010). Higher percentage of yellow *Vibrio* compared to the green ones is reported to be a WSSV protective factor (Tendencia et al., 2011). Shrimp farmers are not aware of the mechanism behind the use of molasses to enhance the growth of the yellow vibrios but it may be related to the biofloc technology. In biofloc technology an organic substrate, like molasses, is used to enhance the growth of heterotrophic bacteria and to control accumulation of toxic nitrogenous wastes (Schneider et al., 2006).

Feeding with natural food, i.e. green algae, has been reported to be a WSSV protective factor (Tendencia et al., 2011). Corre et al. (1999) reported that in shrimp ponds in the Philippines, the density of *Chlorella* is 10^6 cells/ml. However, Cremen et al. (2007) reported a lower density of 10^5 cell/ml of green algae of the class Chlorophycea, with *Nanochloropsis* and *Chlorella* as dominant species, in commercial farms in Iloilo, Philippines. Antibacterial property of the aqueous extract and pure cultures of *Chlorella* spp. have been reported (Das and Pradhan, 2010; Makridis et al., 2006). Aside from the antibacterial property, *Chlorella* has been reported to improve pond water quality (Hernandez et al., 2009; Sivakumar et al., 2011).

This paper evaluated the effect of using GW, adding molasses to enhance the proliferation of yellow vibrios, and seeding of *Chlorella*, on shrimp WSSV load and on water quality, in tank-based experiments.

2. Material and methods

2.1. Experimental shrimp

P. monodon were maintained in 20 ppt rearing water at ambient temperature for 1 week prior to use. The shrimp sent to the Fish Health Section of the Southeast Asian Fisheries Development Center Aquaculture Department for viral detection using polymerase chain reaction techniques were diagnosed to be free from white spot syndrome virus (WSSV), Taura syndrome virus (TSV), infectious hypodermal hemapoietic necrosis virus (IHHNV), yellowhead virus (YHV), infectious myonecrosis virus (IMNV), monodon baculovirus (MBV) and hepatopancreatic virus (HPV) before purchase and before use. The shrimp were fed to satiation with WSSV positive carcass by broadcasting the carcasses into the rearing tank, 24 h before transfer to experimental tanks.

2.2. Culture facility and experimental treatment

Twelve indoor fiberglass tanks (54.5 cm × 40.0 cm × 54.5 cm) were prepared for the 3 treatments with three replicates each: greenwater using tilapia (*greenwater*), use of *Chlorella* as natural food (*chlorella*), and addition of molasses to enhance growth of yellow *Vibrio* (*molasses*), and control using plain seawater (*seawater*). The fiberglass tanks were filled with UV-sterilized seawater diluted with cartridge-filtered (5 μm) freshwater to attain the required salinity of 20 ppt to a volume of 100 l. Ambient temperature was maintained. The tanks were provided with aeration and covered with black cloth and framed netting material to avoid contamination through aerosol and the escape of shrimp.

Tanks for the *greenwater* treatment were stocked with tilapia, *Oreochromis mossambicus* (n = 6; ABW = 55, biomass = 330), 14 days before the experiment and reduced to 3 fish (biomass = 165) per tank, a day before the experiment. The increased biomass was implemented 14 days prior and reduced a day before the experiment in an attempt to hasten production of substances present in greenwater using tilapia in ponds wherein tilapia are stocked 1 month before water is used for shrimp culture.

Tanks for *molasses* was prepared a day before the experiment by adding molasses (5 ppm) into the tanks. The same concentration of molasses was added into the tank daily until termination of the experiment. Molasses available in supermarkets in smaller quantities was used in the experiment.

Tanks for *chlorella* were seeded with the microalgae to a final density of 10^6 cells/ml 1 h before the experiment and daily thereafter. The *Chlorella* density was the density observed for *Chlorella* in shrimp farms (Corre et al., 1999). The *Chlorella* used in this study was isolated from a brackish water pond in Iloilo, Philippines and mass produced in outdoor concrete tanks.

Shrimps (ABW = 4 g) previously fed with WSSV positive shrimp carcass were stocked at 10/tank (biomass = 40). Shrimp were fed daily with commercial pellet at 1% of shrimp biomass. No water change was implemented until 96 h post culture (hpc) to simulate pond conditions in the tanks with different treatments and control. After observations at hpc-96, 50% of the water was changed. Mortality was recorded upon termination at hpc-120. In total, six shrimp samples per treatment were processed for WSSV quantification.

Water temperature, pH, salinity, *Chlorella* count and bacterial flora important in shrimp culture (i.e., total bacterial count, luminous bacteria, vibrios) were determined daily. Ammonia, NH_4, NO_2, NO_3, TSS, phosphate, and bacteria important in the nutrient cycle (i.e., phosphate solubilizing bacteria, nitrogen fixing bacteria, fungi) were analyzed every 2 days and upon termination at hpc-120.

2.3. Water parameters

Temperature and DO were measured using YSI 55 (YSI Instruments); and pH using EH 1000 (Line Seiki Co., Ltd.). The phenate method was used for ammonia analysis (APHA, 1995), flow injection analyzer for ammonium and nitrate, colorimetry for nitrite (ISO 13395, 1996), and the ascorbic acid method (APHA, 1995) for phosphate. TSS was measured using the filtration drying method (Rainwater and Thatcher, 1990). *Chlorella* was counted using a hemacytometer.

2.4. Bacteriological study

2.4.1. Bacterial flora important in shrimp culture

Samples were serially diluted 10-fold using autoclaved (120 °C for 15 min) seawater. Representative dilutions were plated in duplicates onto nutrient agar (NA; Merck) with 1.5% (w/v) sodium chloride (NaCl) for total bacterial count and TCBS (BBL) for the presumptive *Vibrio* count. Agar plates were incubated for 18–24 h at room temperature (approx. 30 °C). Bacterial growth on the agar plates was counted after incubation. Luminous bacteria were counted on NA plates in a darkened room to observe luminescence.

2.4.2. Bacteria important in the nutrient cycle

Water samples were processed as in bacteria important in shrimp culture and plated onto Pikovskaya's agar (PA; Himedia) for the phosphate solubilizing bacterial counts, Jensen's agar (JA; Himedia) for the nitrogen-fixing bacterial count and Czapex Dox agar (CDA; Himedia) supplemented with 1.5% (w/v) yeast extract (BBL) for the fungal count. All inoculated media were incubated at room temperature (approx. 30 °C) and counted 2–6 days after.

2.5. WSSV quantification

The pleopods of 6 shrimp samples were separately fixed in alcohol and kept at −4 °C until WSSV qPCR analysis. Total genomic DNA and associated WSSV viral DNA were extracted from the pleopods using DNAPREP-ALK (Cong ty TNHH Nam Khoa) following the protocol provided by the manufacturer. The DNA quality and concentration were determined by including positive control with known WSSV quantity and pleopod tissue with known weight in the analysis.

Real time PCR method was based on Durand and Lightner (2002). Primers and probes were synthesized by Proligo (Sigma) in Singapore. Briefly, 5 μl extracted DNA sample was added to 20 μl WSSV-TQPCR

mix prepared from PCR buffer 1× (Invitrogen) added 5 mM $MgCl_2$, platinum taq polymerase (Invitrogen, 1 IU/reaction), dNTP (sigma, 200 μM/each), primers (20 pm/reaction) and taqman probes (FAM) specific for WSSV (5 pm/reaction). Another 5 μl extracted DNA sample was also added to 20 μl Deca-TQPCR mix prepared from PCR buffer 1× (Invitrogen) added 5 mM $MgCl_2$, platinum taq polymerase (Invitrogen, 1 IU/reaction), dNTP (sigma, 200 μM/each), primers and taqman probes (HEX) specific for ribosome DNA of decapoda (5 pm/reaction). The qPCR was performed using C1000 thermal cycler (CFX96 Realtime System, BioRad). The amplification program consisted of 3 min and 30 s at 95 °C for initial denaturation, followed by 40 cycles of 95 °C for 15 s for denaturation, and 60 °C for 1 min annealing. Real time PCR for each sample was performed along with a set of standard dilutions WSSV-DNA (from 1000 to 10,000 viral copies/5 μl input volume), and a negative sample. The standard dilutions of WSSV were used to calculate the viral copy number that existed in the tested sample. The amount of the tested sample in mg was calculated based on the threshold cycle (Ct) of the ribosome DNA detected in the sample based on the following formula:

$$SQ(ng) = 10^{LogSQ} \text{ with } LogSQ = (intercept - Ct)/slope$$

The Ct is the Ct of the ribosome DNA detected in the tested sample. Based on previous analysis done on the gill and pleopod samples with known weight, the *intercept* was 32.06 and the *slope* was 3.23 (Hung Van, pers. comm.).

2.6. Statistical analysis

The repeated measure design was used (SPSS v.19). Mauchley's test was used to test for sphericity. If tests were not significant statistically ($P > 0.05$), it was concluded that there was homogeneity in the variances and sphericity was met. MANOVA was applied to variables that violated sphericity. Hpc was used as the repeated measures; treatment was the independent variable. Results of the WSSV quantification were analyzed separately with shrimp as the repeated measures and treatment as the independent variable.

Bacterial counts, total plankton count and WSSV QPCR results were log 10 transformed before analyses. Tukey's test in univariate ANOVA was used to determine the significant difference between hpcs, and between treatments at specific hpc. Pearson's correlation was used to determine factors that affected survival rate across treatments, and per treatment.

3. Results

Shrimp survival was lowest in *greenwater* (10%) and highest in *chlorella* (20%) (Fig. 1). WSSV was significantly lowest in *greenwater* (10^0 WSSV/mg pleopod) and highest in *chlorella* (10^5 WSSV/mg pleopod) (Fig. 2). Across treatments percentage shrimp survival was positively correlated with NH_4 (Table 1). However, varied correlations of the parameters with % survival were observed upon separate analysis of each treatment. The % survival in *chlorella* (Table 2) was positively correlated with QPCR ($P = 0.038$); in *greenwater* (Table 3) negatively with NH_4 ($P = 0.07$) and TAN ($P = 0.022$), and positively with NH_3 ($= 0.002$); and in *molasses* (Table 4) negatively with NH_3 ($P < 0.001$) but positively with NH_4 ($P < 0.001$). No parameter was significantly correlated with % survival in infected shrimp cultured in the control using *seawater* (Table 5).

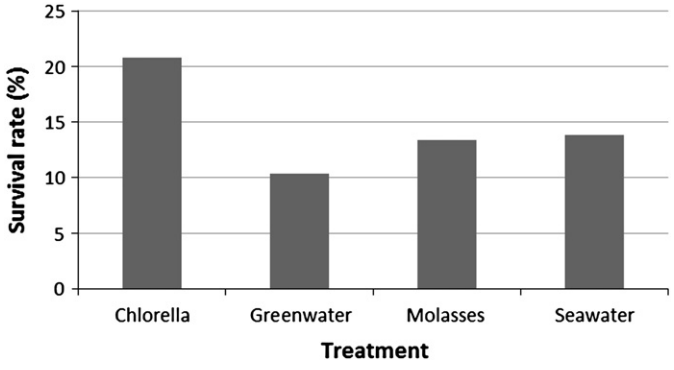

Fig. 1. Shrimp survival rate observed in the tanks stocked with *P. monodon* and cultured using greenwater, with molasses added, seeded with *Chlorella* and plain seawater.

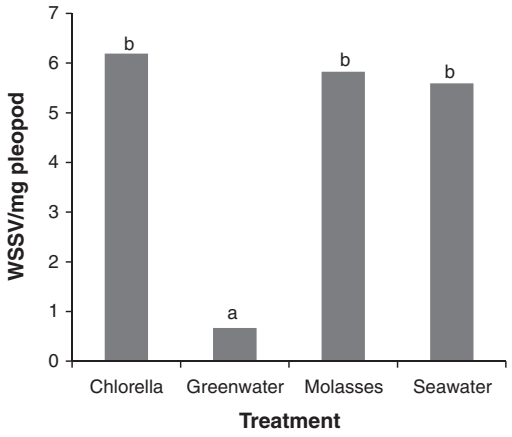

Fig. 2. WSSV viral load observed in the tanks stocked with *P. monodon* and cultured in water seeded with *Chlorella*, using greenwater, with molasses added, and plain seawater. Columns with the same letters are not significantly different ($P > 0.05$).

Initial WSSV load in shrimp after feeding with WSSV positive carcass was 1.40×10^1 WSSV/mg sample. Viral load was significantly lowest in *greenwater* treatment (7.0×10^0 WSSV/mg pleopods). Highest viral load was observed in *chlorella* treatment (4.64×10^5), followed by *molasses* (4.64×10^5) and the control (3.66×10^5).

Among the nitrogenous wastes, significant differences between treatments were observed in NH_3, NH_4, TAN and NO_2. Highest levels were observed in *greenwater* (Fig. 3a–d). Except for NO_3, nitrogenous waste level increased and was highest at hpc-96. Concentration of most of the wastes decreases with increasing hpc and was significantly higher in greenwater at hpc 0 and 48 (Fig. 3a–d), while the trend in *chlorella* and *seawater* was increasing. At hpc-120, NO_2 ($P < 0.05$) was significantly higher in *greenwater*. Across treatments lower concentrations of the nitrogenous waste components were observed at hpc-120. No significant difference in PO_4 and TSS was observed but levels were highest in *greenwater* and lowest in *molasses*. Significant differences were observed in temperature, pH and salinity but all were within the optimum level for shrimp culture. Observed DO were below optimum and were not significantly different between treatments.

Table 1
Pearson's correlation coefficient for survival rate (% surv) and correlated parameters of WSSV infected shrimp cultured across treatments.

	% surv	^0T	NH_4	NH_3	TAN	NO_2	BC
% surv	1	–	–	–	–	–	–
^0T	.320	1	–	–	–	–	–
NH_4	.622*	.535**	1	–	–	–	–
NH_3	.097	.356*	.758**	1	–	–	–
TAN	.439	.435**	.881**	.976**	1	–	–
NO_2	−.279	.139	.298*	.174	.225	1	–
BC	.139	.146	.392**	.461**	.464**	.543**	1

BC = black colonies on TCBS.
* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table 2
Pearson's correlation coefficient for WSSV viral load (QPCR), percentage shrimp survival (% surv) and correlated parameters in WSSV infected shrimp cultured in water seeded with *Chlorella*.

	% surv	QPCR	NH_3	NH_4	TAN	NO_2	NO_3	pH
% surv	1	–	–	–	–	–	–	–
QPCR	.998*	1	–	–	–	–	–	–
NH_3	−.996	−.988	1	–	–	–	–	–
NH_4	−.563	−.611	.334	1	–	–	–	–
TAN	−.975	−.987	.811**	.822**	1	–	–	–
NO_2	−.403	−.348	.454	.642*	.672*	1	–	–
NO_3	a	a	−.081	−.705*	−.486	−.602*	1	–
pH	−.600	−.551	−.087	−.787**	−.540	−.510	.939**	1

a = cannot be computed because at least one of the variables is constant.

Total plankton count (TPC) was significantly highest in *chlorella* and lowest in *seawater* ($P<0.001$). TPC decreased significantly ($P=0.002$) with advancing hpc though the trend was irregular.

Among the bacteria important in shrimp culture, significantly higher total bacterial count (TBC), yellow *Vibrio* on TCBS (YV), presumptive *Vibrio* count (PVC) and the black colonies on TCBS (BC) were observed in *greenwater* ($P<0.05$); while the percentage of green *Vibrio* was significantly lower ($P<0.05$).

Among the bacteria important in the nutrient cycle, significant difference ($P<0.05$) was observed in the phosphate solubilizing bacteria (PSB) between treatments. The level of phosphate solubilizing bacteria (PSB) and the nitrogen fixing bacteria (NFB) varied significantly ($P<0.05$) at different hpcs, but no trend was observed.

4. Discussion

The low survival in all treatments is explained by the high nitrogenous waste concentrations, low oxygen concentrations, and the cannibalistic characteristic of the shrimps. Particularly in greenwater, NH_4 and TAN are negatively correlated with survival rate; the high concentrations observed starting at hpc-0 could further explain for the lowest shrimp survival in *greenwater*. Infected shrimp could have died of ammonium toxicity at early phase of the experiment. The presence of the omnivorous tilapia might also be a contributing factor for the lowest survival in *greenwater*.

Results of the experiment did not provide explanation for the positive correlation between WSSV load and shrimp survival in WSSV infected shrimp cultured in *Chlorella* seeded water. However, the antiviral properties of marine microalgae have been reported (Mansour et al., 2011; So et al., 2009; Vo and Kim, 2010). Furthermore, Rigaux et al. (2012) reported prolonged survival in mice despite increase in viral load and suggested that the inferons (IFN), produced by the host cell in response to infection, have protective immunomodulatory function against pneumonia virus of mice thus promoting survival despite the increase in viral load. Interestingly, Kim et al. (2010) found that hydrolyzed *Chlorella vulgaris* promotes the release of IFN in mice. Ponprateep et al. (2011) suggested that the binding of WSSV particle to a proteinase, i.e. IFN, gives the shrimp protection against WSSV. In the present study, it is possible that *Chlorella* stimulated the production or release of IFN in the shrimp resulting to increased immunocompetence and ability to resist the WSSV virus and consequently to high survival rate. However, this theory needs further study.

Significantly low WSSV copies or load was observed in greenwater. Viral load of shrimp cultured in greenwater decreased from the original 10^1 to 10^0 WSSV/mg sample while those in the other treatments increased 4 or 5 times, suggesting that the use of the greenwater culture system could provide some protection against WSSV. Furthermore, no correlation exists between viral load and shrimp survival in this study, implying that the low survival in greenwater was not due to the viral infection.

Nitrogenous wastes were beyond the ideal level in all cases. Ammonia is excreted by aquatic animals; excess feed, and feces contribute to the nitrogenous load of the water (Lin and Chen, 2003). Observed highest N level in the *greenwater* was expected considering the 2 aquatic animals in the system, tilapia and shrimp, that would excrete NH_3 during respiration. The significantly higher nitrogenous wastes at hpc-0 in *greenwater* were expected since the tanks were stocked with tilapia 2 weeks prior to the start of the experiment. The high N observed in the *chlorella* is contrary to Hii et al. (2011) who reported that microalgae efficiently removes N in the water. The high and increasing N level in the *chlorella* could be attributed to the decomposition of the senescing *Chlorella* in the system. Bouchard et al. (1998) demonstrated that the decomposition of plant detritus could increase nitrogen concentration in the water. The lowest level was observed in the *molasses*. This is in accordance to Schneider et al. (2006) who reported that addition of molasses promotes the growth of heterotrophic bacteria that efficiently remove nitrogenous wastes from solid fish wastes. However, heterotrophic bacterial count in the molasses treatment was not higher than the other treatments. It is possible that molasses has other components responsible for the nitrogen removal from the water other than the heterotrophic bacteria. Results of this study are consistent with Avnimelech (2009) who reported that the use of molasses to promote growth of bacterial bioflocs is more efficient in removing nitrogen from the water than plants. The lower nitrogenous waste level at hpc-120 can be attributed to

Table 3
Pearson's correlation coefficient for percentage shrimp survival (% surv) and correlated parameters in WSSV infected shrimp cultured using greenwater.

	% surv	NH_4	NH_3	TAN
% surv	1	–	–	–
NH_4	−1.000*	1	–	–
NH_3	1.000**	.785**	1	–
TAN	−.999*	.878**	.986**	1

a = cannot be computed because at least one of the variables is constant.
* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table 4
Pearson's correlation coefficient for percentage shrimp survival (% surv) and correlated parameters in WSSV infected shrimp cultured in water with molasses added.

	% surv	NH_3	NH_4	NO_2	TAN	pH	^oT
% surv	1	–	–	–	–		
NH_3	−1.000**	1	–	–	–		
NH_4	1.000**	−.150	1	–	–		
NO_2	a	.367	−.747**	1	–		
TAN	1.000**	−.131	1.000**	−.742**	1		
pH	0.679	−.702*	.442	−.569	.430	1	
^oT	1.000**	−.145	.223	−.185	.221	.439	1

a = cannot be computed because at least one of the variables is constant.
** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Table 5
Pearson's correlation coefficient for WSSV viral load (QPCR), percentage shrimp survival (% surv) and correlated parameters in WSSV infected shrimp cultured in seawater.

	% surv	QPCR	NH_3	NH_4	TAN	NO_3	^oT
% surv	1	–	–	–	–	–	–
QPCR	−.433	1	–	–	–	–	–
NH_3	−.518	−.547	1	–	–	–	–
NH_4	.946	−.703	.308	1	–	–	–
TAN	.736	−.929	.831**	.786**	1	–	–
NO_3	−.525	−.540	−.550	−.407	−.596*	1	–
^oT	.474	−.999*	.637*	.510	.713**	−.878**	1

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

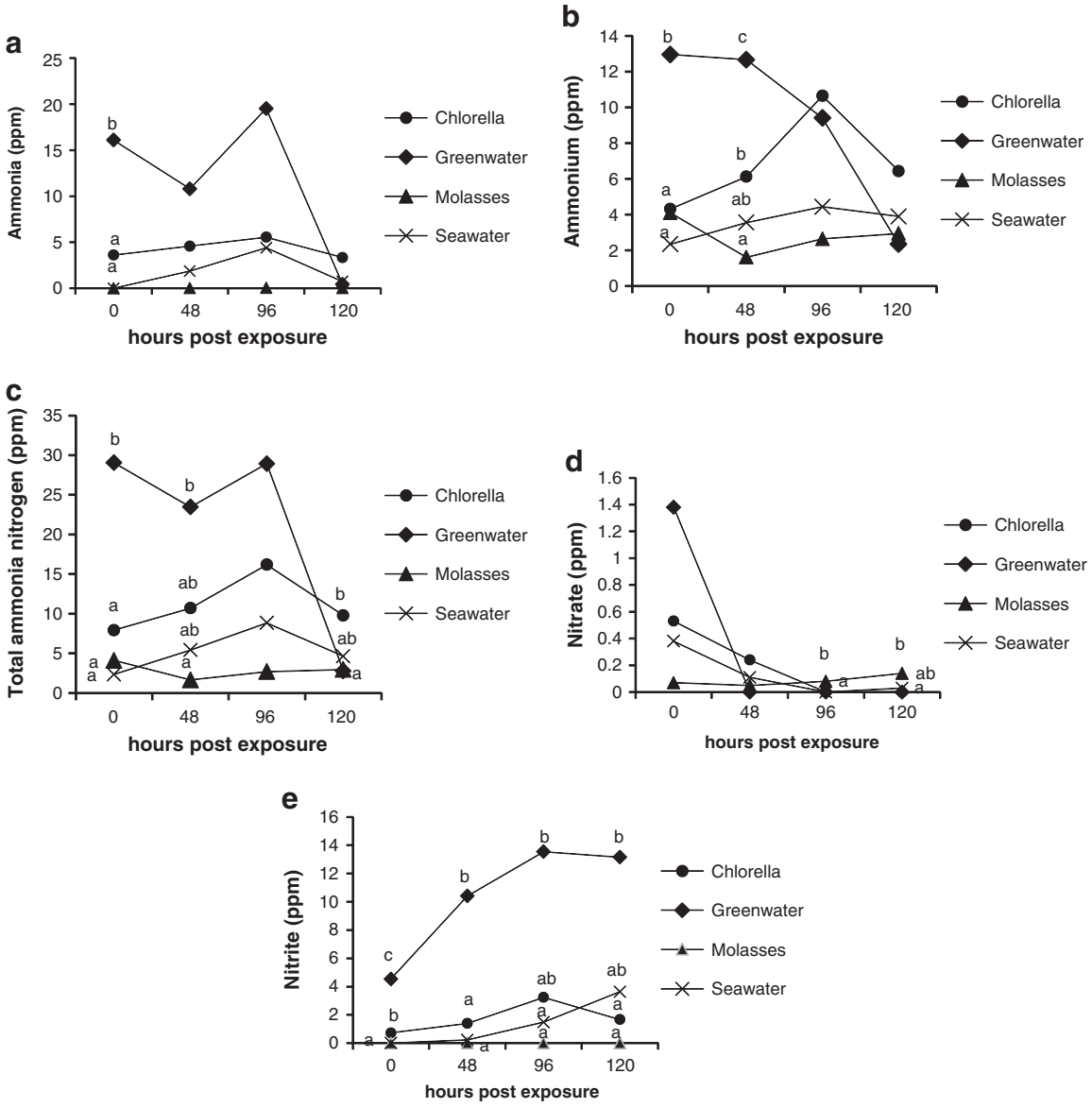

Fig. 3. Observed nitrogenous wastes: a) NH_3, b) NH_4, c) TAN, d) NO_2 and e) NO_3 in tank water stocked with WSSV infected *P. monodon* stocked in *Chlorella* seeded water, greenwater with tilapia, molasses, and seawater at different post exposure times. Points with different letters are significantly different ($P<0.05$).

the water change implemented at hpc-96. Water change is one way of removing nitrogen from the water (Funge-Smith and Briggs, 1998).

Significantly higher total plankton count (TPC) in chlorella and at hpc-0 was expected. The microalgae were seeded in this treatment. The shrimps were newly transferred in the tanks and might not have started ingesting the natural food present. This explains for the highest count observed at this hour. The presence of plankton in the other treatments suggests that planktons are ubiquitous in the water.

The higher total bacterial count (TBC), higher yellow *Vibrio* (YV), higher black colonies on TCBS (BC) and the lower percentage green *Vibrio* (PGV) observed in the *greenwater* could be among the factors explaining the low WSSV load in this treatment. High total bacterial count, high yellow *Vibrio* and lower percentage green *Vibrio* are reported as WSSV protective factors (Tendencia and Verreth, 2011). Shewanella, the black colonies on TCBS have been reported to have probiotic properties against some vibrios (Fuenzalida et al., 2007; Gram and Huss, 1996; Prescott et al., 2005; Zadeh et al., 2010). The black colonies could have acted on the pathogenic vibrios thus making shrimp less susceptible to WSSV.

The use of the greenwater culture could provide protection against WSSV outbreak but nitrogenous wastes in the water were high, while concentrations were low in water with molasses; therefore, pond trials on the use of greenwater in combination with molasses for shrimp culture are suggested. Pond conditions are better than experimental conditions thus might give better shrimp survival. The mechanism behind the decrease in the viral load in shrimp cultured in greenwater, the immunomodulatory effect of *Chlorella* in shrimp, and other components of molasses that might be responsible in the nitrogen removal from the water need further study. Results of this study could serve as baseline data for possible ecological means of WSSV prevention and control.

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References

APHA (American Public Health Association), 1995. Standard Methods for the Examination of Water and Waste Water, 19th ed. American Public Health Association, Washington, DC. 1082 pp.

Austin, L.F., Stuckey, P.A.W., Robertson, I., Effend, D.R., Griffith, W., 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. Journal of Fish Diseases 18, 93–96.

Avnimelech, Y., 2009. Biofloc Technology-A Practical Guide Book. The World Aquaculture Society.

Bouchard, V., Creach, V., Lefeuvre, J.C., Bertru, G., Mariotti, A., 1998. Fate of plant detritus in a European salt marsh dominated by *Atriplex portulacoides* (L.) Aellen. Hydrobiologia 373 (374), 75–87.

Corre Jr., V.L., Janeo, R.L., Caipang, C.M.A., Calpe, A.T., 1999. Sustainable shrimp culture techniques: use of probiotics and reservoirs with "green water". Philippine Council for Aquatic and Marine Research and Development, Los Baños, Laguna, and University of the Philippines Visayas, Miag-ao, Iloilo. 32 pp.

Corre Jr., V.L., Janeo, R.L., Ronquillo, J.D., Kurokura, H., 2005. Use of greenwater technology as biocontrol of luminous bacteria in intensive shrimp (*Penaeus monodon*) grow-out culture. UPV Journal of Natural Sciences 10 (1), 51–60.

Cremen, M.C.M., Martinez-Goss, M.R., Corre, V.L., Azanza, R.V., 2007. Phytoplankton bloom in commercial shrimp ponds using green-water technology. Journal of Applied Phycology 19 (6), 615–624.

Das, B.K., Pradhan, J., 2010. Antibacterial properties of selected freshwater microalgae against pathogenic bacteria. Indian Journal of Fisheries 57 (2), 61–66.

Diggles, B.K., Carson, J., Hine, P.M., Hickman, R.W., Tait, M.J., 2000. *Vibrio* species associated with mortalities in hatchery-reared turbot *Colistium nudipinnis* and brill *C. guntheri* in New Zealand. Aquaculture 183, 1–12.

Durand, S.V., Lightner, D.V., 2002. Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. Journal of Fish Diseases 25 (7), 381–389.

Flegel, T.W., 2009. Current status of viral diseases in Asian shrimp aquaculture. Israeli Journal of Aquaculture—Bamidgeh 61, 229–239.

Fuenzalida, L., Armijo, L., Zabala, B., Hernández, C., Rioseco, M.L., Riquelme, C., Espejo, R.T., 2007. *Vibrio parahaemolyticus* strains isolated during investigation of the summer 2006 seafood related diarrhea outbreaks in two regions of Chile. International Journal of Food Microbiology 117, 270–275.

Funge-Smith, S.J., Briggs, M.R.P., 1998. Nutrient budgets in intensive shrimp ponds: implications for sustainability. Aquaculture 164, 117–133.

Gram, L., Huss, H.H., 1996. Microbiological spoilage of fish and fish products. International Journal of Food Microbiology 33, 121–137.

Hernandez, J.-P., de-Bashan, L.E., Rodriguez, D.J., Rodriguez, Y., Bashan, Y., 2009. Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium Bacillus pumilus from arid zone soils. European Journal of Soil Biology 45 (1), 88–93.

Hii, Y.S., Soo, C.L., Chuah, T.S., Mohd-Azmi, A., Abol-Munafi, A.B., 2011. Interactive effect of ammonia and nitrate on the nitrogen uptake by *Nannochloropsis* sp. Journal of Sustainability Science and Management 6 (1), 60–68.

Junpeng, C., Li, J., Thompson, K.D., Li, C., Han, H., 2007. Isolation and characterization of pathogenic *Vibrio parahaemolyticus* from diseased post-larvae of abalone *Haliotis diversicolor supertexta*. Journal of Basic Microbiology 47 (1), 84–86.

Kim, H.-H., Kim, K.-K., Jeong, H.-J., Kim, H.-M., Hong, S.-H., Uma, J.-Y., 2010. Effects of hydrolyzed *Chlorella vulgaris* by malted barley on the immunomodulatory response in ICR mice and in Molt-4 cells. Journal of the Science of Food and Agriculture 90, 1551–1556.

Lavilla-Pitogo, C.R., Baticados, Ma.C.L., Cruz-Lacierda, E.R., de la Pena, L.D., 1990. Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture 91, 1–13.

Lin, Y.-C., Chen, J.-C., 2003. Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. Aquaculture 224, 193–201.

Lio-Po, G.D., Leano, E.M., Penaranda, M.D., Villa-Franco, A.U., Sombito, C.D., Guanzon Jr., N.G., 2005. Anti-luminous *Vibrio* factors associated with the green water grow-out culture of the tiger shrimp *Penaeus monodon*. Aquaculture 250, 1–7.

Makridis, P., Costa, R.A., Dinis, M.T., 2006. Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched *Artemia metanauplii*. Aquaculture 255 (1–4), 76–81.

Mansour, H., Shoman, S., Kdodier, M., 2011. Antiviral effect of edaphic cyanophytes on rabies and herpes-1 viruses. Acta Biologica Hungarica 62 (2), 194–203.

Ponprateep, S., Tassanakajon, A., Rimphanitchayakit, V., 2011. A Kazal type serine proteinase SPIPm2 from the black tiger shrimp *Penaeus monodon* is capable of neutralization and protection of hemocytes from the white spot syndrome virus. Fish & Shellfish Immunology 31, 1179–1185.

Prescott, L., Harley, J., Klein, D., 2005. Microbiology, 6th ed. McGraw-Hill, New York.

Rainwater, F.H., Thatcher, L.L., 1990. Methods for Collection and Analysis of Water Samples. US Govt. Printing Off., Washington.

Rigaux, P., Killoran, K.E., Qiu, Z., Rosenberg, H.F., 2012. Depletion of alveolar macrophages prolongs survival in response to acute pneumovirus infection. Virology 422 (2), 338–345.

Schneider, O., Sereti, V., Eding, E.H., Verreth, J.A.J., 2006. Molasses as C source for heterotrophic bacteria production on solid fish waste. Aquaculture 261, 1239–1248.

Sivakumar, N., Sundararaman, M., Selvakumar, G., 2011. Efficacy of micro algae and cyanobacteria as a live feed for juveniles of shrimp *Penaeus monodon*. African Journal of Biotechnology 10 (55), 11594–11599.

So, Y.K., Seok, R.K., Oh, M.-J., 2009. In vitro antiviral activities of Korean marine algae extracts against fish pathogenic infectious hematopoietic necrosis virus and infectious pancreatic necrosis virus. Food Science and Biotechnology 17 (5), 1074–1078.

Tendencia, E.A., Dela Peña, M.R., 2010. Effect of different sizes of saline red tilapia hybrid *Oreochromis niloticus* × *O. mossambicus* on the growth of luminous bacteria *Vibrio harveyi*. Philippine Agricultural Scientist 93 (4), 369.

Tendencia, E.A., Verreth, J.A.J., 2011. Temperature fluctuations, low salinity and water microflora are risk factors for WSSV outbreaks in pond culture of *Penaeus monodon*. Israeli Journal of Aquaculture—Bamidgeh 63, 548 (IIC, 7 pages).

Tendencia, E.A., dela Peña, M.R., Fermin, A.C., Lio-Po, G., Choresca, C.H., Inui, Y., 2004. Antibacterial activity of tilapia *Tilapia hornorum* against *Vibrio harveyi*. Aquaculture 232, 145–154.

Tendencia, E.A., Fermin, A.C., dela Peña, M.R., Choresca Jr., C.H., 2006. Effect of *Epinephelus coioides*, *Chanos chanos*, and GIFT tilapia in polyculture with *Penaeus monodon* on the growth of the luminous bacteria *Vibrio harveyi*. Aquaculture 253, 48–56.

Tendencia, E.A., Bosma, R.H., Verreth, J.A.J., 2011. White spot syndrome virus (WSSV) risk factors associated with farming practices in polyculture and monoculture *P. monodon* farms in the Philippines. Aquaculture 311, 87–96.

Thompson, J., Gregory, S., Plummer, S., Shields, R.J., Rowley, A.F., 2010. An in vitro and in vivo assessment of the potential of *Vibrio* spp. as probiotics for the Pacific white shrimp, *Litopenaeus vannamei*. Journal of Applied Microbiology 109 (4), 1177–1187.

Travers, M.A., Basuyaux, O., Le Goic, N., Huchette, S., Nicolas, J.-L., Koken, M., Paillard, C., 2009. Influence of temperature and spawning effort on *Haliotis tuberculata* mortalities caused by *Vibrio harveyi*: an example of emerging vibriosis linked to global warming. Global Change Biology 15 (6), 1365–1376.

Vo, T.-S., Kim, S.-K., 2010. Potential anti-HIV agents from marine resources: an overview. Marine Drugs 8 (12), 2871–2892.

Zadeh, S.S., Saad, C.R., Christianus, A., Kamarudin, M.S., Sijam, K., Shamsudin, M.N., Neela, V.K., 2010. Assessment of growth condition for a candidate probiotic, *Shewanella algae*, isolated from digestive system of a healthy juvenile *Penaeus monodon*. Aquaculture International 18 (6), 1017–1026.