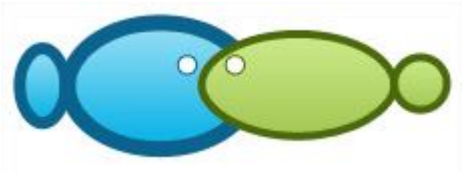

Multivariate analyses of microbial concentration and environmental variables in pond-based penaeid shrimp culture systems

[1]Marz Linnaeous L. Rabadon, [1]Madel F. Damaso, [1,2]Mark Nell C. Corpuz

[1] Center for Research on Aquaculture and Aquatic Resources in Brackishwater Systems, Institute of Fisheries and Aquatic Sciences, Bataan Peninsula State University, Orani Campus, Bataan 2112, Philippines; [2] Graduate School, School of Environmental Science and Management, University of the Philippines Los Baños, College, Laguna 4031, Philippines. Corresponding author: M. L. L. Rabadon, mllrabadon@bpsu.edu.ph

Abstract. Multivariate assessment on bacteria and water quality parameters in tropical shrimp farming are scanty, despite the critical role of these variables in culture management. This preliminary study investigated the bacterial water quality of pond water during one culture cycle, with emphasis on *Vibrio* spp. concentrations isolated from shrimp specimens. The study performed multivariate analyses on bacterial and environmental parameters in pond-based culture of penaeid shrimps in Bataan, Philippines. The bacterial assessment showed increasing total heterotrophic bacterial count and *Vibrio* concentrations in the sequence: stocking phase < mid-cycle < pre-harvest phase. Principal component analysis for bacterial and environmental data viz, temperature, salinity, and dissolved oxygen (in order of importance) identified the most important variables affecting the water quality. It also divulged the maximal correlation with ecological data resulting to the observed differences of the sampling groups (80.32% variation). *Vibrio* species detected from the shrimp culture ponds were composed of known shrimp pathogens viz., *Vibrio parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*, *V. cholerae*; and *V. panuliri*. Such findings provide baseline data for effective water management during grow-out phases.
Key Words: biosecurity, *Penaeus monodon*, PCA, *Vibrio* spp., *Vibrio panuliri*.

Introduction. Shrimp farming, mainly *Penaeus monodon* and *P. vannamei* is a very important economic activity in the Philippines. The recent total annual production of shrimp valued at Php 25 billion (1$ = Php 50) (PSA 2018). The Central Luzon (Philippines) is also the main producer of high-valued *P. monodon* amounting to 21,000-24,000 Mt per annum whilst *P. vannamei* contributes 1,600-1,900 Mt per annum. The *P. monodon* became the country's top export earner, but has fallen due to production failures in the late 1990's as well as the high cost of its production (Yap 1999; Muegue et al 2015). The main culprit in the collapse of the shrimp industry in the early 90's was the widespread occurrence of vibriosis, luminous bacterial infections, and white spot syndrome which were all attributed to environmental degradations (Rosario & Lopez 2005; Dabu et al 2015).

The shrimp farming in Central Luzon utilizes large irregularly-shaped ponds (> 10,000 m^2), implements a semi-intensive polyculture system, and is dependent on tidal influxes from rivers or estuaries for water recharging. The water management on these ponds, however, is being challenged by the polluted riverine water (Rabadon & Corpuz 2021) and poor implementation of aquaculture biosecurity measures (Flores et al 2015) which are linked to food contamination brought about by anthropogenic waste inputs and are known to cause the spread of diseases to farmed species (Pruder 2004). Furthermore, several shrimp producers overlooked the adoption of intensive closed system aquaculture, and eco-friendly protocols that may improve food safety and productivity (Flores et al 2015, 2016).

The monitoring of facultative and obligate bacteria is a vital scheme to assess the sanitary and bacterial quality of water (EPA 1986; Ashbolt et al 2001). The *Escherichia*

coli, a gram-negative bacterium is one of the specific indicators of fecal contamination in a tropical region. Enterro-haemorrhagic *E. coli* have emerged as a serious gastrointestinal pathogen although the mode of transmission is mainly through the consumption of contaminated food (Mead et al 1999). Outbreaks associated with water-borne *E. coli*, are known to cause many types of infections and are spread to humans in a variety of ways. Furthermore, prevalence of pathogenic *Vibrio* spp. has been correlated to water quality and certain environmental conditions and seasonal variations (Williams & La Rock 1985; Barbieri et al 1999; Pfeffer et al 2003).

There is no baseline data on the periodic concentrations of total viable heterotrophic bacteria, fecal coliform, and *Vibrio* species in shrimp grow-out culture cycle. Moreover, research on bacterial water quality associated with multi-environmental variables in pond-based shrimp culture has never been done in the Philippines, until the initiation of this study. To bring new information, this study evaluated the bacterial concentrations and environmental parameters in pond-based shrimp farming, with emphasis on temporal variabilities of bacterial concentration (heterotrophic and fecal coliform, and *Vibrio* spp.). Moreover, the study shed light on the ordination among microbial data with several key environmental parameters, and characterized the composition of *Vibrio* specimens based on molecular analyses.

Material and Method

Description of the study sites. Water samples were collected from a brackishwater fishpond in Orani, Bataan, Philippines (14°48.5' N; 120°32.6' E). The fishpond has two main compartments for grow-out production (each having settling pond compartment). The flow-in and discharge of pond water is tidal-influenced. The wetland where the ponds are located has small patch of mangroves predominated by *Rhyzophora* sp. (Manliclic et al 2018) and is highly influenced by riverine water influx from the Orani River, in which the dowstream are impacted by anthropogenic pollution (Rabadon & Corpuz 2021). The wetland where the fishpond is located supports several subsistence and artisanal fisheries. The studied areas have two pronounced seasons: a dry season from November to May, and a wet season during the rest of the year.

Sample collection. The study commenced from June 2019 until December 2019. Three periodic samplings were carried-out: two days after seeding/stocking (PS), a median date between seeding and harvest (Mid), and two days before harvest (PH). Water quality parameters including dissolved oxygen (DO), salinity, turbidity, depth, temperature, pH, hardness, NO_3^-, NO_2^-, and NH_3 were measured *in situ* twice a day, and monitored every three days. Composite water and shrimp specimens were collected during grow-out period following the protocols of Noriega-Orozco et al (2007). During sampling, water samples were prepared from a mixture of surface and bottom water collected from the middle of the ponds. A total of 15 shrimp specimens were aseptically collected from the selected ponds in respective sizes during each periodic sampling based on the methods of Jayasinghe et al (2010). Water samples and shrimp specimens were placed in sterile ice boxes, transported under low temperature, and immediately subjected to bacterial analyses. Microbiological assessment were perfomed at the Science Laboratory of Bataan Peninsula State University Orani Campus.

Bacterial isolation and quantification. General culture media (NA) and MacConkey (MC) plates were prepared according to the manufacturers' specifications (HiMedia®). The glass wares, micropipettes, and other materials (heat resistant) for microbiological methods including the prepared media were autoclaved under 121°C; 15 psi for 15 min. A 1-mL inoculant from the pooled water samples were serially diluted following ten-fold dilution (10^{-1} to 10^{-5}) using sterile dH_2O. One to two dilution factors were utilized for the enumeration of the total heterotrophic (THC) and fecal coliform (TC) bacteria counts. A volume of 100 µL from each of the chosen dilution factors were inoculated through standard spread plate method on the prepared culture plates. Each dilution factor utilized were spread-plated in triplicates. Thereafter, the inoculated plates were incubated in an inverted position at room temperature.

All samples were handled aseptically. Water samples were pooled with equal amounts (v) and agitated vigorously. Shrimp muscle and brain fluids were homogenized, eluted and serially diluted based on the method of Jayasinghe et al (2010) with few modifications. Briefly, shrimp samples were amalgamated separately for muscle and brain fluid samples using microtube pestle and pooled with equal amounts (w) for each individual sample. Thereafter, a series of 6 10-fold dilutions (dilution factor: 10^{o} to 10^{-6}) enriched in alkaline peptone water (APW) were prepared in order to estimate the most probable number (MPN) of total heterotrophic and presumptive *Vibrio* spp. population (Noriega-Orozco et al 2007; Ganesh et al 2010).

The glass wares, micropipettes, and other materials (heat resistant) including culture media were autoclaved under 121°C; 15 psi for 15 min. Prepared samples were spread-plated on NA plates, MC plates and thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates. The NA plates and TCBS were incubated for 12-24 h before conducting the quantification (CFU mL^{-1}) of the THC and presumptive *Vibrio* counts (VC) respectively. The total plate counts were obtained through manual counting using grid method. The total plate counts were obtained through manual counting using grid method. Total plate counts were used to determine the CFU mL^{-1} of samples using the formula:

$$\text{CFU } mL^{-1} = \frac{\text{No. of colonies x dilution factor}}{\text{Final volume plated}}$$

Purification and conservation of isolates. Distinct and dominant colonies were selected (~20%) from plates containing 20~200 colonies. Colony coloration (i.e. green and yellow) and other morphological characteristics were considered in the selection of the colonies for purification and conservation for further testing and identification. Selected isolates were re-inoculated on TCBS agar and purified through streak-method. Purified isolates were stored in low temperatures through stab culture submerged in mineral oil as cryoprotectant.

Identification of pure Vibrio spp. isolates. Inoculants from the purified isolates were used as working cultures for further identification processes. The purified isolates were classified through a biochemical key (Jayasinghe et al 2010) and further identified through molecular methods. Briefly, the biochemical tests include (not in hierarchical sequence): oxidase test, VP test, growth tolerance on NaCl, and O-nitrophenyl-beta-D-galactopyranoside (ONPG) test. Thereafter, classified isolates were transported to the Philippine Genome Center of the University of the Philippines Diliman for assistance on further molecular identification. Processes performed include deoxyribonucleic acid (DNA) extraction, 16S rRNA gene amplification and purification, and capillary sequencing technology. To achieve better results, samples with low success rates were reprocessed.

Data analyses. Normality of data (Shapiro-Wilk test) and homogeneity of variance (Levene's test) were verified. Variables did meet the assumptions and were subjected to univariate parametric tests. Mean bacterial count was subjected to analysis of variance (ANOVA), followed by Tukey's post-hoc test if found significantly different ($p < 0.05$). Principal component analysis (PCA) was used to examine the association of environmental parameters with bacterial abundance based on culture phase group variabilities. PCA was performed on nine environmental parameters using the correlation matrix. PCA also identified the most important variables that contribute the most to group differences. All data analyses were performed using PaST® v 3.0 (Hammer et al 2001) and SPSS v 21.

Results and Discussion. The range of water quality parameters in the sampling sites are shown in Table 1. Optimal values for culture were observed in all water quality parameters (see Manliclic et al 2018) except for the detected NH_3 levels during post-seeding and after harvesting. Water turbidity for midyear sampling had become quite excessive (Boyd & Tucker 1992) as inferred by increased in plankton abundance. Nevertheless, the present result is comparable to the limnological study conducted in similar brackishwater fishpond published elsewhere (Manliclic et al 2018).

Table 1

Fluctuation of water quality parameters of pond water during the study

Water quality parameters	*Sampling periods*		
	Post-seeding	*Mid-cycle*	*Pre-harvesting*
Temperature (ºC)	29.00-31.60	32.90-33.80	34.10-34.20
DO (mg L^{-1})	5.90-10.90	6.00-6.70	8.20-8.00
pH	8.09-8.33	7.40-7.50	7.32-8.40
Salinity (ppt)	5.00	5.00-6.00	10.00
Hardness (mg L^{-1})	425.00	425.00	425.00
NH_3 (mg L^{-1})	0-0.25	-	0-0.25
NO_3 (mg L^{-1})	-	-	-
NO_2 (mg L^{-1})	-	-	-
Depth (cm)	0.76-1.25	0.43-0.83	1.05-1.25
Secchi disc visibility depth (cm)	30.10-33.20	20.50-25.00	34.10-34.40

Note: (-) = 0 value or undetectable.

Bacterial concentration analysis. Figure 1 shows the variation in the levels of THC, TC, and VC on biological and water samples in three shrimp cycle period. The bacterial concentrations (CFU mL^{-1}) in the shrimp ponds ranged from 390 to 2.5 x 10^4 for THC, 0 (no growth) to 4 x 10^3 for VC, and 0 (no growth) to 1.4 x 10^3 for TC. Highest THC concentrations were measured in pre-harvest samples ($p < 0.05$). Significant differences were found on VC between post-seeding, mid-cycle and pre-harvest periods in the increasing sequence: PS < Mid < PH. According to Noriega-Orozco et al (2007), biological growth conditions are favorable for *Vibrio* species during harvest time. The accumulation of wastes in ponds towards harvest period plays a factor for the progression of VC (Kannapiran et al 2009). Nonetheless, no significant differences were observed in TC, but the presence of fecal coliforms on food production system still pose great health risk. According to Ferreira et al (2011), the ideal level for TC for shrimp ponds is at 1.0 x 10^3 CFU mL^{-1} or lower. During the study, TC levels fluctuations were within the said ideal limit.

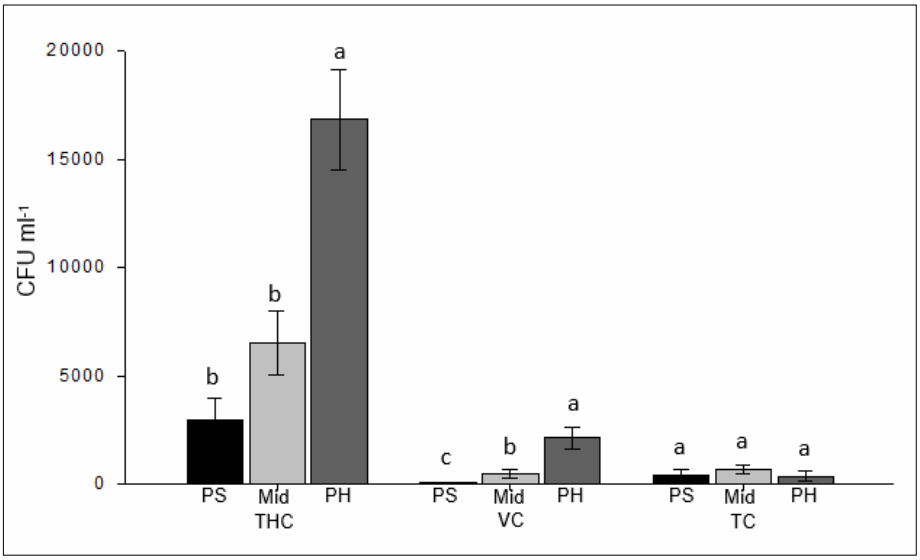

Figure 1. Mean values (SD = error bars) and differences of bacterial concentrations during the periodic sampling. Different superscripts represent significant differences at $p < 0.05$ within bacterial groups. Total heterotrophic bacteria count (THC), *Vibrio* concentration (VC) and total fecal coliform count (TC). Post-seeding (PS), mid-cycle (Mid) and pre-harvesting (PH).

According to farm records, there is no history of antibiotic usage. The shrimp ponds apply traditional semi-intensive culture system relying on tidal influxes from the Orani river for water supply. This culture method was observed to be the most common practice in the province and nearby areas without proper education on biosecurity measures (Flores et al

2015). To properly evaluate the status of the shrimp industry in the area, this study evaluates the status of bacterial concentrations to understand implications on the current status and provide baseline data for proper management regimes.

Multivariate analyses. Principal component analysis for significant environmental variables extracted from correlation matrix showed that PC 1 and PC 2 accounted for 80.32% of the total variation. Among the nine water quality parameters, six environmental variables were found significant based on Jolliffe's cut-off value of 0.71. The PC1 and PC2 accounted for 80.32% of the total variation (Figure 2). In PC1, the pooled bacterial loading were greater during PH period as compared to PS and Mid (right-left reading). The variation was primarily explained by the influence of temperature, salinity, and DO (in order of importance). The present finding also indicate changes in *Vibrio* concentration during the aquaculture cycle, with strong correlation to high temperature, salinity, and DO levels. The THC was also highly correlated to the level of NH_3, in which the main sources can be from fish excretion and feeds (Hargreaves & Tucker 2004). The high THC is also attributed to the presence of high organic and dissolved salts in the water from feeds, or from contamination from animal and human excreta, discharge from sewage facilities and other anthropogenic activities (Karikari & Ansa-Asare 2006; Shittu et al 2008). In the case of TC on the ponds, PCA and ANOVA expressed that there was no relevant relationship to water quality and no significant variation relative to culture period. Several studies claims that fecal coliforms are inversely correlated with salinity (Anderson et al 1979; Mallin et al 1999; Karbasdehi et al 2017). However, stable concentration of fecal coliforms in this study suggests that the naturally nutrient-rich culture water in ponds, which supports the environmental conditions for fecal coliforms remain unaffected by influx of marine water.

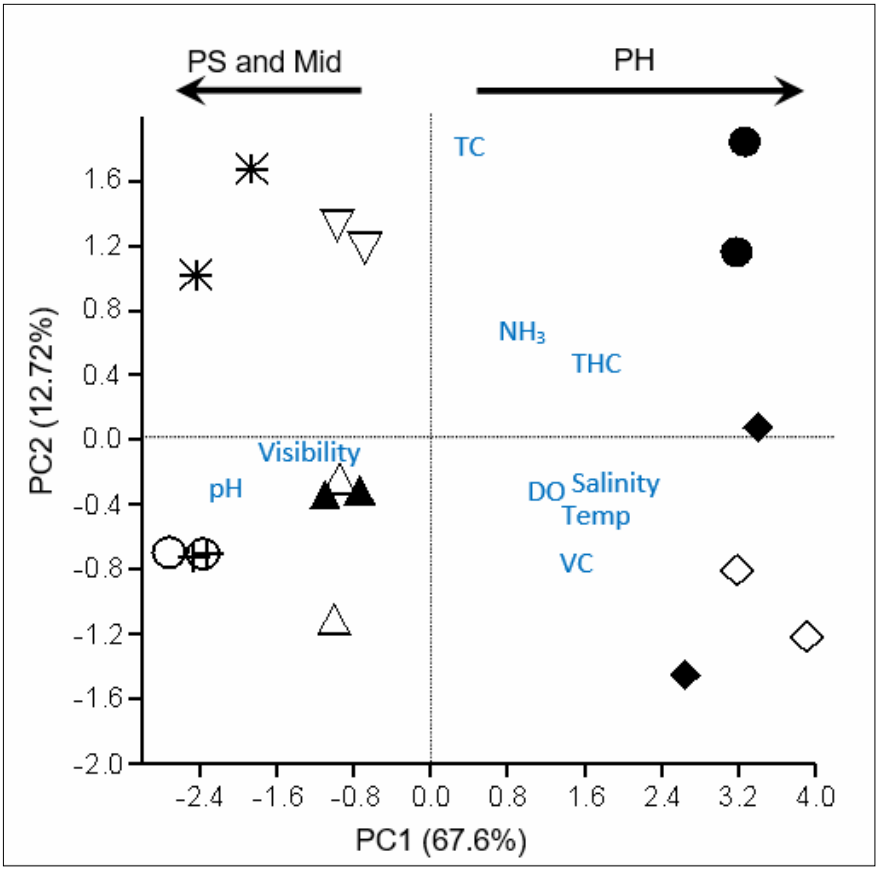

Figure 2. PCA biplot for multivariate parameters in shrimp pond showing points during post-seeding (PS) (O = *Penaeus monodon*; + = *Penaeus vannamei*; * = water), mid-cycle (Mid) (Δ = *P. monodon*; ▲= *P. vannamei*; ∇ = water), and pre-harvest (PH) (◇ = *P. monodon*; ♦ = *P. vannamei*; ● = water).

Higher THC and VC were recorded towards pre-harvest period. This is in congruence with the observation reported in several studies attributing it to the increase of organic load as a result of artificial feeding (Horsley 1997; Dalmin et al 2002; Kannapiran et al 2009). There have been studies conducted to improve water quality as well as mitigate the increase of *Vibrio* concentration in shrimp ponds. The use of selected strains of *Bacillus* as probiotic bacteria has shown great potential in controlling *Vibrio harveyi* causing luminous vibriosis and *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) in shrimps (Moriarty 1998; Truong et al 2021). The study of Kurniaji et al (2021) also indicated that the polyculture of shrimp and seaweed affected the shrimp health positively and reduced *V. harveyi* population.

Vibrio identification. After establishing risk implications of the bacterial concentrations in a river-fed shrimp culture system, an additional part of this study aims to identify and preserve *Vibrio* species associated with shrimp culture in Bataan. This will be vital for future studies for the proper management and development of strategies and low-cost technologies or immunotherapies that will be applicable for utility to the farmers in Bataan and nearby areas. Briefly, a total of 46 isolates were characterized, purified and preserved for future use. Distinct isolates were further analyzed for molecular identification. Results show that isolates matched (≥ 99%) with *V. parahaemolyticus*, *V. vulnificus, V. alginolyticus, V. cholerae, V. panuliri*, and *Vibrio* sp. Among the six species, the first three are known shrimp pathogens (Alapide-Tendencia & Dureza 1997; de Souza Valente & Wan 2021) and *V. cholerae* has been notorious for its zoonotic nature that pose great risk to food safety. Another interesting data obtained from this study is the first isolation of *V. panuliri* from shrimp. *V. panuliri* is a novel marine bacterium that was first isolated from marine lobsters (Kumari et al 2014). The etiology and potential pathogenicity of *V. panuliri* is yet to be established, and will be the basis for succeeding studies.

Conclusions. River water quality indices indicate better values during dry season for shrimp culture and for water quality in general. Increase in *Vibrio* spp. levels complemented with the growth-out shrimp operation following increasing sequence: pre-seeding (pre-stocking) < mid-cycle < post-harvesting. The findings may have implications on the effective land and water management during grow-out phases and appropriate post-harvest schemes for shrimp to reduce bacterial growth. The coliform levels were within optimum range, although risks are still relevant as the presence of coliform was confirmed in shrimp specimens.

The routine monitoring of microbial communities is vital for the refinement of management regimes necessary for specific conditions during shrimp grow-out operation. Data presented in this paper can serve as baseline data for improved bacterial water quality monitoring, and deduced risk implications based on scientific evaluations. The use of (bacterial) water quality indices complemented with plankton communities in other fishponds and rivers are open for future investigations.

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Conflict of interest. The authors declare that there is no conflict of interest.

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Authors:
Marz Linnaeous L. Rabadon, Center for Research on Aquaculture and Aquatic Resources in Brackishwater Systems, Institute of Fisheries and Aquatic Sciences, Bataan Peninsula State University, Orani Campus, Bataan 2112, Philippines, e-mail: mllrabadon@bpsu.edu.ph
Madel F. Damaso, Center for Research on Aquaculture and Aquatic Resources in Brackishwater Systems, Institute of Fisheries and Aquatic Sciences, Bataan Peninsula State University, Orani Campus, Bataan 2112, Philippines, e-mail: mfdamaso@bpsu.edu.ph
Mark Nell C. Corpuz, Center for Research on Aquaculture and Aquatic Resources in Brackishwater Systems, Institute of Fisheries and Aquatic Sciences, Bataan Peninsula State University, Orani Campus, Bataan 2112, Philippines; Graduate School, School of Environmental Science and Management, University of the Philippines Los Baños, College, Laguna 4031, Philippines, e-mail: mnccorpuz@bpsu.edu.ph

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