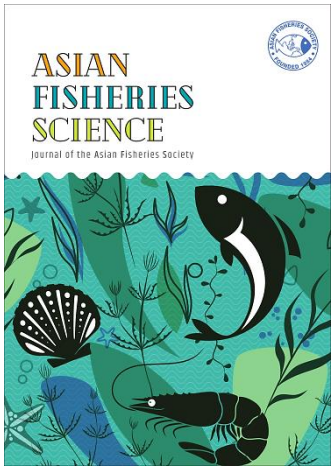

Bacterial Microbiota of Hatchery-Reared Freshwater Prawn *Macrobrachium rosenbergii* (de Man, 1879)

M.A. MONGHIT-CAMARIN[1,2], E.R. CRUZ-LACIERDA[1,*], R.V. PAKINGKING, JR.[3], M.L. CUVIN-ARALAR[4], R.F. TRAIFALGAR[1], N.C. AÑASCO[5], F.W. AUSTIN[6], M.L. LAWRENCE[6]

[1]Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miag-ao, Iloilo 5023, Philippines

[2]Fisheries Department, College of Fisheries, Mindanao State University-Marawi, Lanao del Sur 9700, Philippines

[3]Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan 5021, Iloilo, Philippines

[4]Binangonan Freshwater Station, Southeast Asian Fisheries Development Center, Aquaculture Department, Binangonan, Rizal, Philippines

[5]Institute of Marine Fisheries and Oceanology, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miag-ao, Iloilo 5023, Philippines

[6]Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Starkville 39762, MS, USA

*E-mail: eclacierda@up.edu.ph | Received: 08/06/2020; Accepted: 01/09/2020

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Abstract

Quantitative and qualitative analyses of bacterial microbiota associated with hatchery of freshwater prawn *Macrobrachium rosenbergii* (de Man, 1879) were conducted over three larval cycles, along with important water quality parameters. Physicochemical parameters (temperature, DO, salinity, pH and total ammonia) of culture water were within the optimum level required for the hatchery phase of *M. rosenbergii*. Aerobic plate count (APC) (log_{10} CFU.mL^{-1}) ranged from 4.2 ± 0.18–8.7 ± 0.01 in rearing water and (log_{10} CFU.g^{-1}) 3.3 ± 0.12–9.1 ± 1.1 in eggs and larvae. Significant differences in APCs among larval stages were observed. Ten genera and 17 species were identified with the predominance of Gram-negative bacteria constituting 63 % of all isolates (n = 706). *Acinetobacter baumannii, Acinetobacter lwoffii, Chryseobacterium indologenes, Enterobacter aerogenes, Enterobacter cloacae*, *Vibrio furnissii, Vibrio cholerae* non-01, *Plesiomonas shigelloides, Pseudomonas aeruginosa, Pseudomonas pseudoalcaligenes, Bacillus cereus, Staphylococcus haemolyticus, Staphylococcus warneri* and *Staphylococcus xylosus* were predominantly present in culture water. These bacteria likewise dominated in eggs and larvae except for the absence of *Ps. aeruginosa, Ps. pseudoalcaligenes, V. cholerae* non-01, *Staphylococcus epidermidis* and *Staph. xylosus* in eggs, clearly indicating that resident bacteria in water affect the composition of bacteria in eggs and larvae. Some of these are opportunistic pathogens. Thus, control measures to reduce influx of pathogenic microbes in the system by maintaining good water quality and good farm management practices through disinfection of culture facilities, rearing water, *Artemia* cysts, the practice of good hygiene of personnel, regular water exchange and feed regulation among others and cautionary use of antibiotics can be adopted.

Keywords: quantitative analysis, species composition, qualitative, normal flora, pathogen

Introduction

Aquaculture is the world's fastest-growing sector of agriculture with an average annual growth rate of 5.3 % in 2001–2018 (FAO, 2020). From the total global fish production of 179 million tons (2018), 82 million tons originate from aquaculture activities. Out of this total aquaculture production, 3 % come from the culture of freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879) (FAO, 2020). *Macrobrachium rosenbergii* has become a widespread subject to be studied and farmed commercially because of its fast growth, large size, good quality meat, omnivorous feeding habit and well-established reputation for both domestic and export markets around the world (Narmatha et al., 2017). Currently, there is a surge in interest to the farming of freshwater prawn since this species is seemingly less susceptible to diseases as compared to the penaeid shrimps (Pillai and Bonami, 2012). However, in the past decade, global demand for this commodity has increased, culture systems have intensified and eventually disease outbreaks have become a serious issue in the economic viability of freshwater prawn culture (Sharshar and Azab, 2008; Pillai and Bonami, 2012). Microbial diseases affecting all aspects of freshwater prawn farming have been considered as the major constraint in the sustainability of the industry (Sihag and Sharma, 2012).

In the hatchery, production success is hampered by mass mortalities caused by bacterial infection. Reduced hatchery production of post larvae has been linked to high *Vibrio* population in the larvae and the rearing water (Soundarapandian and Babu, 2010). Vibrios are also known to cause high or mass mortalities in penaeid shrimp culture (Lavilla-Pitogo et al., 1998). *Vibrio* spp. are ubiquitous in prawn culture environments and have been documented as a component of the normal microbiota of crustaceans. Consequently, some *Vibrio* spp. have been identified as primary pathogens of crustaceans, including *M. rosenbergii* (Sharshar and Azab, 2008). Mesophilic Enterobacteriaceae group which include pathogenic strains of *Escherichia coli* spp., *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Enterobacter* spp. and *Citrobacter* spp. composed the majority of bacteria implicated in the disease outbreak of *M. rosenbergii* and seafood-borne bacteria which are of human health importance (Yathavamoorthi et al., 2010).

There is growing awareness of the influence of the bacterial composition of the gut and culture medium on the health and growth of the host (Oxley et al., 2002). Early works have shown improvement in prawn production through manipulation of the bacterial species composition of the rearing water and in the digestive tracts of prawns (Moriarty, 1999). However, until now, the autochthonous bacterial composition of the freshwater prawn and its culture environment has not been fully elucidated. Characterisation of the different types and quantity of bacterial populations in cultured freshwater prawn in relation to the culture environment are considered useful indicators to determine the success of production and the quality of harvested prawns (Lalitha and Surendran, 2004). Also, detailed information pertinent to the bacterial quantity and species composition in apparently healthy *M. rosenbergii* and its rearing environments is essential. Furthermore, such information is important for prawn growers to be adept with predicting possible disease epizootics and concomitant opportunity to instigate management action to prevent these outbreaks. Pertinent information on the bacterial microbiota of the hatchery-produced freshwater prawn in the Philippines is currently not available. Thus, the present study was undertaken to investigate quantitatively and qualitatively the bacteria present in the rearing water, eggs and larvae of *M. rosenbergii*.

Materials and Methods

Sampling site

The study was conducted at Binangonan Freshwater Station (BFS) of the Aquaculture Department, Southeast Asian Fisheries Development Centre (SEAFDEC AQD), Binangonan, Rizal. The BFS has verified and demonstrated the hatchery and grow-out culture of *M. rosenbergii* (Cuvin-Aralar et al., 2011). Following the protocols prescribed by Cuvin-Aralar et al. (2011), the broodstocks were kept in concrete tanks at a density of 20–60 L per individual and a sex ratio of one male to five females. Sufficient aeration and moderate flow-through water were provided. Breeders with similar eggs of similar stage of development based on egg colour were placed together in one tank at a density of one breeder per 1.5 m^2. Upon spawning and soon after hatching of eggs, the spent breeders were returned to the broodstock tanks. The larvae were then transferred to rearing tanks with filtered artificial brackishwater (Valenti and Daniels, 2000) with 12 ppt salinity. The prepared artificial brackishwater was allowed to mature for at least a week prior to use. The combination of *Artemia* nauplii and egg custard were used as feeds for the freshwater prawn larvae. Excess feed was siphoned out before feeding.

Physicochemical parameters of rearing water

Physicochemical parameters of rearing water were recorded prior to microbiological examination. Temperature and dissolved oxygen (DO) were measured using YSI 55 DO meter and pH was measured using Thermo Scientific pH meter**.** Salinity was determined with a refractometer (Atago, Japan). Ammonia was determined using the Nessler method adapted from the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). All determinations were performed between 8:00 and 9:00 AM daily, before any exchange of water.

Bacteriological analyses

Microbiological investigations were performed for the rearing water, eggs and for each of the larval stages of freshwater prawn for three larval cycles. Water, eggs and larval samples were collected between 8:00 and 9:00 AM. The collection of samples was performed before changing the water in the rearing system.

Twenty-five randomly selected *M. rosenbergii* broodstocks of the same developmental stage were collected for the analysis. Five of the broodstocks were randomly selected and were used as the source of eggs for bacterial assessment. The eggs were collected from their brood pouches using sterile forceps and were analysed for bacterial composition and quantity. The remaining 20 berried broodstocks were then equally distributed to five different incubation tanks (4 broodstock per tank) with 50 L volume capacity filtered freshwater until the eggs hatched. Upon hatching of eggs, larvae (150 per L) were transferred to five larval rearing tanks (100 L capacity).

Larvae were fed with *Artemia* nauplii twice daily from Day 2 and chicken egg custard twice daily was introduced from Day 7 onwards. Fifty percent of the water was changed daily in spawning tanks and in rearing tanks after the 2nd larval stage, i.e., 3 days

after hatching as shown in Table 1 (Cuvin-Aralar et al., 2011).

The first larval sample was collected on the day of hatching of eggs from rearing tanks and subsequent samples were taken until the larvae metamorphosed into its last larval stage. Twenty-five larvae (5 larvae.unit^{-1}) were collected in sterile beakers.

Table 1. Water and feeding management during larval rearing of giant freshwater prawn, *Macrobrachium rosenbergii*.

Day	Time	Feed type	Water exchange (%)
0	no feeding	no feeding	50 %
1	no feeding	no feeding	None
2	8:00 AM	5 AN.mL^{-1}	None
	5:00PM	5 AN.mL^{-1}	
3	8:00 AM	5 AN.mL^{-1}	50 %
	5:00 PM	5 AN.mL^{-1}	
4	8:00 AM	5 AN.mL^{-1}	50 %
	5:00 PM	5 AN.mL^{-1}	
5	8:00 AM	5 AN.mL^{-1}	50 %
	5:00 PM	5 AN.mL^{-1}	
6	8:00 AM	5 AN.mL^{-1}	50 %
	5:00 PM	5 AN.mL^{-1}	
7	8:00 AM	3 AN.mL^{-1}	
	11:00 AM	EC	50 %
	2:00 PM	EC	
	5:00 PM	3 AN.mL^{-1}	
8-30	8:00 AM	3 AN.mL^{-1}	
	11:00 AM	EC	50 %
	2:00 PM	EC	
	5:00 PM	3 AN.mL^{-1}	

*AN- *Artemia* nauplii; *EC- Egg custard.

Bacterial quantification

Tryptone soy agar (TSA, HiMedia, India) was used for primary isolation and enumeration of total aerobic heterotrophic bacteria. For bacterial quantification, the plates were incubated at ambient temperature (28 °C) for 48 h (Prakash and Karmagam, 2013).

To quantify bacteria from culture tank water

Samples (100 mL) from the five larval rearing tanks were collected aseptically using sterile containers. The five water samples were pooled, serially diluted to 10^{-6} using sterile normal saline solution (NSS) and 100 µL of each diluted sample was spread in triplicate on TSA plates. After incubation, the plates containing 30–300 colonies were counted and results were calculated as colony-forming units (CFU) per mL of water (Prakash and Karmagam, 2013). Samplings were performed once daily per larval stage before changing the water in the rearing system until day 30 of culture.

To quantify bacteria from Macrobrachium rosenbergii *eggs*

One gram of *M. rosenbergii* eggs was randomly collected from the broodstocks and washed by vigorous agitation in three changes of 10 mL sterile NSS to remove surface microflora. The collected samples were homogenised in nine volumes of sterile NSS, serially diluted to 10^{-8} dilution, and 100 µL of each diluted sample was spread in triplicate on TSA plates. After plate counting, results were calculated as CFU.g^{-1} (Phatarpekar et al., 2002).

To quantify bacteria from Macrobrachium rosenbergii *larvae*

Larvae (n = 25) from each of the larval stages (1-11) were randomly collected from the hatchery tanks, pooled and similarly processed following the method described above for egg samples. Results were calculated as CFU.g^{-1} (Phatarpekar et al., 2002).

Isolation and identification of bacteria

Morphological and colonial characteristics of bacterial colonies on the TSA plates were recorded. The bacterial colonies were divided into different types according to the colony characteristics of shape, size, elevation, structure, surface, edge, colour and opacity. Accordingly, 3–5 representatives of each colony type were randomly picked from plates containing 300 colonies regardless of the dilution, and the isolated colonies were further subcultured to obtain a pure culture. Bacterial cultures were stocked in nutrient broth (NB) containing 15 % glycerol at –80 °C (Pakingking et al., 2015) and were deposited at the Regional Fish Health Laboratory of Bureau of Fisheries and Aquatic Resources (BFAR)-Caraga, Philippines.

Identification of the bacterial isolates was conducted at the College of Veterinary Medicine (CVM) Diagnostic Laboratory Services, Mississippi State University (MSU), Mississippi, USA. Permits such as Export Commodity Clearance from BFAR, Quezon City, Philippines and certification and an endorsement letter from the CVM Diagnostic Laboratory Services, MSU, Mississippi, USA allowing the transport of the bacterial isolates were secured prior to the transport of the samples. Initial characterisations of the bacterial isolates were performed following the criteria specified in Bergey's Manual of Systemic Bacteriology (Holt et al., 1994) using Gram-staining and biochemical tests such as oxidase, catalase and indole tests. The bacterial isolates were also grown on blood agar plates (BD Difco) to observe the haemolytic activity. Further identification of the bacterial isolates was done using the Sensititre Automated Reading and Incubation System (ARIS) 2x (Thermo Scientific, USA). In this system, inoculum was prepared from 18 h bacterial culture on blood agar plates using the sensititre sterile water (T3339) to make a 0.5

McFarland standard using the Nephelometer (Thermo Scientific, USA). With the aid of the Sensititre AIM Automated Inoculation Delivery System (Thermo Scientific Sensititre AIM Automated Inoculation Delivery System, Part No. V3020), 100 µL of the inoculum was inoculated into the Gram-negative or Gram-positive identification plates. Each test plate is capable of identifying three separate isolates. After overnight incubation of the plates in the machine, taxonomic identification of both Gram-negative and Gram-positive bacteria up to the species level was generated.

Statistical analysis

Aerobic plate counts (APCs) were expressed as CFU.g^{-1} for larvae and eggs, and as CFU.mL^{-1} for water. APCs were $\log_{10}$ transformed before statistical analysis was done. Data were statistically analysed using Sigma Plot version 12.5. One-way analysis of variance (ANOVA) was used to determine any significant differences for the physicochemical parameters, while Kruskal-Wallis was used for the bacterial counts. Duncan's multiple comparison was used to compare between groups. Correlation between the water quality parameters and bacterial load in culture water, eggs and larvae were also analysed using the two-tailed Pearson correlation analysis. All data were considered significant at $P < 0.05$ level.

Results

Physicochemical parameters of the rearing water

Data on physicochemical characteristics of eggs, larval stage and water at the time of sampling are presented in Table 2. Among replicate tanks, no significant differences were noted in water quality parameters ($P > 0.05$). Furthermore, no significant correlation between the water quality parameters and bacterial load was observed. Water temperature, DO, pH, salinity and total ammonia levels were within the optimum range required for rearing of *M. rosenbergii* larvae (New, 2002).

Quantitative data

Results of the quantitative estimation of APC in the eggs and larvae of *M. rosenbergii* and culture water are presented in Table 3. In general, APC in egg and larval samples ranged from 10^3 to 10^9 CFU.g^{-1}. In the case of culture water, mean APC ranged from 10^4 to 10^8 CFU.mL^{-1}. As shown in Table 3, APC in eggs and larvae varied among days of culture but no distinct pattern was noted as to which particular days APC would increase or decrease. However, the highest APC was observed for the larval stage 2 with mean APC ($\log_{10}$ CFU.g^{-1}) of 9.1 ± 1.1. Significant increase in aerobic plate counts was also observed in the culture water, particularly during day 30, with the highest mean APC ($\log_{10}$ CFU.mL^{-1}) of 8.7 ± 0.01.

Bacterial composition

A total of 706 bacterial isolates were characterised biochemically using conventional methods and Sensititre ARIS 2x. These bacteria were identified to species level (Table 4). The bacterial isolates belong to 10 bacterial genera and 17 species. The bacterial genera were predominantly Gram-negative. Moreover, nine bacterial species were documented in all sample types (culture water, eggs and larvae) namely *Aeromonas caviae*, *Acinetobacter baumannii*, *Acinetobacter lwoffii, Chryseobacterium indologenes*, *Enterobacter aerogenes, Enterobacter cloacae*, *Plesiomonas shigelloides, Serratia marcescens* and *Vibrio furnissii*. Predominant species from the egg samples were *A. caviae, V. furnissii* and *Staphylococcus warneri* with a percentage composition of 28 %, 15 % and 11 % respectively. *Pseudomonas pseudoalcaligenes, E. aerogenes* and *A. baumannii* were the dominant bacteria in the larval samples with a percentage composition of 20 %, 15 % and 13 %, respectively. Furthermore, *V. furnissii, V. cholerae* and *Staphylococcus xylosus* were the dominant bacteria found in the culture water samples with a percentage composition of 21 %, 17 % and 13 %, respectively. Detected *V. cholerae* were further classified as non-01 strains using the rapid detection test kit for *V. cholerae*. All the 17 identified species were found both in water and larval samples. However, only 12 of the identified species were found in eggs.

Discussion

This is the first comprehensive report on the bacteriology of hatchery-reared freshwater prawn larvae in the Philippines. Notably, the physicochemical parameters during the course of our sampling were within the normal range required for freshwater prawn larval culture systems (New, 2002). No information on the correlation between the physicochemical parameters and the bacterial load associated with hatchery-reared freshwater prawn is available for comparison. Moreover, in the present study, no significant correlation was observed between the culture water temperature, DO, pH and total ammonia with that of the quantitative counts in culture water, eggs, and larvae. This could be due to the reason that the water parameters were constant during the entire 30-day culture period.

The present study showed that the bacterial load in eggs, larval samples and tank water differed among days of culture. A general trend of increasing bacterial load in culture water was observed in the present study. Moreover, increasing bacterial cell count of larval sample with advancing age of larvae was also observed. The same results were noted by

Table 2. Physicochemical characteristics of the rearing water during the larval rearing of giant freshwater prawn, *Macrobrachium rosenbergii*.

Day	Temperature (°C)	DO (mg.L^{-1})	Salinity (ppt)	pH	Total ammonia (mg.L^{-1})
Day 0 (eggs)	25.47 ± 0.02	6.60 ± 0.11	0	7.30 ± 0.51	0.11 ± 1.08
Day 1 (Stage 1)	25.43 ± 0.15	6.61 ± 0.32	12	7.20 ± 0.86	0.11 ± 0.43
Day 3 (Stage 2)	25.97 ± 0.15	6.99 ± 0.21	12	7.13 ± 0.75	0.17 ± 0.33
Day 5 (Stage 3)	26.10 ± 0.10	6.58 ± 0.21	12	7.00 ± 0.75	0.16 ± 0.29
Day 7 (Stage 4)	25.27 ± 0.23	6.50 ± 0.44	12	6.90 ± 0.81	0.15 ± 0.03
Day 9 (Stage 5)	25.93 ± 0.06	6.78 ± 0.15	12	6.80 ± 0.15	0.14 ± 0.02
Day 12 (Stage 6)	25.90 ± 0.10	6.80 ± 0.01	12	7.20 ± 0.57	0.11 ± 0.14
Day 17 (Stage 7)	25.10 ± 0.51	6.59 ± 0.16	12	7.13 ± 0.42	0.19 ± 0.05
Day 19 (Stage 8)	25.53 ± 0.49	6.68 ± 0.28	12	7.50 ± 0.59	0.18 ± 0.14
Day 22 (Stage 9)	25.87 ± 0.31	6.58 ± 0.11	12	7.40 ± 0.51	0.17 ± 0.04
Day 25 (Stage 10)	26.20 ± 0.52	6.60 ± 0.30	12	7.20 ± 0.44	0.15 ± 0.03
Day 30 (Stage 11)	25.10 ± 0.17	6.51 ± 0.17	12	7.20 ± 0.23	0.11 ± 0.12

Values are means (± SD) of three larval runs at five rearing tanks per run.

Table 3. Aerobic plate counts (mean ± SD) in the culture water, eggs and larvae of giant freshwater prawn *Macrobrachium rosenbergii*. Data represent the mean Log_{10} (± SD) CFU.g^{-1} and CFU.mL^{-1} of viable bacteria in the eggs, larvae and water collected per sampling.

Day	Mean body weight (g)	Eggs (CFU.g^{-1})	Larvae (CFU.g^{-1})	Culture water (CFU.mL^{-1})
Day 0 (eggs)	NA	4.1 ± 0.99^{ab}	NA	4.2 ± 0.18^{a}
Day 1 (Stage 1)	0.02	NA	3.30 ± 0.12^{a}	6.30 ± 0.96^{bc}
Day 3 (Stage 2)	0.02	NA	9.10 ± 1.10^{d}	5.10 ± 0.94^{ab}
Day 5 (Stage 3)	0.03	NA	5.90 ± 0.41^{bc}	7.10 ± 1.70^{cd}
Day 7 (Stage 4)	0.03	NA	7.80 ± 1.80^{cd}	8.20 ± 1.40^{cd}
Day 9 (Stage 5)	0.04	NA	6.90 ± 0.16^{cd}	7.96 ± 0.03^{cd}
Day 12 (Stage 6)	0.06	NA	6.80 ± 1.90^{c}	6.90 ± 0.11^{cd}
Day 17 (Stage 7)	0.07	NA	8.30 ± 0.59^{cd}	8.10 ± 0.19^{cd}
Day 19 (Stage 8)	0.08	NA	6.60 ± 0.50^{cd}	8.10 ± 0.40^{cd}
Day 22 (Stage 9)	0.09	NA	6.20 ± 2.60^{bc}	5.10 ± 0.16^{ab}
Day 25 (Stage 10)	0.10	NA	6.00 ± 2.10^{bc}	7.30 ± 2.40^{cd}
Day 30 (Stage 11)	0.15	NA	8.30 ± 0.43^{cd}	8.70 ± 0.01^{d}

Values are means of three larval runs at five rearing tanks per run. Values with the same superscripts are not significantly different at $P < 0.05$. NA: not applicable.

Table 4. Bacterial composition and percentage distribution in the rearing water, eggs and larvae of freshwater prawn *Macrobrachium rosenbergii*.

Bacteria	Gram stain reaction	Water		Egg		Larvae		Total	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
Aeromonas caviae	-	3	1	65	28	6	3	74	11
Acinetobacter baumannii	-	9	3	15	6	27	13	51	7
Acinetobacter lwoffii	-	11	4	9	4	9	4	29	4
Chryseobacterium indologenes	-	25	9	16	7	22	11	63	9
Enterobacter aerogenes	-	18	7	9	4	30	15	57	8
Enterobacter cloacae	-	13	5	9	4	6	3	28	4
Plesiomonas shigelloides	-	6	2	8	3	6	3	20	3
Pseudomonas aeruginosa	-	8	3	-	-	18	9	26	4
Pseudomonas pseudoalcaligenes	-	8	3	-	-	40	20	48	7
Serratia marcescens	-	3	1	9	4	5	3	17	2
Vibrio cholerae non-01	-	45	17	-	-	9	4	54	8
Vibrio furnissii	-	58	21	36	15	7	3	101	14
Bacillus cereus	+	3	1	9	4	5	3	17	2
Staphylococcus epidermidis	+	6	2	-	-	2	1	8	1
Staphylococcus haemolyticus	+	7	3	19	8	2	1	28	4
Staphylococcus warneri	+	8	3	26	11	2	1	36	5
Staphylococcus xylosus	+	35	13	-	-	2	1	37	5
Unidentified		5	2	3	1	4	2	12	2
Total		271	100	233	100	202	100	706	100

Values are means of three larval runs at five rearing tanks per run.

Miyamoto et al. (1983) and Anderson et al. (1989). APCs of ca. $\leq 10^4$ CFU.g^{-1} were quantified in the eggs of freshwater prawn. These values were lower compared to Phatarpekar et al. (2002), wherein the APCs ranged from $2.4 \pm 0.4 \times 10^5$ to $8.6 \pm 1.6 \times 10^6$ CFU.g^{-1}. On the other hand, the mean APC ($\log_{10}$ CFU.g^{-1}) of 9.1 ± 1.1 obtained for the larvae was comparable to the previous report of Al-Harbi (2003) ($5.0 \pm 1.5 \times 10^7$ CFU.g^{-1}). Similarly, the mean APC ($\log_{10}$ CFU.mL^{-1}) of 8.7 ± 0.01 quantified in water samples was similar to the results of Al-Harbi (2003) ($2.2 \pm 0.8 \times 10^7$ CFU.mL^{-1}). Additionally, the APCs quantified in the culture water in the current study were within the ranges previously reported by Suluja et. al (2005) and Phatarphekar et al. (2002). The differences noted for bacterial counts in the culture water and larval samples in the present study could be attributed to the type of culture system used (Al-Harbi and Uddin, 2004). We used the static renewal system wherein water in the tanks was partially replaced. Whereas studies conducted in other countries have used the clear water culture system, wherein water in each tank was changed almost completely either once or twice daily (Al-Harbi and Uddin, 2004). Nonetheless, because all *M. rosenbergii* eggs and larval samples collected during the course of our sampling appeared to be healthy, and no mortalities were recorded, it can be inferred that APCs of $\leq 10^9$ CFU.g^{-1} in eggs and larvae and $\leq 10^8$ CFU.mL^{-1} in the water could be considered normal baseline bacterial quantities for aquaculturists actively engaging in freshwater prawn culture in the Philippines. However, more *in vitro* and *in vivo* studies are warranted to elucidate this issue.

Laboratory-hatched brine shrimp (*Artemia*) has been suggested as a source of bacteria in the culture water environment (Austin and Allen, 1982). Although this suggestion was not examined in the present study, Austin and Allen (1982) reported that bacterial numbers in dried cysts were relatively low compared with the counts obtained during the hatching processes when there was a rapid multiplication of bacteria of some taxa reaching 1.2×10^7 CFU.mL^{-1} in the water. This clearly indicates that the bacterial populations from laboratory-hatched *Artemia* could enrich the culture water and may thereby influence the viable bacterial counts. Austin and Allen (1982) further pointed out that there was no evidence of intimate colonisation of the nauplii by bacteria and that, the loosely attached surface microbiota were removed readily by washing hatched nauplii with sterile seawater. However, the high bacterial load in the culture water of the freshwater prawn examined in the current study could not be linked to dried *Artemia* cysts because as part of the BFS SEAFDEC AQD's freshwater prawn hatchery management, the decapsulated cysts were thoroughly disinfected with 30 ppm formalin. This procedure reduces if not completely eradicates the bacterial load of the hatching medium during incubation (Cuvin-Aralar et al., 2011). Also, in accordance with the BFS freshwater prawn hatchery management, water exchange frequency in the tanks was strictly followed as an attempt to regulate the bacterial population in the culture water. The quantities of egg custard fed to prawn larvae was also regulated accordingly. Although egg custard is presumed to be free of bacteria, it has been reported that excessive feeding of prawn larvae with egg custard could enable bacteria existing in the culture water to readily utilise the organic substances from uneaten feeds to rapidly proliferate and consequently give rise to disease epizootics (Zobell and Feltham, 1938; Phatarpekar et.al, 2002). However, it is worth noting that high bacterial population is not necessarily a disadvantage, especially if the bacteria present in the culture water are not pathogenic. High quantity of beneficial microbiota, i.e. probiotic bacteria, in the culture water may indicate a potential for organic matter recycling, self-cleaning potential and re-mineralisation (Al-Harbi, 2003). Because ambient temperatures ranging from 28–30 °C favour the proliferation of resident bacteria in the culture water, strict adherence to freshwater prawn hatchery practices including disinfection of culture water, other hatchery paraphernalia and *Artemia* cysts during decapsulation is imperative for the sustainability of freshwater prawn aquaculture.

Gram-negative bacteria including *A. caviae*, *A. baumannii*, *A. lwoffii, C. indologenes, E. aerogenes, E. cloacae, P. shigelloides, Ps. aeruginosa, Ps. pseudoalcaligenes, Se. marcescens, V. cholerae* non-01 and *V. furnissii* dominated the overall composition of the bacterial microbiota in the freshwater prawn eggs, larvae and culture water. Anderson et al. (1989) observed a decrease in bacterial population with age of the larvae in some of the larval tanks. However, no distinct pattern was observed between bacterial composition and larval stage in the present study. All of the identified species present in the rearing water were also found in the eggs and larvae of *M. rosenbergii* except for the nil isolation of *Ps. aeruginosa, Ps. pseudoalcaligenes, V. cholerae* non-01, *Staph. epidermidis* and *Staph. xylosus* in the egg samples. This clearly indicated that the resident bacteria of the rearing water congruently influenced the microbiota of spawned eggs and larvae. Moreover, all bacteria identified in the larval samples were represented in the eggs of *M. rosenbergii* system, although in different percentages of incidence. A decrease in the bacterial composition of eggs may be attributed to the presence of membrane, which holds them together in the brood chamber. The membrane may act as a barrier and prevent the attachment and subsequent proliferation of bacteria present in the water onto the egg surface. It has been observed that when eggs come in contact with tank water upon their release, bacteria present in the water attach and proliferate onto egg surfaces (Sahul Hameed, 1993; Phatarpekar et al., 2002). The preening of eggs by the berried female with its pleopods might also help in restricting the bacterial population (Phatarpekar et al., 2002).

Notably, the dominance of *V. furnissii* (21 %) in the rearing water was documented in the present study. This observation could be attributed to the water salinity (12 ppt) used in this study which favoured the proliferation of halophilic *Vibrio* spp. Moreover, the warm temperature of the rearing water may have also favoured the growth of *Vibrio* spp. Vibrios are also known as part of the normal microbiota of brackish and marine waters (Chen et al., 2011). However, *V. furnissii* is not regarded as a pathogen of freshwater prawn, hence, its presence in the eggs and larval samples is not likely linked to the possible occurrence of disease epizootics (delves-Broughton and Poupard, 1974; Lightner and Lewis, 1975; Yasuda and Kitao, 1980; Lewis et al., 1982; Al-Harbi, 2003). Additionally, *V. cholerae* non-01 was detected in the culture water and larval samples, similar to data obtained by Al-Harbi and Uddin (2004). Although not also considered a pathogen of *M. rosenbergii*, the presence of *V. cholerae* in the water and larval samples should be noted because of its zoonotic potential to pond technicians through direct contact with infected animal or culture water (Pakingking et al., 2015).

Molecular identification of the isolates was not conducted in the present study. However, studies have shown the reliability and accuracy of Sensititre ARIS 2x as an identification system. Doing and Rioux (2006) showed that an essential agreement of 98 %, after discrepant analysis, was obtained between the ARIS 2X and the antimicrobial susceptibility testing (AST) methods evaluated. Categorical error rates attained with the ARIS 2X were within acceptable limits, and a very major error rate of <1.5 %, was comparable to recent studies evaluating the Sensititre ARIS 2X and MicroScan System. The Sensititre ARIS 2X System could, therefore provide an excellent platform to consolidate microbiology test methods (Chapin and Musgnug, 2004).

Conclusion

The results of the present study indicate that the resident bacteria of the rearing water correspondingly influence the bacterial microbiota of freshwater prawn eggs and larvae. The results provide a basis for further research in exploring the possibility of rearing larvae in lower salinities to reduce *Vibrio* population in water. Furthermore, the resident bacteria in the culture water of freshwater prawn identified in this study were mostly opportunistic pathogens which can proliferate beyond the threshold levels and cause disease outbreaks in prawn larvae especially if the host is stressed. It is, therefore, necessary to regulate the bacterial load in the freshwater prawn aquaculture system by maintaining good water quality and good farm management practices through disinfection of culture facilities, rearing water and *Artemia* cysts, the practice of good hygiene of hatchery personnel, regular water exchange and feed regulation among others. These practical safety measures managed the resident bacterial microbiota in the rearing water, eggs, and larvae and consequently prevented the proliferation of potential pathogens. The use of antibiotics may also be explored but with regulation and caution because of known negative health implications. The detection of *A. caviae, B. cereus, V. furnissii, V. cholera* non-01, *Ps. aeruginosa, C. indologenes, Ps. pseudoalcaligenes* and *P. shigelloides* in the current study should not be equally ignored in conjunction with their involvement in zoonosis.

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