

Application of Indoor Recirculation Aquaculture System for White Shrimp (*Litopenaeus vannamei*) Growout Super-Intensive Culture at Low Salinity Condition

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Abstract

The use of close aquaculture system including Recirculation Aquaculture System (RAS) has been implemented to allow a more stable water quality, good hygiene and efficient use of water resources in wide shrimp aquaculture production. This study aims to optimize shrimp stocking density and to evaluate microbial community profile in super-intensive culture of Pacific white shrimp (*Litopenaeus vannamei*) using RAS technology at low salinity. Before stocking, post larvae shrimp was gradually acclimatized from salinity level of 32 ppt to 5 ppt within 14 days. Different stocking density of 500 PL/m³, 750 PL/m³ and 1,000 PL/m³ were tested in four replicates. During 84 days grow out period, no differences in water quality parameters were observed. At the end of grow out period, significant differences were found in final body weight (14.87 ± 0.24 g, 13.09 ± 0.78 g, 11.32 ± 0.71 g), survival (70 ± 1.42%, 53.67 ± 4.16%, 44 ± 1.35%), specific growth rate (7.12% BW/day, 6.95% BW/day, 6.79% BW/day), and feed conversion ratio (1.32 ± 0.09, 1.45 ± 0.16, 2.05 ± 0.24) for the 500 PL/m³, 750 PL/m³, and 1,000 PL/m³ treatment group, respectively. However, similar total productivity of 5.20 kg/m³, 5.24 kg/m³, and 4.99 kg/m³ was observed for 500 PL/m³, 750 PL/m³, and 1,000 PL/m³ treatment group, respectively. The implementation of RAS can allow a stable community structure of culturable bacteria even at high shrimp density of up to 1,000 PL/m³, with the observed bacterial abundance of 1.28 × 10³ to 5.28 × 10⁴ CFU/mL and 9.49 × 10⁴ to 2.27 × 10⁶ CFU/mL in shrimp and culture water, respectively. It is suggested that the application of RAS at the optimal shrimp density of 500 PL/m³ allowed a high shrimp culture productivity of up to 5.20 kg/m³ within 84 days grow out period.

Keywords: *Litopenaeus vannamei*; Culturable bacteria; Community structure; Low salinity; Recirculation aquaculture system; Stocking density

Introduction

Indonesia is the largest archipelagic country in the world with 5.8 million km² of ocean and 91,181 km of coastline, which contain marine and fishery resources as potential for natural resource-based economic development [1]. Recently, Indonesia is world's second largest aquaculture producer country after China. In average, the aquaculture production in Indonesia increased 27.8% per year [2], where 16.5% of the total production are dominated by shrimp industry. Pacific white shrimp, *Litopenaeus vannamei*, is one of the main commodities [2] due to the availability of its Specific Pathogen Free (SPF) broodstock/larvae [3] as well as its ability to grow in high stocking densities and cope with wide variations of water salinity [4]. Even though the global farmed shrimp production has increased in the recent years, major producing countries has experienced a decline in output due to disease-related problems including vibriosis caused by pathogenic bacteria *Vibrio sp.* It has been suggested that the number of *Vibrio sp.* which exceeds the threshold of 10⁴ CFU/mL indicating susceptibility to vibriosis. Vibriosis often occurred when the organic content in the pond is high. Therefore, maintenance of water quality is critical in wide shrimp production [5].

Conventional improvement of shrimp production through the expansion of land and use of open systems has several constraints including unstable water quality and high susceptibility to infectious diseases that often lead to the unpredicted shrimp productivity. On the other hand, the use of close system Recirculation Aquaculture System (RAS) offers a more flexible, predictable, hygienic, and environmentally friendly shrimp production even at high density, contributing to the more sustainable shrimp industry [6]. RAS technology has the advantage of highly efficient use of water resources, mainly due to

the water treatment process through water circulation along the RAS components. It is also including physical and biological filtration units, which can solve the problems raised from high level of organic content in the system and also minimize the risk of infection by pathogenic bacteria that may exist in new untreated seawater [6].

Related to the disease problem, one alternative strategy to address the vibriosis problem has been provided in the form of the bacterial storage compound poly-β-hydroxybutyrate (PHB), the polymer of the short-chain fatty acid β-hydroxybutyrate (β-HB), as carbon reserve and intracellular energy source for a large variety of bacteria, including the genera of *Bacillus*, *Pseudomonas*, *Rhizobium* and *Alcaligenes*. PHB is water insoluble and biologically degrades into β-HB upon entrance in the gastrointestinal tract [7]. Several studies have confirmed the protective effect of PHB against a variety of bacterial diseases in farmed aquatic animals against, albeit through undefined mechanisms [8-11]. Interestingly enough, an increased growth performance in crustaceans has also been observed as a result of dietary PHB supplementation for prawn [9,12] and shrimp [13] culture. Therefore, this study aims to

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optimize the stocking density and to evaluate community structure of culturable bacteria in super-intensive culture of Pacific white shrimp (*Litopenaeus vannamei*) fed with PHB supplemented diet in RAS technology at low salinity condition.

Materials and Methods

System preparation and conditioning

This study was took place at the Laboratory of Aquatic Ecology, School of Life Sciences and Technology, Institut Teknologi Bandung. The RAS system consists of 100 L settlement tank, 100 L protein skimmer, 50 L activated carbon tank, 300 L biofilter and 12 of 100 L shrimp culture tanks (Figure 1). Following the inoculation of nitrifying bacteria consortium into the biofilter tank, biofilter conditioning was carried out by adding 5 mg/L of ammonium chloride (NH₄Cl) followed by daily measurement of ammonium (NH₄), nitrite (NO₂), and nitrate (NO₃) level. When ammonia and nitrite level have reached 0 mg/L, 10 mg/L ammonium chlorides were added. The biofilter was then ready for use once the ammonia and nitrite level have decreased to 0 mg/L once again.

Experimental design

The water in the culture tank is sterilized prior use by using 30 mg/L of chlorine (NaClO) for 1 day, then neutralized using 30 mg/L of sodium thiosulfate (Na₂S₂O₃) for 1 day [14]. Before stocking, 8 days old shrimp post larvae (PL8) was gradually acclimatized from salinity level of 32 ppt to 5 ppt within 14 days. The shrimp was then cultured for 84 days at three different stocking shrimp density of 500 PL/m³, 750 PL/m³ and 1,000 PL/m³, each in four replicates. Poly-β-hydroxybutyrate (PHB)-supplemented diet at 0.5 g PHB/kg [11-13] was used as the sole shrimp diet in this study. RAS is operated continuously for 24 hours without water replacement, with the exception of water addition to compensate water evaporation that may gradually increase the salinity level of the culture water. Siphoning was conducted daily to remove most sediment from the culture tank as well as settlement tank [15,16].

Water quality parameters

Water quality parameters including dissolved oxygen (DO), temperature, pH, NH₃⁺, NO₂⁻, NO₃⁻ and salinity level were measured biweekly during the 84 days of grow out period. DO level and temperature

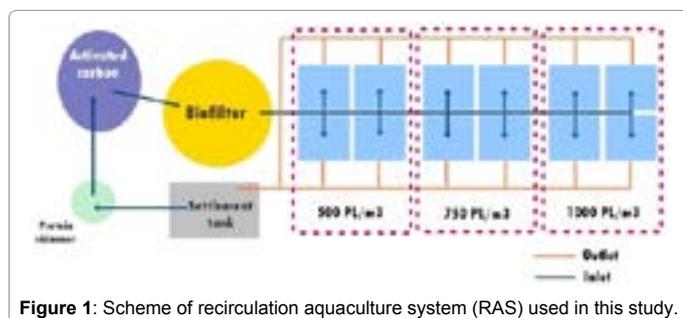


Figure 1: Scheme of recirculation aquaculture system (RAS) used in this study.

Mean Body Weight	Feeding Rate (%)	Survival Rate (%)	Feeding Tray Monitoring Intervals (hours)
<1	10.0	100	3.5
1-3	8.0	98	3.5
3-5	6.0	96	2.5
5-7	5.0	94	2
7-9	4.0	92	2

Table 1: Feed management applied during 84 days grow out period [15].

was measured using DO meter while pH level was measured by using pH meter. The NH₄⁺, NO₂⁻, and NO₃⁻ levels were measured following Nessler, Diazotation, and Nitrate-HCl spectrophotometer method [17]. Salinity measurement was done using hand refractometer.

Feeding regime and maintenance of the system

Feeding management was done by calculation of daily feed amount based on estimation of mean body weight (MBW), survival estimation and feeding rate by following below equation:

$$\text{Daily Feed(g)} = \text{SD} \times \text{MBW} \times \text{FR} \times \text{SR}$$

where SD is the initial stocking density, MBW is the average mean body weight of shrimp (gram), SR is estimated survival rate (%), and FR is feeding rate (%). Detailed feeding regime used for this trial is presented in Table 1. Daily feeding frequency was four times a day at 09:00, 12:00, 16:00 and 21:00.

Proximate analysis

Proximate analysis was done at PT. Saraswanti Indo Genetech, Bogor, Indonesia, to determine the water, ash, total protein and lipid content, carbohydrates, and fatty acid content of the diet. Water content measured using drying method. The ash content was measured using heating at 600°C temperature and then weighed until constant [16]. Protein content was measured using Kjeldhal method while the lipid content was measured using Soxhlet method, fatty acid analysis was conducted by using gas chromatography, while carbohydrate content (including nitrogen free extract and crude fiber) was calculated by following this equation:

$$\text{Carbohydrate level (\%)} = 100\% - (\text{moisture content} + \text{fat content} + \text{ash content} + \text{protein content})$$

Biological parameters

Sampling of shrimp was done every two weeks to evaluate the biological parameters including shrimp growth and survival. At the end of grow out period, the final shrimp total biomass was measured. The shrimp specific growth rate (SGR), survival as well as feeding efficiency in term of Feed Conversion Ratio (FCR) were also calculated using the following formulas [18]:

$$\text{Mean Body Weight (gr)} = W/\Delta N$$

where W is weight shrimp and total shrimps

$$\text{Survival (\%)} = N_t/N_0 \times 100\%$$

where N₀ is initial shrimp number, N_t is final shrimp number, t is culture period (day)

$$\text{Specific growth rate (\%)} = [\text{Ln}(W_2 - W_1)] / (T_2 - T_1) \times 100\%$$

where W₁ is initial body weight (g) at time T₁ (day), and W₂ is final body weight (g) at the time T₂ (day)

$$\text{Feed Conversion Ratio (FCR)} = \Sigma W_{\text{feed}} / \Delta W \times 100\%$$

where ΣW_{feed} is the total feed given during culture (g), and ΔW is the total weight of shrimp in each tank (g)

$$\text{Biomass (gr)} = \text{Density} \times \text{Average } \Delta W$$

where ΔW are total of weight shrimps.

Microbiological parameters

Three shrimps were sampled from each replicate tank and pooled,

rinsed and homogenized in 9 g/L NaCl sterile saline solutions. Subsequently, 100 µl of each whole-body sample homogenate was plated on Nutrient Agar Seawater plates. The inoculated plates were incubated at 27 ± 1°C for 24 h and the total number of viable heterotrophic bacteria was counted. One hundred microliters of each whole-body sample homogenate was also inoculated on Thiosulfate Citrate Bile Salts Sucrose agar plates to count the total number of *Vibrio* [19].

Pure bacterial samples then going through further identification process using molecular approach. Bacterial DNA extraction, polymerase chain reaction / PCR amplification, and bacterial rRNA 16S gene sequencing were done at the Macrogen Inc., Korea, using commercial QiagenDNEasy blood and tissue kit, using 27F/1492R primer, using 785F/907R primer, respectively. Nucleotide Blast versus Genbank and National Centre for Biotechnology Information (NCBI) data was used for sequence homology search for the test sequences [19]. These results were validated by phylogenetic analysis performed by Maximum likelihood with 1000 bootstrap on MEGA 6.0.

The abundance of culturable bacteria, the proportion of each different species, as well as the Shannon-Wiener diversity index were calculated. Predominant culturable bacteria is determined when its proportion of abundance is higher than 10% [20]. Subsequently, similarity dendrogram was produced with UPGMA clustering method using Sorensen's coefficient with MVSP (Multi Variate Statistical Package) v32 software.

Statistical analysis

Comparison of shrimp survival, final MBW, total biomass, SGR, and FCR between treatment groups were done by using One-way Analysis of Variance analysis, where grouping of treatments was based on significant differences in mean values according to Duncan test (0.05) level of confidence.

Result and Discussion

Bio-filter conditioning

Conditioning of biofilter was performed in 10 days. Three days following addition of 5 mg/L NH₄Cl, the NH₄⁺ level had been decreased to 0 mg/L, while NO₂⁻ was accumulated to 40 mg/L. This indicated that at day 3, only Ammonia Oxidizing Bacteria (AOB) had been activated.

Parameters	500 PL/m ³	750 PL/m ³	1,000 PL/m ³	Tolerance level
DO (mg/L)	5.95-8.24	5.75-8.14	5.67-8.32	4-12 [23]
Temperature (°C)	28.35-30.40	28.20-30.38	28.40-30.45	26-30 [23]
pH	6.80-8.00	6.78-7.90	6.70-7.90	6.5-8.5 [23]
NH ₄ ⁺ (mg/L)	0-0.52	0-0.71	0-0.67	≤0.8 [24]
NO ₂ ⁻ (mg/L)	0-0.53	0-0.66	0-0.64	≤1.0 [24]
NO ₃ ⁻ (mg/L)	0-62.86	0-66.12	0-58.20	<200 [25]

Table 2: Physicochemical water quality parameters during 84 days of culture period.

Nutrient parameters	Content (% dry weight)
Water	5.77 ± 0.63
Ash	10.65 ± 0.11
Total Lipid	8.19 ± 0.18
Total Protein	39.81 ± 0.28
Carbohydrate	35.58 ± 0.7

Table 3: Proximate analysis of shrimp diet.

Parameters	Density		
	500 PL/m ³	750 PL/m ³	1,000 PL/m ³
Individual MBW(g)	14.87 ± 2.42 ^a	12.94 ± 2.72 ^b	11.32 ± 3.60 ^c
Total Biomass (g)	520 ± 17.53	524 ± 16.72	499 ± 27.54
Survival Rate (%)	70 ± 1.42 ^a	53.67 ± 4.16 ^c	44 ± 1.35 ^b
Feed Conversion Ratio	1.32 ± 0.05 ^a	1.45 ± 0.08 ^b	2.05 ± 0.12 ^c
Specific Growth Rate (%BW/day)	7.12 ± 0.01 ^a	6.95 ± 0.01 ^b	6.79 ± 0.01 ^c

Table 4: Biological parameters during 84 days grow out period.

At day 4, NH₄Cl was added at a higher concentration of 10 mg/L. At day 7, the NH₄⁺ level had been decreased to 0 mg/L, while NO₂⁻ level started to decrease and NO₃⁻ level was increased to 80 mg/L. This indicated that both the AOB and Nitrite Oxidizing Bacteria (NOB) had been activated. The oxidation of NH₄⁺ was faster than the oxidation of NO₂⁻ due to the relatively faster growth of AOB (± 8 h) compared to NOB (± 12 h) [21]. In addition to that, NOB needed the NO₂⁻ as the oxidation product by AOB as its growth substrate. Following 10 days of biofilter conditioning period, the biofilter was ready to use as both NH₄⁺ and NO₂⁻ level had reached 0 mg/L, while NO₃⁻ level reached 100 mg/L.

Water quality parameters

Water quality management is one of primary consideration in any shrimp farming, particularly in systems with higher stocking density. It is suggested that degradation of water quality is detrimental to shrimp growth and survival. During 84 days of grow out period, all physicochemical water quality parameters in all shrimp densities were in tolerance level for shrimp culture (Table 2). This suggests that the continuous use of RAS can maintain stable water quality parameters even at high stocking densities of up to 1,000 PL/m³ with high organic load to the system. However, slightly lower inorganic nitrogen compounds of ammonia and nitrite were observed in the lowest shrimp density compared to the higher shrimp densities.

Proximate analysis of shrimp diet

Li et al. [22] suggested that properly high dietary lipid is able to improve the performance of *L. vannamei* at low salinity. It is reported by Zhu et al. [23] that 8% lipid diet allowed a slightly higher growth than 6% or 10% lipid diets in shrimp culture at low salinity of 2 psu. The diet used in this study contained 8.19 ± 0.18% lipid, which was assumed to allow an optimum shrimp growth at low salinity of 5 ppt [22,23]. It is also suggested that an increase in dietary protein level in the diet of *L. vannamei* is necessary to increase the production of *L. vannamei* at low salinities, with a recommended 40% of dietary protein for shrimp culture to improve growth and health [22]. In this study, the diet was formulated to contain 40% total protein level and had the actual total protein level of 39.81 ± 0.28% (Table 3). Overall, the diet used in this study supported a normal, if not optimal, shrimp growth at low salinity and that the resulted shrimp growth in this study using different high stocking density was not impaired by the low salinity level.

Biological parameters

During 84 days of grow out period, significant differences were found in final MBW, survival, SGR and FCR between shrimp groups with different density (Table 4). In term of growth, lower MBW were observed with the increasing shrimp density, where the lowest shrimp density of 500 PL/m³ produced shrimp with final MBW of 15 g, while the higher densities resulted in lower final MBW of 11 to 13 g. Similar to the growth parameter, a significantly higher survival (up to 70%) was

observed in the lowest shrimp density compared to the higher shrimp densities, where the highest shrimp density gained the lowest survival of only about 44%. However, there was no significant difference in the total biomass produced between different shrimp density, with the total productivity value of up to 5.20 kg/m³, 5.24 kg/m³, and 4.99 kg/m³ for the shrimp density of 500 PL/m³, 750 PL/m³, and 1,000 PL/m³, respectively. In term of feed utilization efficiency, it was showed that the lowest FCR value of 1.32 was obtained in the lowest shrimp density and the values were increased with the increasing shrimp density of up to 2.05 in the 1,000 PL/m³ density.

In general, it can be suggested that the application of RAS system at the shrimp density of 500 PL/m³ can maintain good water quality during the 84 days of grow out period and allow high productivity of up to 5.20 kg/m³ with high feed utilization efficiency and thus is highly potential to be implemented at the industrial level.

Dynamic abundance of culturable bacteria

The dynamic of the abundance of culturable bacteria in both culture water and shrimp during the culture period are shown in Table 5. In general, the number of culturable bacteria was calculated as much as 10³ CFU/mL in the culture water and 10⁵ CFU/mL in shrimp. The dynamic of the amount of bacteria present in water and shrimp depends on the organic content in the tank culture. Input of organic content can be derived from feeding and residual metabolism [24].

Community structure of culturable bacteria

A total of nine species of culturable bacteria in water samples and six species of culturable bacteria in shrimp samples were observed in different proportions (Figures 2 and 3). The identified bacteria were *Staphylococcus saprophyticus*, *Shewanella amazonensis*, *Aeromonas*

caviae, *Shewanella litorisediminis*, *Acinetobacter dioresistens*, *Bowmanella pacifica*, *Bacillus altitudinis*, *Microbacterium kitamiense*, and *Pseudomonas aeruginosa*.

In general, there was one bacterial species, *S. litorisediminis*, which was always observed with >10% of abundance both in the culture water and shrimp in all treatment groups, especially until the 8th week of culture period. Some species of *Shewanella* genus are considered as a good probiotic candidate as they exhibit antibacterial activity that may improve survival rate of shrimp culture [25]. The existed *S. litorisediminis* is suspected to be one of the indigenous bacteria in the shrimp, however very limited knowledge exists on the presence and role of *S. litorisediminis* in shrimp culture. Other species of *Shewanella* genus observed in this study is *S. amazonensis*, which has the ability to reduce heavy metals such as iron, manganese oxide, thiosulfate, and sulphur-containing substances [26].

During the last 1-2 weeks of shrimp culture, a high relative abundance of *A. radioresistens* was observed in both shrimp and culture water. This bacterial species was reported to be one of the predominant strains with the PHB accumulating ability in a bacterial community from activated sludge [27]. The PHB supplementation in the shrimp diet used in this study may have opened niches for this bacterial species. *P. aeruginosa* is one of the bacteria that has the ability as a probiotic in shrimp. Research has been done that the feed given a mixture of *P. aeruginosa* can suppress the number of bacteria *Vibrio* sp. both in water and shrimp [28].

Shannon-Wiener diversity index

Diversity of species is determined by number of species (species richness) and relative abundance (species of evenness) [28]. Diversity

Density	Culturable bacterial load in culture water (CFU/mL)	Culturable bacterial load in shrimp culture (CFU/mL)
500 PL/m ³	8.30 × 10 ² – 4.20 × 10 ³	2.50 × 10 ³ – 3.49 × 10 ⁵
750 PL/m ³	1.33 × 10 ³ – 5.81 × 10 ³	5.50 × 10 ³ – 4.63 × 10 ⁵
1,000 PL/m ³	2.17 × 10 ³ – 6.32 × 10 ³	4.40 × 10 ³ – 3.36 × 10 ⁵

Table 5: The abundance of culturable bacteria following 84 days of culture period.

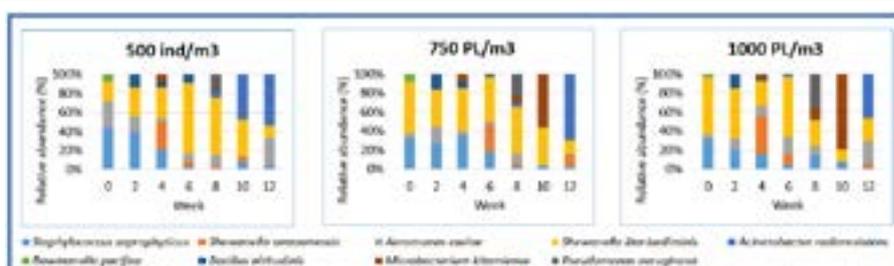


Figure 2: Relative abundance of culturable bacteria in culture water.

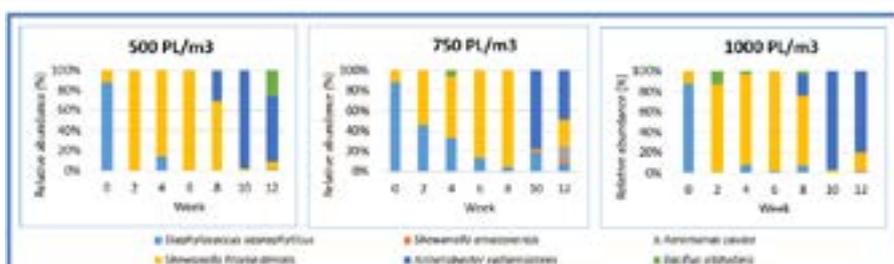


Figure 3: Relative abundance of culturable bacteria in shrimp culture.

Density	H' index of culture water	H' index of shrimp culture
500 PL/m ³	1.621	1.113
750 PL/m ³	1.728	1.167
1,000 PL/m ³	1.819	1.002

Table 6: Shannon-Wiener diversity index of water and shrimp culturable bacteria.

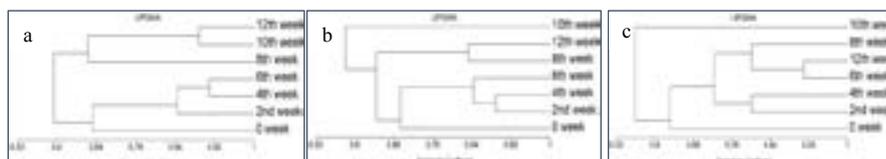


Figure 4: Dendrogram of similarity of culturable bacteria in culture water at stocking density of (a) 500 PL/m³ (b) 750 PL/m³ and (c) 1,000 PL/m³.

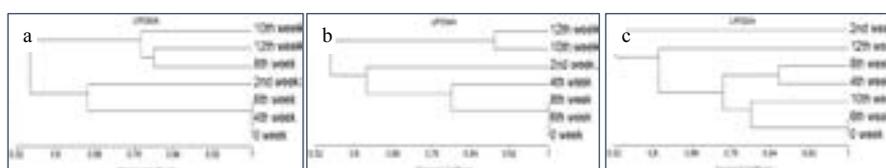


Figure 5: Dendrogram of similarity of culturable bacteria in shrimp culture at stocking density of (a) 500 PL/m³ (b) 750 PL/m³ and (c) 1,000 PL/m³.

can be affected by the competition between species within the habitat. The similarity of niches such as the physical or chemical conditions of the environment can trigger a species to adapt in a new environment [29,30]. The diversity index is considered low when the value less than one, medium if the value is between one to three, and high if higher than three [31]. The value of the diversity index in all three treatments with different stocking density is shown in Table 6. The overall results suggested that the diversity index value of culturable bacteria in culture water and shrimp are both in the medium category.

Sorensen similarity index

Similarity analysis is often used to monitor the stability of culturable bacteria community structure. In this study, the values of the Sorensen similarity index were >0.5, suggesting similar community structures in both shrimp and culture water between all treatment groups [32]. Construction of dendrogram of culturable bacteria in culture water in the 500 PL/m³ treatment group have two large groups (*clade*), while 750 PL/m³ treatment group have three large group with tenth week as an outgroup. Similarly, the 10th week occurred as the outgroup in the 1,000 PL/m³ treatment group. Dendrogram of similarity culturable bacteria in water is shown in Figure 4. On the other hand, construction of dendrogram in shrimp at the 500 PL/m³ treatment group has two large groups, while 750 PL/m³ treatment group has three large groups. Similarly, the 1,000 PL/m³ treatment group has three groups with the second week as an outgroup. Dendrogram of similarity culturable bacteria in shrimp is shown in Figure 5. The overall results of this study suggested that the culturable bacterial community profile in both shrimp and culture water during 84 days of grow out period using RAS at low salinity were similar between different shrimp stocking density.

Conclusion

In this study, similar total productivity levels were observed in all treatment groups with different stocking density. All physicochemical water quality parameters in all treatment groups were in tolerance level for shrimp culture. Furthermore, stable community structure of culturable bacteria was also observed during the entire shrimp culture

period even at high shrimp density of up to 1,000 PL/m³. Based on the overall results it is suggested that RAS can be applied for shrimp super-intensive culture at the optimal density of 500 PL/m³ that allowed a high shrimp culture productivity of up to 5.20 kg/m³ within 84 days grow out period.

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