Application of eDNA method to analyze bacterial community structures in the recirculation aquaculture systems of *Litopenaeus vannamei*

Zulkisam Pramudia\(^1,2,3\), A A Amin\(^2,3\), A T Yanuar\(^2,3\), Y A D Susanti\(^1,2,3\), U Yanuhar\(^4\), S M Ulfa\(^3\), A S Huda\(^5\) and A Kurniawan\(^2,3,4\,6\)

\(^1\)Postgraduate Program of Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Jawa Timur
\(^2\)Coastal and Marine Research Centre, University of Brawijaya, Malang, Jawa Timur
\(^3\)Microbial Resources and Biotechnology Research Group, Interdisciplinary Graduate School, University of Brawijaya
\(^4\)Faculty of Fisheries and Marine Science, University of Brawijaya, Malang
\(^5\)ASH consulting, Probolinggo
\(^6\)Corresponding author: andi_k@ub.ac.id

**Abstract.** One of the main focuses in microbial ecology is the analysis of microbial communities in water, especially concerning bacteria. The results of the analysis related to the abundance of bacteria will be essential knowledge for the development of aquaculture, especially those relating to the dynamics of pond water quality. This study aims to analyze the total abundance of bacteria and water quality dynamics in the water of *Litopenaeus vannamei* aquaculture that applies a recirculation system. The study was conducted for three months on a concrete pond plot with an area of 2500 m\(^2\). Samples of bacterial abundance were taken from pond water, while water quality checks were carried out at ponds and pond outlets. Sampling was carried out at weeks 1, 3, 5, and 8 during the cultivation phase. Bacterial abundance was calculated using Environmental DNA (eDNA), which refers to DNA extracted from the environment without isolating the target organism. The water quality parameters observed were pH, conductivity, DO, turbidity, temperature, and salinity. This study suggested that the abundance of bacteria from the first to the 8th week were 15.3x10\(^7\); 17.1x10\(^7\); 36.6x10\(^7\); and 35.4x10\(^7\), respectively. Moreover, DO ranged from 3.2 ppm to 7.6 ppm; temperature ranged from 30 °C to 33.3°C; turbidity ranged from 12 NTU to 57 NTU; salinity ranged from 19 ppt to 24 ppt; conductivity ranged from 2.93 s/m to 3.54 s/m, and pH ranged from 6.6 to 7.0. This study indicates that the recirculation aquaculture system in Lamongan, East Java may obtain the optimum water quality and controlling the abundance of bacteria to support the aquaculture of *Litopenaeus vannamei*.

1. **Introduction**

Studies related to microbial ecology are closely related to the analysis of microbial communities in water, whose study results help develop aquaculture systems such as the recirculation aquaculture system (RAS) [1]. One of the main focuses in this study related to microbial ecology is analyzing the abundance of bacteria in water in the cultivation of fishery commodities such as vannamei (*Litopenaeus vannamei*). Vannamei shrimp itself is a prima donna export commodity from Indonesian fishery products [2]. Therefore, to understand and modify aquatic systems such as vannamei ponds, bacteria in water in the RAS system of vannamei is one of the critical parameters [3].
Various methods to analyze the abundance of bacteria in ecosystems have been developed. Methods often used include culture-based methods such as agar plate methods or cell identification-based methods such as DAPI staining and molecular analysis [4]. To be able to get good analytical results, a practical but also sensitive analytical method is needed. One of the methods developed to analyze microbial communities is the eDNA method. Bacterial abundance may be analyzed using Environmental DNA (eDNA), which refers to DNA extracted from the environment without isolating the target organism [5].

Water quality parameters are one of the crucial things in the success of RAS [6]. The water quality condition in aquaculture cannot be separated from the abundance of bacteria in the water [7, 8]. Therefore, studies of bacterial abundance using methods such as eDNA need to be combined with dynamic analysis of pond water quality to evaluate whether RAS can work well in aquaculture such as vannamei aquaculture. This study aims to develop an eDNA method in analyzing the abundance of bacteria in the RAS and evaluate the dynamics of water quality in the RAS for cultivating vannamei. This study indicates that the eDNA method can be modified to analyze the abundance of bacteria in pond water. Moreover, the results of this study show that the quality of pond water can be maintained to support vannamei cultivation in Indonesia.

2. Materials and methods
The following is an explanation related to the materials and methods in this study.

2.1. Study area and sampling
This study was conducted on vannamei shrimp ponds using the Recirculation Aquaculture System in Lamongan Regency, East Java. In this pond, vannamei shrimp (Litopenaeus vannamei) was cultivated with an initial stocking density of 250 ind/m². The cultivation period (Day of Operation), which is the focus of the research, is until the 60th day (8th week).

2.2. eDNA analysis
In this study, the abundance of bacteria in water was carried out by modifying the eDNA method. Bacterial abundance analysis was carried out for samples taken at weeks 1, 3, 5, and 8. The total bacterial number in the soil was estimated by using environmental DNA (eDNA) with the low stirring method [9]. One g of soil sample was mixed with 8.0 mL of eDNA buffer and 1 mL of 20% sodium dodecyl sulfate (SDS) solution. Thus, the suspension stirred at 1,500 rpm for 20 min, followed by centrifugation of the suspension at 6,000 × g for 10 min. The supernatant was mixed with a mix of chloroform-isoamyl alcohol (24:1 (v/v), and centrifuged at 18,000 × g for 10 min. The crude nucleic acid performed by 500 µL of aqueous phase and 300 µL of isopropanol mixture, then centrifugation at 18,000 × g for 20 min. The remained was dissolved in 1× TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Quantification of the eDNA was performed based on the intensity of the band after electrophoresis of 1% agarose gel using KODAK 1 D 3.6 Image Analysis Software (Eastman Kodak Company, CT, USA).

2.3. Water parameter analysis
Measurement of water quality parameters is carried out once a week. The parameters measured are pH, conductivity, DO, turbidity, temperature, and salinity. These water quality parameters were measured using a water quality checker model WQC-22A from the DKK-TOA brand. Measurements were carried out three times, and the results were the average value of these repetitions.

3. Results and discussion
3.1. Bacteria abundance
Bacterial abundance in water in vannamei culture with RAS was analyzed in this study (Figure 1). In principle, the analysis of the eDNA method to determine the abundance of this bacterium is an analysis by utilizing the estimated number of bacteria by utilizing the amount of eDNA isolated from environmental samples (i.e., vannamei pond water). Although this method has been reported to be successful in estimating the abundance of bacteria in the soil [10], it has not been widely used to analyze
bacteria in water, especially pond water in aquaculture. However, from Figure 1, it can be seen that the extraction method performed (by modifying the eDNA method for bacteria in the soil) can collect a sufficient amount of eDNA to carry out an analysis of bacterial abundance in water.

Figure 1. Profile of gel electrophoresis analysis results from bacterial eDNA in water (A is the marker, B is week 1, C is week 3, D is week 5, and E is week 8)

Based on the profile of eDNA extracted from water samples, the value of the abundance of bacteria in water in vannamei cultivation with RAS was calculated. The results are shown in Table 1. The abundance analysis results using eDNA showed that the value of bacterial abundance in water in vannamei cultivation with RAS tended to increase and then stabilize. The bacterial abundance values for weeks 1, 3, 5, and 8 in this study were $15.3 \times 10^7$, $17.1 \times 10^7$, $36.6 \times 10^7$, and $35.4 \times 10^7$, respectively. The abundance of these bacteria was generally similar to that reported with other studies analyzing the abundance of bacteria in water in vannamei culture [11].

The increase in the number of bacteria in the 1st to 5th week can be caused by the increase in the availability of nutrients due to the decomposition of organic matter in the pond [12]. The increase in organic matter is a consequence of the increase in the dose of feed and the excretion results along with the age of the vannamei [13]. Organic matter in water is a source of nutrients supporting bacterial growth, generally carried out through a biodegradation process [14]. The number of bacteria that decreased slightly or tended to be stable at week 8 could indicate that the abundance of bacteria had reached a saturation point in the aquatic system. This balance of bacterial abundance is needed as a form of stability in the aquatic system in ponds [15, 16]. The stability of this aquatic system is one of the essential parameters to support the success of the aquaculture business carried out. The results of the analysis of the abundance of bacteria show that the life of bacteria in pond water is closely related to the dynamics of pond water quality.

Water quality dynamics in vannamei culture with RAS were analyzed weekly in this study (Figure 2). Water quality parameters are essential prerequisites for achieving success in aquaculture, including vannamei cultivation [17]. The water quality parameters measured in this study are water quality parameters that can be easily measured in the field and are usually used as a standard for monitoring the health of shrimp ponds. The measurement results in this study show DO values ranged from 3.2 ppm to 7.6 ppm; temperature ranged from 30 °C to 33.3°C; turbidity raged from 12 NTU to 57 NTU; salinity ranged from 19 ppt to 24 ppt; conductivity ranged from 2.93 s/m to 3.54 s/m, and pH ranged from 6.6 to 7.0.
Table 1. Value of bacterial abundance in pond water of RAS

| Week | bacterial abundance ($10^7$) |
|------|-----------------------------|
| 1    | 15.3                        |
| 3    | 17.1                        |
| 5    | 36.6                        |
| 8    | 35.4                        |

Figure 2. Water quality parameters during vannamei aquaculture with RAS (2a is DO, 2b is pH, 2c is temperature, 2d is conductivity, 2e is salinity, and 2f is turbidity)
To get good yields, the water quality in ponds must meet specific standards for vannamei cultivation [18]. These standards are the conditions needed for the vannamei to grow optimally. One way to optimize these water quality parameters is to implement an aquaculture system to maintain water quality. In the pond that is used as the object of this research, the system applied is RAS. The water quality measurements indicate that the water quality parameters meet the water quality standards for vannamei cultivation [19, 20]. This result indicates that the RAS used in the vannamei ponds in Lamongan, East Java studied in this study succeeded in maintaining the water quality in the ponds.

Water quality in vannamei ponds will be largely determined from a load of organic waste in the ponds. Without decomposition by microbes, especially bacteria, water quality will decrease drastically along with the increase in organic load during vannamei cultivation [21]. Bacterial activity will help degrade organic matter so that existing organic matter can be decomposed. The success of this decomposition will reduce the need for water replacement during the vannamei cultivation process. This bacterial activity may be seen from the increase in the number of bacteria, which can be known through the analysis of bacterial abundance. The results of this study indicate that the abundance of bacteria continues to increase, and water quality can be maintained to meet vannamei cultivation standards. Furthermore, this study indicates that the analysis of bacterial abundance using the eDNA method can be used as a basis for understanding the abundance of bacteria in water from vannamei culture.

4. Conclusion
Bacterial abundance and water quality dynamics during vannamei cultivation with the Recirculation Aquaculture System were analyzed in this study. The results of water quality measurements show that the application of RAS can make the measured water quality parameters meet the vannamei cultivation standards. This study also showed that bacteria in water in vannamei cultivation ranged from $15 \times 10^7$ to $36 \times 10^7$. Furthermore, modification of the eDNA method was successfully carried out in this study to be used to analyze bacterial communities in water, especially in aquaculture. Thus, this study is the first study to report the application of eDNA to analyze the abundance of bacteria in water in vannamei cultivation in Indonesia.

5. Reference
[1] Ekawati A W, Ulfa S M, Dew C S U, Amin A A, Salamah L N, Yanuar A T, Kurniawan A 2021 Aquaculture Studies, 21, 93-100.
[2] Wahidin D, and Purwhagen K 2018 Heliyon, 4(7), e00683.
[3] Pramudia Z, A Kurniawan, A A Amin 2019 Semnaskan-UGM Prosiding. XVI; 18 – 22.
[4] Hamada S, Fujita S 1987 Histochemistry. 1983;79(2):219-26.
[5] Adhikari D, Kai T, Mukai M, Araki KS, Kubo M 2014 Current Topics in Biotechnology, 8, 81-91.
[6] Susanti Y A D, Pramudia Z, Amin A A, Salamah L N, Yanuar A T, Kurniawan A 2021 Rekayasa 14 (1). 121-127.
[7] Kurniawan A, Yamamoto T 2019 Int Journal of Microbiology, 2019, 1–7.
[8] Perwira I Y, Ulinuha D, Al Zamzami I M, Ahmad F H, Kifly M T H, Wulandari N 2020 IOP Conference Series: Earth and Environmental Science, 493, 012025.
[9] Aoshima H, Kimura A, Shibutani A, Okada C, Matsumiya Y, Kubo M 2006 Appl Microbiol Biotechnol. 71(6):875-80.
[10] Espina L 2020 PLoS ONE 15(8): e0237748.
[11] Qin Y, Hou J, Deng M, Liu Q, Wu C, Ji Y, He X 2016 Scientific Reports, 6(1).
[12] Darwin C H, Suneetha K, Kavitha K, Govinda R V 2017 Advanced Science and Research 2:123-129.
[13] Lee C, Kim S, Lim S J, Lee K J 2017 Fisheries and Aquatic Sciences, 20 (1).
[14] Emerenciano M, Gaxiola G, Cuzo, G 2013 Biomass Now - Cultivation and Utilization. doi:10.5772/53902
[15] Alfiansah YR, Hassenrück C, Kunzmann A, Taslihan A, Harder J and Gärdes A 2018 Front. Microbiol. 9:2457.
[16] Chen W Y, Ng T H, Wu J H, Chen J W, Wang H C 2017 Scientific Reports, 7(1).
[17] Nguyen, Tram A T, Kim A T, Nguyen, and Curtis Jolly. 2019 Sustainability 11(19): 5277.
[18] Eddiwan E, Sukendi S, Siregar Y I, Saam Z. 2020 IOP Conference Series: Earth and Environmental Science, 430, 012039.
[19] Ni M, Yuan J, Liu M, Gu Z 2018 Aquaculture Reports, 11, 53–58.
[20] Musa M, Lusiana E, Buwono N, Arsad S, Mahmudi M 2020 Biodiversitas Journal of Biological Diversity. 21 (10): 4695-4701.
[21] Gilbert J A, Neufeld J D 2014 PLoS Biol 12(12): e1002020.

6. Acknowledgments
The authors would like to thank Prof. Motoki Kubo from the Bioengineering Laboratory, Ritsumeikan University, Japan, for the facilities provided for conducting eDNA analysis. This research was supported by a research grant from the Ministry of Education, Culture, Research and Technology, Republic of Indonesia.