Diversity and fluctuation of cultivable *Vibrio* bacteria populations in an integrated multi-trophic aquaculture (IMTA) system of *Holothuria scabra*, *Chanos chanos* and *Gracillaria* sp.

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**Abstract.** Sea cucumber is one of the vital aquaculture commodities in the global market. Progressive demand and limiting the supply of sea cucumber from natural sources drove sea cucumber aquaculture development. An integrated multi-trophic aquaculture system (IMTA) was a polyculture system in aquaculture to improve production by promoting nutrient utilisation through different tropical levels. This system combined sea cucumber (*Holothuria scabra*), milkfish (*Chanos chanos*), and seaweed (*Gracillaria* sp.) in a rearing tank system. Several factors affect the success of IMTA processes, including microorganisms. Microorganisms in the aquaculture environment play a crucial role in elements cycling, energy flow and farmed-species health. Diseases caused by the bacteria, mainly from the *Vibrionaceae* family, were often chronic than acute and may cause a high mortality percentage. This study aimed to investigate the *Vibrio* bacterial diversity and fluctuation in the water of IMTA rearing tanks. The result indicated that the *Vibrio* bacterial diversity from the polyculture system (IMTA) was generally higher than the monoculture system. *Vibrio* bacteria populations from different culture systems have been found to consist of varied bacterial species. However, the predominant species was the same. The polyculture system is suggested as a sustainable and eco-friendly culture system.

**Keywords:** aquaculture system; bacteria; diversity; IMTA; microorganism; *Vibrio* spp.

1. Introduction

Sea cucumbers are high fishery product species. Some ecological importance of sea cucumber can improve sediment quality, modify water chemistry, increase biodiversity, play significant roles in nutrient cycling, transfer an organic matter from detritus to higher trophic levels. Most of them are omnivorous deposit-feeder [1]. The commercial benefits of sea cucumber are important food and flavouring food source, functional foods and nutraceuticals. The use of sea cucumber as a food product or a dietary supplement (functional foods and nutraceuticals) has led to more expansion into worldwide nutraceutical markets.

Consequently, the broad expansion and more benefits of these products have driven overfishing practices globally. Furthermore, these overexploited sea cucumber populations have increased market value as well as stimulated efforts to develop aquaculture production systems and technology [1,2]. The ability and trophic position of sea cucumbers in the marine ecosystem are considered vital processors of surface sediment and would be proper candidates for coculture in the IMTA system [3]. Integrated multi-trophic aquaculture (IMTA) system is a developed aquaculture systems that recycles bioproducts (waste) from one species into inputs (energy, fertilisers and food) for another by combining organic extractive species (suspension and deposit feeders) with inorganic extractive species (seaweed) in the production of fed aquaculture species (finfish/shrimp) [3,4]. Bacteria populations in aquaculture
environments are affected by many factors, such as water quality, animal density, inorganic nutrients, fish faeces, and feedstuff remnants. Those factors are several problems in aquaculture practices that should be well managed to reduce mass mortality caused by infectious diseases and increase production quality. The accumulation of nitrogen related metabolites and the decomposition of unutilised feed is the primary waste of cultured organisms such as ammonia. The efficiency of nitrogen metabolism using bacteria and other trophic organisms in aquaculture systems plays a vital role in sustainable water quality management for the long term [3, 5].

Microorganisms serve a critical role in aquaculture environment, because responsible several processes including recycling nutrients, degrading organic waste, and occasionally infecting and dispatching farmed aquatic animals. However, some microorganisms have a mutual symbiosis with farmed aquatic animals and protect against diseases [6]. Controlling and monitoring the total number of opportunistic bacteria such as Vibrio spp. as well as the development of microbial infection in aquaculture environments hold great potential to manage health status and production quality. Vibrios are Gram-negative curved rod bacteria that can be found in a wide range of aquatic and marine environments. Some species are vital for natural ecosystem because several natural processes such as the carbon cycle, nitrogen cycle, and osmoregulation relies on vibrio species. [7, 8]. On the other hand, they can cause mortality and infectious diseases in cultured aquatic animals under low water quality. Several studies have revealed that Vibrio is associated with infection in molluscs [9, 10], fish [11, 12], shrimp [13] and sea cucumber [14, 15], such as V. alginolyticus, V. harveyi, V. vulnificus, V. parahaemolyticus, V. metschnikovii, V. cholerae, V. mimicus, and V. fluvialis.

In this study, we cultivate, identify, and investigate Vibrio spp. diversity in the IMTA system and semi-intensive aquaculture system for six months in the dry and rainy seasons, as well as how its correlations. We also observe how the different aquaculture systems influence the Vibrio diversity and the total Vibrio populations in both aquaculture systems. We hypothesised firstly, that the Vibrio diversity index in the IMTA system would show significantly higher than the Vibrio diversity index in}

Figure 1. Illustration design of integrated multi-trophic aquaculture system (IMTA) consisted of sea cucumber (Holothuria scabra), milkfish (Chanos chanos) and seaweed (Gracillaria sp.) (IMTA illustration design created with BioRender.com).
the Semi-intensive aquaculture system. Secondly, there would be a positive correlation between the total number of *Vibrio* colonies, sampling months and season.

2. Material and methods

2.1. Cultured species conditions

The IMTA system was located at the Research and Development Division for Marine Bioindustry – National Research and Innovation Agency (BRIN), North Lombok, West Nusa Tenggara, Indonesia. The concrete tanks used in this study have been operated for sea cucumber aquaculture using a semi-intensive system and an IMTA system since 2011 and periodically monitored to ensure stable and healthy organisms conditions. Those concrete tanks were used to rearing sea cucumber in two different aquaculture systems, which were a semi-intensive aquaculture system and an integrated multi-trophic aquaculture system (IMTA) consisted of sea cucumber (*Holothuria scabra*), milkfish (*Chanos chanos*), and seaweed (*Gracillaria* sp.) (figure 1).

The concrete tank size for a semi-intensive aquaculture system was 10 m$^2$ in area and 1 m in depth. Meanwhile, the concrete tank size for the IMTA system was 2.77 m$^2$ in area and 1.8 m in depth. The new seawater replacement for the semi-intensive aquaculture system was 50% volume per three days, and 20% of volume per week was applied for the IMTA system. All of the tanks were continuously aerated. A formulated feed consisted of *Enhalus acoroides* seagrass (40%), cow manure (30%) and *Pennisetum purpureum* napier grass (30%) was given *ad libitum* during the rearing period. Milkfish were fed once a day, 1% of body weight. Several water quality parameters (pH, temperature, salinity, and dissolved oxygen) were measured regularly.

2.2. Bacterial isolation and characterisation

*Vibrio* bacteria were isolated from the rearing water in the semi-intensive aquaculture system and the IMTA system. Those water samples were collected aseptically with a sterile 50 mL syringe twice a month for six months (August 2017 – January 2018). The dry season was from August to October 2017, and the rainy season was from November 2017 to January 2018 was the rainy season. Then, the samples were brought directly to the Microbiology Laboratory and adequately prepared for further detailed analysis. Enumeration of bacteria was conducted by using serial dilutions and the spread plating method. Serial dilutions of the samples were performed using 9 ml of natural sterile seawater. *Vibrio* was isolated by inoculating 100 µL of the original sample and adding 9 ml of natural sterile seawater to obtain 10$^{-1}$, 10$^{-2}$, 10$^{-3}$ and 10$^{-4}$ diluted water samples. Then, it was spread over the three plates containing selective Thiosulphate Bile Salt Sucrose agar (TCBS) medium (Hi-Media Laboratories, Mumbai, India) in sterile seawater, followed by incubation at 35 °C for 20 – 24 h. The colony counts were performed after 24 - 48 h, and the population density was expressed as Colony Forming Units (CFU) per ml. For each sample, a representative number of colonies was selected randomly to isolated. Then, those selected colonies were biochemically characterised based on the classical morphological and biochemical tests such as 0%, 3%, 6%, 8%, and 10% NaCl growth, motility, indole production, MRVP test, TSI (Triple Sugar Iron) test, lysine and ornithine decarboxylase, and fermentation of sugars (sucrose, glucose and

| Aquaculture system | Temperature (°C) | Salinity (‰) | pH | Dissolved oxygen (DO) (mg/L) | Total *Vibrio* (CFU/mL) |
|--------------------|-----------------|---------------|----|---------------------------------|------------------------|
| Semi-intensive     | 26±1.07$^a$    | 34±1.6$^a$    | 8.0±0.3$^a$ | 7.3±0.4$^a$                       | 6.33x10$^{3a}$        |
| IMTA               | 28±0.8$^b$     | 34±0.9$^a$    | 8.3±0.6$^a$ | 7.7±0.7$^a$                       | 1.3x10$^{4a}$         |

Data are presented as means ± SD. Different letters indicate significant differences between the semi-intensive aquaculture and IMTA systems ($P < 0.05$), t-test analysis.
fructose) as described in Bergey’s Manual of Systematic Bacteriology. We measured *Vibrio* bacterial diversities based on the Shannon diversity index, using the $H' = - \sum p_i \ln(p_i)$ formula (Shannon, 1984). Shannon’s index of evenness was calculated according to $E = H'/\ln S$, where $\ln S$ represents $H'_\text{max}$. The Shapiro-Wilk test was used in the first step to estimate the normality and homogeneity of data. Statistical differences between the bacteria diversity index in the semi-intensive aquaculture and IMTA systems were performed by t-test analysis. Pearson’s correlation was used to measure the total number of bacteria colonies in two different aquaculture systems and sampling months. $P$-value < 0.05 and $P$ value < 0.01 being accepted as a significant level using IBM SPSS version 27 software for windows.

### 3. Results and Discussion
#### 3.1 Environmental parameters
Water quality data showed no remarkable result among salinity, pH, and dissolved oxygen parameters. Except for the temperature and the total number of *Vibrio* colonies, they were $26 \pm 1.07 \, ^\circ C$ and $6.33 \times 10^3 \, \text{CFU/mL}$, respectively. Meanwhile, temperature and total *Vibrio* in the IMTA system were $28 \pm 0.8 \, ^\circ C$ and $1.3 \times 10^4 \, \text{CFU/mL}$, respectively (table 1). These data demonstrated that different temperatures in the IMTA system and the Semi-intensive aquaculture system might cause a greater number of *Vibrio* populations. Several previous studies have revealed that environmental factors, particularly temperature and salinity, have been linked to total *Vibrio* and bacteria proliferation rate [16-18]. However, the total number of Vibrio populations in tropical regions such as Indonesia were not significantly affected by temperature and salinity due to a narrow range of temperature and salinity observed during sampling months [17].

Moreover, the higher total number of *Vibrio* population could be affected by a higher concentration of dissolved organic matter as a degradation product by bacteria or deposit feeders from feedstuff remnants and fish faeces in the tank of the IMTA system [4,17]. The different temperatures between the IMTA and semi-intensive aquaculture systems may be caused by the higher frequency and volume of new seawater replacement in the semi-intensive aquaculture system than in the IMTA system. This way could maintain the seawater temperature in the tank of the semi-intensive aquaculture system remain stable and similar to natural seawater temperature. However, according to [19], environmental variables...
such as temperature and increasing nutrient concentrations have a direct impact on semi-intensive aquacultures. Meanwhile, the higher seawater temperature in the tank of the IMTA system might be led by the warming of intense sunlight during the day as well as lower frequency and the volume of new seawater replacement.

3.2 Shannon index diversity of vibrio bacteria
There was a significant difference in the Vibrio diversity index between the IMTA and the semi intensive aquaculture systems ($P < 0.01$). The Shannon diversity index ($H'$) value of Vibrio bacteria populations in the semi-intensive aquaculture system was 1.71, followed by the Shannon evenness index (E) value was 0.72. Meanwhile, the diversity of Vibrio bacteria in the IMTA system showed that the Shannon diversity index value of Vibrio bacteria populations was 2.21 and 0.75 was for the Shannon evenness index (E) value, respectively (Figure 2). The Shannon diversity index of Vibrio species in the IMTA system was significantly higher than the diversity index of Vibrio species in the semi-intensive aquaculture system. However, the Shannon evenness index value expressed that both aquaculture systems had high bacterial diversity because the range of the Shannon evenness index is 0-1. This result might be caused by higher temperature, Vibrio species associated with different trophic organisms, and excessive organic matter, mostly from fish bioproducts and uneaten food, in the IMTA system. Based on several previous studies, temperature and salinity greatly induce bacteria proliferation in the environment [20][21]. In addition, this results in accordance with a previous study that higher organic matter in integrated aquaculture or polyculture system such as the IMTA system played a major role in the bacterial proliferation rate dan diversity [4].

3.3 Identification of isolated vibrio species
The main microorganisms in water column included Aeromonas and Vibrio, which were detected in all seasons (summer and rainy). Variation of Vibrio bacteria from the rearing water in two different aquaculture systems showed that Vibrio species' total number and diversity in the semi intensive aquaculture system was less varied. In contrast, diversity and the total number of Vibrio species in the IMTA system varied greatly compared to the semi-intensive aquaculture system (figure 3). Variation of Vibrio bacteria in both aquaculture systems showed a varied trend. Organic enrichment, increasing water nutrients and specific bacteria associated with different trophic organisms might lead to a greater diversity of bacterial species in the IMTA system. There were two predominant Vibrio species in the IMTA and semi-intensive aquaculture systems, namely Vibrio cholerae and Vibrio alginolyticus. Another predominant species was Vibrio metschnikovii. According to [22], V. alginolyticus and V. cholerae live in the marine environment and are associated with fish, shrimp, algae and other aquatic organisms. Vibrio cholerae is a gram-negative, oxidase-positive, motile, catalase-positive and indole producing bacteria. This species has been known as an important human pathogen that can cause cholera, a severe diarrhoeal disease outbreak, after consuming contaminated shellfish or other aquatic organisms. They grow in widely salinity range such as seawater, brackish water, and freshwater [22, 23]. Vibrio alginolyticus has been identified as a bacteria pathogen related to Vibriosis diseases in marine organisms such as abalone [24, 25], sea cucumber [15], and seahorse [26].

Furthermore, Vibrio metschnikovii has been described as a bacteria pathogen in humans related to chronic human diseases such as severe septic shock and cardiac arrest. Even though human infections with this bacteria are rare, they can be fatal as well [27]. Most Vibrio bacteria well-known are harmless; however, some species are harmful to humans and other living organisms, particularly V. alginolyticus and V. cholerae [20]. However, as inorganic extractive species in aquaculture systems, Vibrio species and seaweeds such as Gracillaria sp. can utilise either suspended or dissolved organic matter also minimizing sedimentation and organic matter accumulation depend on several environmental factors (light, dissolved oxygen and nutrient availability) [4]. In the IMTA system large numbers of bacteria in the water were filtered out by seaweed, used it for their growth and resulting of reduced number of Vibrio colonies within the normal concentration range to about $10^3 – 10^4$ CFU/mL. Seaweed used in this colonies within the normal concentration range to about $10^3 – 10^4$ CFU/mL. Seaweed used in this environmental control services [28]. Meanwhile, using sea cucumbers as an organic extractive species
in the IMTA system has been shown to offer ecological advantages, since deposit-feeding can minimize total system waste (feces and uneaten food) and nutrient loading through direct consumption and sediment bioturbation [1].

Figure 3. Variety of bacteria species and their colonies total number in the semi-intensive aquaculture system (A) and the IMTA system (B). Asterisk signs (*) showed that they were the predominant species.
3.4 Correlation of the total number of vibrio colonies and sampling months

The variation trend of the total number of *Vibrio* colonies in the IMTA system was inconsistent, and no significant correlation with variation seasons in different sampling months. However, the significant Pearson's correlation \( (P < 0.01) \), 0.893 of Pearson's correlation index value was detected between the number of bacteria colonies and sampling months in the semi-intensive aquaculture system (Figure 4). In August, the total number of *Vibrio* in the IMTA system was higher than the semi intensive system. However, a sharp decrease in the total number of *Vibrio* in September and interestingly, this pattern was found similar for the following month, in dry and rainy seasons for the IMTA system. This result showed that the ability of seaweed and sea cucumber to reduce the *Vibrio* population in the IMTA system was effective. According to previous study, sea cucumbers (*H. scabra* and *A. japonicus*) may absorb shrimp feces and uneaten food, lowering the organic load such as particulate organic carbon and nitrogen. However, the effectiveness of sea cucumbers to reduce aquaculture waste will be determined by the physiological performance of species [1].

In contrast, a graph pattern of the total number of *Vibrio* in the semi-intensive aquaculture system showed a significantly positive correlation during sampling months. This result may be led by higher frequency and greater volume of new seawater replacement in the semi-intensive aquaculture system than the IMTA system. In tropical regions such as Indonesia, the rainy season cause high contamination of dissolved organic matter and nutrient from the river, groundwater inputs and aquaculture effluents into the coastal waters. Consequently, the total number of heterotrophic bacteria, including *Vibrio*, increase extremely compared to the dry season. This result agreed with previous research that reported that a high concentration of nutrients close to the shore reflected input considerably from land [29]. A heavy rainy season in December and January that a heavy rainy season has led to an increasing trend in the total number of *Vibrio* population in the semi intensive aquaculture system. Our limitations are total heterotrophic bacteria populations, limited information and data for nutrient and dissolved organic matter concentration, sea cucumber, milkfish, and seaweed biomass. Future studies need to investigate nutrient and dissolved organic matter concentration, cultured organism biomass or production, and their correlations.

![Figure 4](image)

**Figure 4.** Correlation and fluctuation between the total number of *Vibrio* colonies and sampling months in the IMTA and The semi-intensive aquaculture systems. Two asterisk signs (*) showed a significant Pearson's correlation between the total number of bacteria colonies and sampling month \( (P < 0.01) \).
4. Conclusions
We have demonstrated a positive correlation between the total of Vibrio colonies and seasonal variation in the semi-intensive systems. In contrast, there was no significant correlation between total Vibrio colonies and seasonal variation in the IMTA system. The Vibrio diversity in the IMTA and the semi-intensive aquaculture systems differ significantly; however, predominant species in those both aquaculture systems are similar. Notable fluctuation of total Vibrio colonies and remarkable Vibrio diversity index in the IMTA system during sampling months has proven that temperature and organic matter, mostly coming from excessive fish faeces and feedstuff remnants, were likely to influence the higher value of Vibrio diversity index and fluctuation of total bacteria colonies. However, those factors do not influence the total Vibrio colonies in the IMTA system. This present finding gives information that the IMTA system, combined with sea cucumber, milkfish, and seaweed, could be a promising solution for recycling dissolved organic matter and controlling the bacteria populations. Furthermore, the IMTA system is suggested to be one of the sustainable aquaculture systems and making a significant contribution to the culture’s health. The significantly different temperatures in both aquaculture systems could not lead to a remarkable difference in the total of Vibrio populations of two different aquaculture systems. Significant differences in Vibrio diversity index in both aquaculture systems may be led by higher organic matter and Vibrio bacteria associated with different trophic organisms in the IMTA system.

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