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Prevalence of *Vibrio parahaemolyticus, Vibrio cholerae,* and *Vibrio alginolyticus* in a White-leg Shrimp (*Litopenaeus vannamei*) Farm in Sarawak

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ABSTRACT

Vibriosis in shrimp farms poses potential risks to the industry's sustainability and the consumers' health. Little is known about the dynamics and variation of *Vibrio* spp—population in shrimp production in Sarawak, Malaysia. The apparent prevalence of three *Vibrio* species, *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus*, were investigated in water (n=12), sediment (n=12), shrimp (n=12), and effluent (n=12) samples collected from two ponds in a coastal shrimp farm during one production cycle. Multiplex-PCR using specific primer sets showed the presence of the three *Vibrio* species. Based on the results, *V. parahaemolyticus* was the most prevalent in all four samples, with a contamination rate of 97.92% (95% CI: 89. 10 to 99.89%), while *V. cholerae* and *V. alginolyticus* had a contamination rate of 47.92% (95% CI: 34.37 to 61.67%) and 25.0% (95% CI: 14.92 to 38.78%), respectively. High *Vibrio* load in the shrimp farm is due to favourable environmental factors, such as optimal temperatures, salinity, and pH ranges for the growth of these species. The study's findings offer important preliminary insights into the prevalence and distribution of these pathogenic *Vibrio* spp., within a shrimp farm in Kuching, Sarawak. This study serves as a potential model for monitoring *Vibrio* spp. prevalence in other shrimp farms across Sarawak, thereby addressing the scarcity of data on prevalence in the region.

Keywords: Microbial load, most probable number, multiplex PCR, shrimp farm, *Vibrio* spp.

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INTRODUCTION

The shrimp industry is a significant contributor to the aquaculture industry in Southeast Asia, with an estimated total area of marine shrimp farms in Malaysia alone at between 5,100 and 5,200 hectares as of 2012 and a total market value of RM1.13 billion in 2020 (Department of Fisheries Malaysia, 2012; Gilbert, 2023). However, the industry has faced challenges due to disease outbreaks, including vibriosis caused by *Vibrio* spp. These heterotrophic bacteria are prevalent in freshwater and marine environments and can inhabit a wide range of environments as free-living cells or symbionts. Approximately 110 species of the genus *Vibrio* have been identified to date, with many of these species being pathogenic to animals and humans (Farmer & Janda, 2015). *Vibrio* spp. Human infections often result from contact with polluted water or ingesting raw or undercooked seafood contaminated with the bacteria (Dutta et al., 2021). *Vibrio parahaemolyticus*, in particular, has been identified as a common cause of food poisoning cases in Malaysia (Sani et al., 2013). *Vibrio* spp. infection can pose serious health risks to immunocompromised individuals, including senior citizens and children, and can result in septicaemia and death (Song et al., 2020).

Some *Vibrio* spp, such as *V. cholerae*, are pathogenic and infectious, while others, such as V. *parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*, are known as opportunistic pathogens (Haifa-Haryani et al., 2022; Valente & Wan, 2021). Shrimp infected with *Vibrio* spp. may exhibit clinical signs, including lethargy, a lack of moulting, abnormal swimming behaviour, reduced food intake, and potentially anorexia as indicated by empty guts and an off-white body colour (Valente & Wan, 2021). *V. parahaemolyticus* that causes acute hepatopancreatic necrosis disease (AHPND), for instance, causes the infected shrimp to exhibit an empty stomach and midgut, along with a hepatopancreas (HP) that appears pale to white (To et al., 2020). Meanwhile, *V. alginolyticus* is the causative agent of flaming bacterial disease that causes the infected shrimp to glow in the dark (Alifia et al., 2021). On the other hand, a few strains of *V. cholerae* are the causative agent of *Vibrio*-caused bacteremia, which is characterised by reduced hemocytes and lethargy in the infected crustaceans (Valente & Wan, 2021). Severe vibriosis in shrimp can even result in mortality. While *Vibrio* spp. is prevalent in the environment, its infection can be controlled when it occurs at low levels. However, environmental factors such as temperature and salinity can trigger the rapid growth of these bacteria, leading to outbreaks of vibriosis that can decimate an entire shrimp population in an infected pond, resulting in significant production losses (Mastan & Begum, 2016; Semenza et al., 2017). Contamination of seafood products with high concentrations of *Vibrio* spp. can also threaten consumer health. Therefore, early detection and enumeration of *Vibrio* spp. is essential for monitoring outbreaks in aquaculture farms, assessing the potential danger to public health, and preventing economic losses.

Currently, there is limited data available on the prevalence of *Vibrio* spp. in aquaculture farms in Sarawak, particularly in shrimp farms. Similar to other states in Malaysia,

shrimp farmers in Sarawak face significant challenges, with disease outbreaks being a major concern. However, the lack of official data poses a challenge, as most incidents are communicated verbally and not formally reported (Baker-Austin et al., 2018). Given Sarawak's goal to increase shrimp production to RM1 billion in export value by 2030, these disease challenges could significantly impede progress (Ling, 2022). Additionally, official data on disease incidents and *Vibrio* spp is scarce. prevalence in Sarawak, attributed to the absence of surveillance systems, under-reporting, or failure to report incidents, impedes a comprehensive understanding of the extent of these challenges and hinders the development of effective strategies for disease management and prevention in Sarawak's shrimp farming industry (Baker-Austin et al., 2018).This study aims to detect and isolate *Vibrio* spp. from water, sediment, shrimp, and effluent samples collected from a shrimp farm in Kuching, Sarawak.

This study has investigated the apparent prevalence of three *Vibrio* spp., *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus*, in water, sediment, shrimp, and effluent samples collected from two ponds in a coastal shrimp farm. One production cycle of shrimp starts from the stocking of post-larvae shrimps to the harvesting of mature shrimps. To our knowledge, this report provides the first detection and quantification of these three *Vibrio* spp. in one cycle of shrimp production on a shrimp farm in Sarawak, Malaysia. This information offers novel perspectives on the challenges the shrimp aquaculture sector encounters in Sarawak. These challenges might diverge from those in other documented locations owing to the distinct geographical and climatic factors unique to the region. These factors contribute to variations in temperature, humidity, precipitation, soil properties, composition, and other environmental variables, consequently influencing the microbial composition in the region (Patel et al., 2023). The findings of this study will yield preliminary important information on the prevalence and distribution of this pathogenic *Vibrio* spp. in a shrimp farm in Kuching, Sarawak. Furthermore, it can serve as a model for prevalence studies in other shrimp farms around Sarawak. This approach will contribute to obtaining more comprehensive data on the prevalence and distribution of *Vibrio* spp. in Sarawak. Subsequently, these insights will aid in developing effective strategies for preventing and controlling vibriosis.

MATERIALS AND METHODS

Sample Collection

Samples were collected from Persatuan Nelayan Kawasan (PNK) Satang Biru, Lembaga Kemajuan Ikan Malaysia (LKIM), Telaga Air, Sarawak (0"N 110°11'51., 1°40'59, 1). Four sample types (water, sediment, shrimp, and effluent) were collected from two shrimp ponds (P1 and P2) every two weeks, from stocking to harvesting, which took approximately three months. Both earthen shrimp ponds have a surface area of one

hectare with a 1-meter depth. The sampling was done in August–October of 2020 for P1 and October–December of 2020 for P2. Every sampling was performed between nine and ten o'clock in the morning, and the entire period covered the warm season and the start of the rainy season.

The sampling method was adapted from the method by Kaysner et al. (1990) with modifications. Forty-eight (n=48) samples of twelve each of water (n=12), sediment (n=12), shrimp (n=12), and effluent (n=12) were obtained throughout the sampling process. During each sampling, each sample type was collected at three different spots (sampling points) to make up the triplicates. At least 1 L of surface water and effluent samples were collected in sterile polypropylene bottles at each sampling point. Shrimp were collected using a net and placed in sterile plastic bags. The shrimp were collected from the post-larvae stage, progressing through the juvenile and sub-adult stages until they reached the harvestable adult stage at the end of the sampling period. Meanwhile, sediment was collected using an ethanol-sterilised polyvinyl chloride (PVC) pipe and placed in sterile 50 mL microcentrifuge tubes. All samples were brought directly to the lab in an ice box and processed within 24 hours of sampling (Kaysner et al., 1990).

In addition to sample collection, the pond water's physical and chemical parameters (temperature, salinity, and pH) were also measured and recorded. These parameters were chosen because the water's temperature, salinity, and pH are commonly reported as one of the many factors influencing the distribution of *Vibrio* spp. (Valente & Wan, 2021). Water temperatures and pHs were measured on-site during every sampling using Fisherbrand™ accumet™ AP125 Portable pH/Ion/mV/Temperature Meter Kit (Thermo Fischer Scientific, U.S.). Meanwhile, water salinity was measured using a salinity refractometer (Hisamatsu, Japan). The measurements were taken triplicate at three different pond spots and expressed as the mean values.

Enrichment and MPN Method

Before enrichment, the triplicate water and effluent samples were mixed in equal volumes in sterile microcentrifuge tubes. The triplicate sediment and shrimp samples were each mixed and homogenised using a sterilised conventional blender. One gram (solid samples) or 1 mL (liquid samples) of each sample was then thoroughly mixed with 9 mL of alkaline peptone water (APW) (Merck, Darmstadt, Germany) and pre-enriched by incubating overnight at 37°C. The suspension was then serially diluted 10-fold from 10^{-1} to 10^{-5} in APW. Using the three-tube most probable number (MPN) method outlined in the Bacteriological Analytical Manual (BAM), 1 mL of each dilution was transferred into triplicate MPN tubes containing 10 mL APW broth. All tubes were incubated at 37°C under aerobic conditions for 24 hours. Prior to extracting the genomic DNA from the MPN tubes after incubation, the turbidity of the tubes was observed (Blodgett, 2000). Sterile APW broth was used as

the blank control throughout this experiment. Sterile APW was included in the experiment as the negative control.

Genomic DNA Extraction

Bacterial DNA from the turbid MPN tubes was extracted using the boiled-cell method with slight modifications (Queipo-Ortuno et al., 2008). One millilitre of each culture was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 10,000 rpm for 5 min. The supernatant was removed, and the resulting pellet was resuspended in 100 µL sterile distilled water. The suspension was then boiled for 10 min and cooled at -21°C for 5 min. The DNA extract was centrifuged at 10,000 rpm for 10 min and then stored at -21°C for analysis.

Multiplex Polymerase Chain Reaction (mPCR)

Multiplex PCR was conducted using three sets of primers that specifically targeted *V. parahaemolyticus, V. cholerae,* and *V. alginolyticus,* respectively, as designed by Kim et al. (2015). The primers used are shown in Table 1. Each 15 µL of the PCR mixture consisted of the following components: 7.5 µL exTEN 2X PCR Master Mix (1st BASE), 0.6 µL of each primer with a concentration of 10 µM, 2.0 µL DNA template, and 1.9 µL sterile distilled water.

Table 1
Sequences, sources, and expected amplicon sizes of primer pairs used to target each Vibrio *spp. in this study (Kim et al., 2015)*

Target species	Primer name	Primer sequence (5'→ 3')	Amplicon size (bp)
Vibrio parahaemolyticus	VP 1155272 F	AGCTT ATTGG CGGTT TCTGT CGG	297
	VP 1155272 R	CKCAA GACCA AGAAA AGCCG TC	
Vibrio cholerae	VC C634002 F	CAAGC TCCGC ATGTC CAGAA GC	154
	VC C634002 R	GGGGC GTGAC GCGAA TGATT	
Vibrio alginolyticus	VA 1198239 F	ACGGC ATTGG AAATT GCGAC TG	199
	VA 1198239 R	TACCC GTCTC ACGAG CCCAA G	

PCR was run by using T100™ Thermal Cycler (Bio-Rad, USA) according to the following conditions: Initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec; final extension at 72°C for 10 min (Kim et al., 2015). The PCR products were electrophoresed on 1.5% (w/v) agarose gel at 80 V for two hours. GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific, USA) was used as the molecular weight marker. A cocktail of *V. parahaemolyticus* ATCC 27969, *V. cholerae* KCDC 13589, and *V. alginolyticus* ATCC 17749 was used as the positive control. In contrast, sterile APW processed parallel with the samples was used as the negative control to replace the DNA template.

From the results of multiplex PCR, MPN values were analysed based on the MPN table for three tubes and expressed as MPN mL^{-1} for water and effluent samples and MPN g^{-1} for sediment and shrimp samples (Man, 1983).

Statistical Analysis

Data was analysed using GraphPad Prism (version 9.5.1, Dotmatics, San Diego, CA). The prevalence of *Vibrio* spp. was calculated with a 95% confidence interval (CI). Fisher's exact test was used to compare the overall prevalence of the three *Vibrio* spp. with the significant level set at $P < 0.05$. Spearman's rs correlation statistic was performed using PAST 4.13 (University of Oslo, Norway) software to test the association between the environmental and chemical parameters (i.e., temperature, pH, and salinity) with each *Vibrio* spp. load.

RESULTS

Multiplex PCR

The expected sizes for the PCR products of *V. parahaemolyticus, V. cholerae,* and *V. alginolyticus* are 297 bp, 154 bp, and 199 bp, respectively. The agarose gel electrophoresis (AGE) of the PCR products showed the presence of bands according to the expected sizes (Figure 1). During the optimisation steps, the annealing temperature of 60°C was ideal for specific amplifications using the three primer sets. On the other hand, PCR products using annealing temperatures lower than 58°C did not produce any band during the AGE.

Based on positive PCR bands, the prevalence of *V. parahaemolyticus, V. cholerae,* and *V. alginolyticus* in four types of samples taken from the shrimp farm, from the stocking period to the harvesting period, were summarised in Table 2.

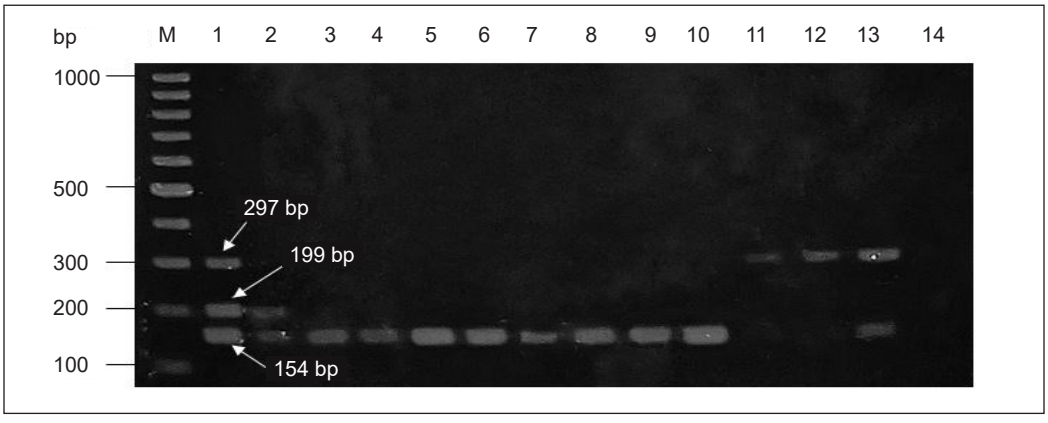

Figure 1. Gel electrophoresis of multiplex PCR of *Vibrio* spp. through amplification of random sequences for identification of *V. parahaemolyticus* (297 bp), *V. alginolyticus* (199 bp), and *V. cholerae* (154 bp). Lane M: 100 bp DNA ladder; Lane 1: positive control (cocktail of *V. parahaemolyticus* ATCC 27969, *Vibrio cholerae* KCDC 13589, and *V. alginolyticus* ATCC 17749); Lane 2–13: representative positive samples; Lane 14: negative control.

Table 2
Prevalence of V. parahaemolyticus, V. cholerae, *and* V. alginolyticus *in water, sediment, shrimp, and effluent samples taken from stocking to harvesting period based on MPN-mPCR method*

Sample	Prevalence (%)			
	n[a]	*V. parahaemolyticus*	*V. cholerae*	*V. alginolyticus*
Water	12	100.0[b] (75.75–100)[c]	58.33 (31.95–80.67)	0.0 (0–24.25)
Sediment	12	100.0 (75.75–100)	33.33 (13.81–60.94)	0.0 (0–24.25)
Shrimp	12	91.67 (64.61–99.57)	16.67 (2.96–44.80)	66.67 (39.06–86.19)
Effluent	12	100.0 (75.75–100)	83.33 (55.20–97.04)	33.33 (13.81–60.94)
TOTAL	48	97.92 (89.10–99.89)	47.92 (34.47–61.67)	25.0 (14.92–38.78)

Note. [a] Number of samples; [b] Percentage of positive samples; [c] 95% confidence interval (%)

Throughout the sampling in the shrimp farm, 48 samples were collected from two ponds during six samplings. Of the entire samples examined, the prevalence of *V. parahaemolyticus, V. cholerae,* and *V. alginolyticus* was 97.92% (n=47, 95% CI: 89.10 to 99.89%), 47.92% (n=23, 95% CI: 34.47 to 61.67%), and 25.0% (n=12, 92% CI: 14.92 to 38.78%), respectively. Among the three *Vibrio* spp., *V. parahaemolyticus* had the highest prevalence, while *V. alginolyticus* had the lowest (P<0.05).

In comparison between samples, *V. parahaemolyticus* was the most prevalent in all four samples. In water samples, the prevalence of *V. parahaemolyticus* and *V. cholerae* were 100% (n=12, 95% CI: 75.75 to 100%) and 58.33% (n=7. 95% CI: 31.95 to 80.67%), respectively, while *V. alginolyticus* was not detected. *V. parahaemolyticus* was again the most prevalent in sediment samples, with 100% (n=12) positive detection, followed by *V. cholerae* with 33.33% (n=4, 95% CI: 13.81 to 60.94%). Meanwhile*, V. alginolyticus* was again not detected in the sediment samples. In shrimp samples, *V. alginolyticus* had the second highest detection after *V. parahaemolyticus,* with 66.67% (n=8, 95% CI: 39.06% to 86.19%) prevalence. In effluent samples, *V. parahaemolyticus, V. cholerae,* and *V. alginolyticus* were positively detected with a prevalence of 100% (n=12, 95% CI: 75.75 to 100%), 83.33% (n=10, 95% CI: 55.2 to 97.04%), and 33.33% (n=4, 95% CI: 6.6 to 60.0%), respectively.

Based on statistical analysis, it has been found that the overall prevalence among the three *Vibrio* spp. is significantly different (P<0.05). However, the prevalence of *V. parahaemolyticus* between sample types was not significantly different. The prevalence of *V. cholerae* significantly differs between sediment and effluent, as well as between shrimp and effluent samples. Meanwhile, the prevalence of *V. alginolyticus* significantly differed between shrimp and water samples and between sediment and shrimp samples.

The three physical and chemical parameters of the water samples were measured and recorded throughout the sampling period. Based on Figure 2, the water temperatures for P1 and P2 remained between 29°C and 34°C from stocking to harvesting. The salinity in P1 was 22–26 ppt, while the salinity in P2 was slightly lower, at 20–25 ppt. Meanwhile, the pH levels for water in both ponds ranged from acidic to neutral (pH 5.9 to pH 7.9).

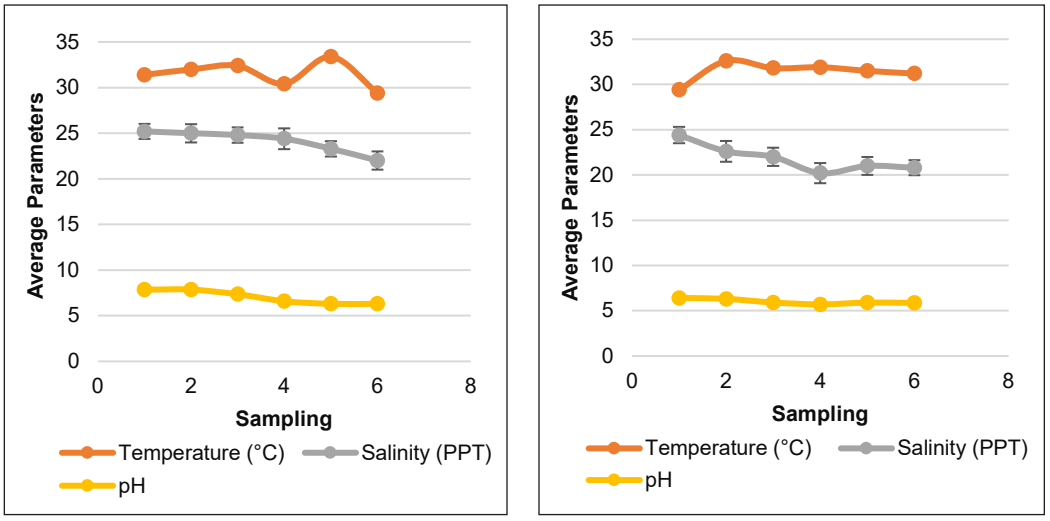

Figure 2. Average temperature, salinity, and pH of pond water in: (a) P1; and (b) P2 throughout the sampling periods

Concentration of *Vibrio* spp. in P1

Vibrio parahaemolyticus

Based on the MPN-mPCR method, it was found that the highest concentration of *V. parahaemolyticus* in water, sediment, and shrimp samples in P1 from stocking (Sampling 1) to harvesting (Sampling 6) was more than 1,100 MPN/mL (Table 3). Although a slight decrease in the detection of *V. parahaemolyticus* in the three samples was observed after the first sampling, the concentration eventually rose to more than 1,100 MPN mL^{-1} at the last sampling. In the effluent samples, the highest concentration of *V. parahaemolyticus* was detected during sampling 5 with 110 MPN mL^{-1}. The concentration of *V. parahaemolyticus* in the effluent samples at P1 is the lowest compared to the other three sample types.

Based on Figure 3, it was observed that the concentration of *V. parahaemolyticus* in water, sediment, and shrimp samples increased to its highest concentration towards harvesting, and it has the highest concentration as compared to the other two *Vibrio* spp. Only in the effluent samples did the concentration of *V. parahaemolyticus* become the lowest compared to *V. cholerae* and *V. alginolyticus* at the end of the sampling period.

Based on the MPN-mPCR result in Table 3, the highest and lowest concentrations of *V. cholerae* in water samples were >1,100 MPN mL^{-1} and <3 MPN mL^{-1}, respectively. The concentration of *V. cholerae* increased to more than >1,100 MPN mL^{-1} on the second sampling but then decreased and remained low at >3 MPN mL^{-1} until harvesting. In the sediment and shrimp samples, the highest detection was observed on sampling two at 38 MPN g^{-1} and 180 MPN mL^{-1}, respectively. However, the concentrations of *V. cholerae* in

Table 3
Microbial load of Vibrio *spp. (MPN g^{-1} or MPN mL^{-1}) in samples collected from P1 throughout one production cycle*

Sampling	Sample	*V. parahaemolyticus*			*V. cholerae*			*V. alginolyticus*		
		Min[a]	Med[b]	Max[c]	Min	Med	Max	Min	Med	Max
1	Water	420	>1,100	-	45	160	420	-	<3	9.5
	Sediment	210	1,100	4,100	3.6	11	38	-	<3	9.5
	Shrimp	-	<3	9.5	-	<3	9.5	180	1,100	4,100
	Effluent	8.7	36	9.4	4.5	16	42	-	<3	9.5
2	Water	18	93	420	420	>1,100	-	-	<3	9.5
	Sediment	40	210	430	3.6	11	38	-	<3	9.5
	Shrimp	1.3	7.4	20	9	43	180	3.7	15	42
	Effluent	1.3	7.2	42	420	>1,100	-	-	<3	9.5
3	Water	180	1,100	4,100	1.3	7.2	18	-	<3	9.5
	Sediment	4.5	16	42	-	<3	9.5	-	<3	9.5
	Shrimp	8.7	36	94	3.6	14	42	1.3	7.2	18
	Effluent	4.5	15	42	1.7	3.6	18	-	<3	9.5
4	Water	180	1,100	4,100	-	<3	9.5	-	<3	9.5
	Sediment	4.5	15	42	-	<3	9.5	-	<3	9.5
	Shrimp	3.6	11	38	-	<3	9.5	0.17	3.6	18
	Effluent	8.7	35	94	-	<3	9.5	-	<3	9.5
5	Water	420	>1,100	-	0.17	3.6	18	-	<3	9.5
	Sediment	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Shrimp	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Effluent	8.7	38	110	40	210	430	0.15	3	11
6	Water	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Sediment	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Shrimp	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Effluent	4.5	15	42	90	290	1,000	8.7	35	94

[a] Minimum MPN g^{-1} or MPN mL^{-1} value; [b] Median MPN g^{-1} or MPN mL^{-1} value; [c] Maximum MPN g^{-1} or MPN mL^{-1} value
(- = No minimum or maximum value)

both samples in the following weeks decreased and remained less than 3 MPN g^{-1} until the harvesting. Unlike *V. parahaemolyticus,* the concentration of *V. cholerae* in the effluent samples was one of the highest among the four sample types. The highest detection was at more than 1,100 MPN mL^{-1}. Although the concentration decreased in sampling three and four, it increased again until harvesting to a maximum value of 1,000 MPN mL^{-1} (Table 3).

From Figure 3, the concentration of *V. cholerae* in water, sediment, and shrimp samples steadily decreased and remained low towards the end of the sampling period, unlike *V. parahaemolyticus*. However, in the effluent samples, the concentration of *V. cholerae* increased until it became more prominent than *V. parahaemolyticus* and *V. alginolyticus.*

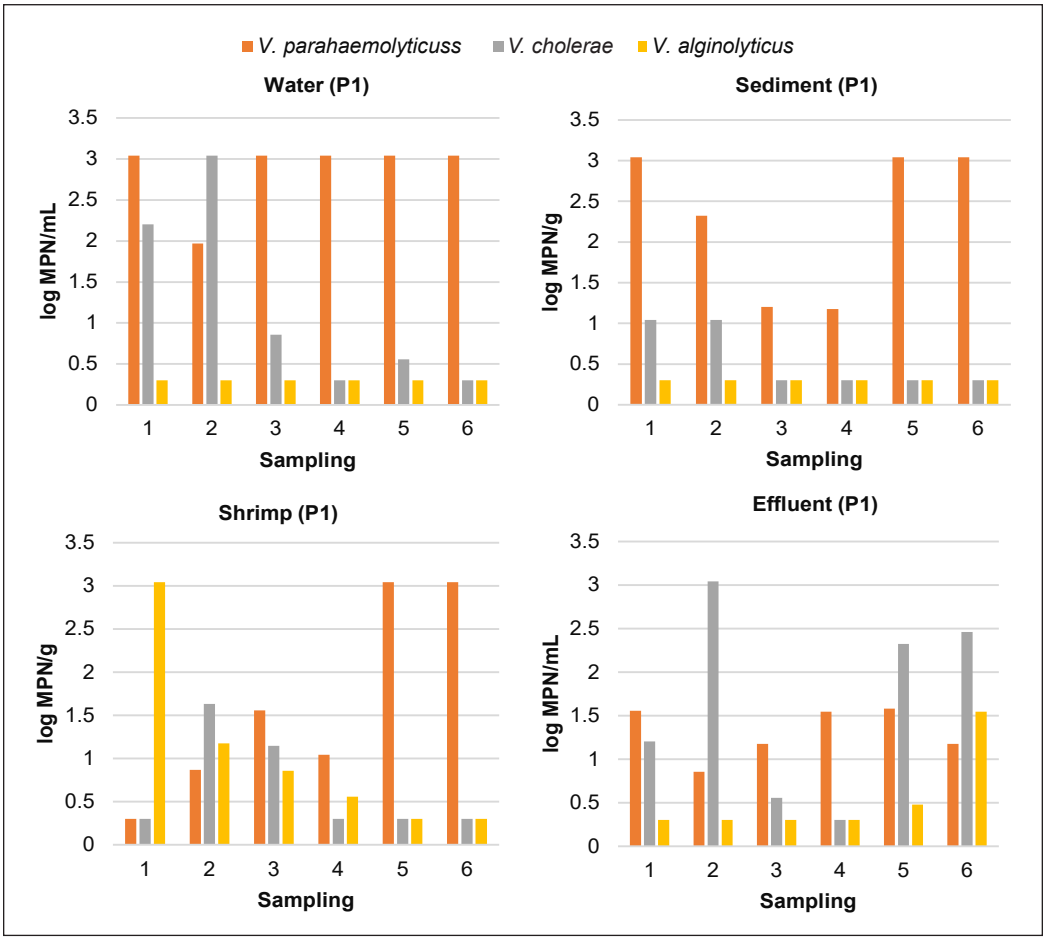

Figure 3. Estimated microbial load of *Vibrio* spp. in water, sediment, shrimp, and effluent samples collected from stocking (Sampling 1) to harvesting (Sampling 6) in P1. The concentrations are expressed in log median MPN mL^{-1} for water and effluent samples and log median MPN g^{-1} for sediment and shrimp samples. For values with no minimum or maximum limit, the log median is derived from the lowest or highest estimated number

In water and sediment samples, the concentration of *V. alginolyticus* detected was <3 MPN mL^{-1} and <3 MPN g^{-1}, respectively, and remained low throughout the sampling period. *V. alginolyticus* was not detected during the MPN-mPCR of both samples from stocking to harvesting (Table 3). *V. alginolyticus* was detected in the shrimp sample on sampling one with the highest concentration of 4,100 MPN g^{-1}. However, the concentration decreased until it became as low as <3 MPN g^{-1} in samples five and six. Meanwhile, in the effluent samples, *V. alginolyticus* concentration only increased in sampling five until it reached a maximum concentration of 94 MPN mL^{-1} during harvesting (sampling six).

Based on the graphs in Figure 3, the concentration of *V. alginolyticus* in water and sediment samples remained the lowest among the three *Vibrio* spp. from stocking to harvesting. Meanwhile, in shrimp samples, *V. alginolyticus* started as the highest *Vibrio*

spp. detected, but the concentration steadily decreased towards harvesting until it became one of the lowest *Vibrio* spp. detected. In contrast, the concentration of *V. alginolyticus* in effluent samples increased towards harvesting to become the second-highest *Vibrio* spp. detected in the samples.

Concentration of *Vibrio* spp. in P2

Vibrio parahaemolyticus

Similar to P1, the highest concentration of *V. parahaemolyticus* in water samples collected from P2 was more than 1,100 MPN mL^{-1} (Table 4). The concentration remained relatively constant throughout the sampling period, from stocking to harvesting. The highest concentration of *V. parahaemolyticus* in sediment samples was as high as >1,100 MPN g^{-1}. However, a decrease was observed in sampling three, with a maximum value of 94 MPN g^{-1} of *V. parahaemolyticus* detected. The concentration increased again in the following weeks and remained constant at >1,100 MPN g^{-1} until harvesting. Similar to shrimp samples from P1, the concentration of *V. parahaemolyticus* detected in shrimp samples from P2 started low at 42 MPN/g but eventually increased to >1,100 MPN g^{-1} towards the harvesting. Meanwhile, the concentration of *V. parahaemolyticus* in the effluent samples increased from 420 MPN mL^{-1} in sampling 1 to >1,100 MPN mL^{-1} towards sampling 6.

Figure 4 shows that the concentration of *V. parahaemolyticus* in all four sample types increased to a maximum value of >1,100 MPN mL^{-1} or > 1,100 MPN g^{-1} towards harvesting. In addition, *V. parahaemolyticus* had the highest detection compared to the other two *Vibrio* spp. being studied.

The highest concentration of *V. cholerae* in water samples was >1,100 MPN mL^{-1}, which is on sampling two, as seen in Table 4. However, the concentration decreased until <3 MPN mL^{-1} towards harvesting. Meanwhile, the highest concentrations of *V. cholerae* in sediment and shrimp samples were 38 MPN mL^{-1} and 180 MPN mL^{-1}, respectively. Similar to water samples, the concentration of *V. cholerae* in both samples then decreased to <3 MPN mL^{-1} or MPN g^{-1} towards harvesting. On the other hand, the concentration of *V. cholerae* in the effluent samples increased to >1,100 MPN mL^{-1} on sampling three and decreased to 18 MPN mL^{-1} on harvesting.

Based on the graphs in Figure 4, the concentration of *V. cholerae* in water, sediment, and shrimp samples remained relatively low compared to *V. parahaemolyticus.* Although increments were observed in the middle, the concentration decreased towards the end of sampling. In effluent samples, the concentration of *V. cholerae* started high, even higher than the concentrations of *V. parahaemolyticus* and *V. alginolyticus.* However, the concentration decreased towards harvesting.

The concentrations of *V. alginolyticus* in both water and sediment samples remained constant at <3 MPN mL^{-1} or MPN g^{-1} from stocking to harvesting (Table 4). Meanwhile,

Table 4
Microbial load of Vibrio *spp. (MPN g^{-1} or MPN mL^{-1}) in samples collected from P2 throughout one production cycle*

Sampling	Sample	*V. parahaemolyticus*			*V. cholerae*			*V. alginolyticus*		
		Min[a]	Med[b]	Max[c]	Min	Med	Max	Min	Med	Max
1	Water	420	>1,100	-	0.15	3	9.6	-	<3	9.5
	Sediment	8.7	29	94	-	<3	9.5	-	<3	9.5
	Shrimp	3.7	15	42	-	<3	9.5	3.6	14	42
	Effluent	37	150	420	180	1,100	4,100	90	290	1,000
2	Water	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Sediment	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Shrimp	8.7	35	94	-	<3	9.5	-	<3	9.5
	Effluent	420	>1,100	-	1.3	7.4	20	3.7	15	42
3	Water	180	1,100	4,100	0.15	<3	9.6	-	<3	9.5
	Sediment	8.7	35	94	1.2	6.1	18	-	<3	9.5
	Shrimp	4.5	15	42	-	<3	9.5	-	<3	9.5
	Effluent	180	1,100	4,100	420	>1,100	-	-	<3	9.5
4	Water	180	1,100	4,100	0.15	<3	11	-	<3	9.5
	Sediment	420	>1,100	-	1.3	7.2	18	-	<3	9.5
	Shrimp	37	150	420	-	<3	9.5	-	<3	9.5
	Effluent	420	>1,100	-	8.7	36	94	-	<3	9.5
5	Water	180	1,100	4,100	-	<3	9.5	-	<3	9.5
	Sediment	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Shrimp	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Effluent	180	1,100	4,100	3.6	14	42	-	<3	9.5
6	Water	180	1,100	4,100	-	<3	9.5	-	<3	9.5
	Sediment	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Shrimp	420	>1,100	-	-	<3	9.5	-	<3	9.5
	Effluent	420	>1,100	-	1.3	7.2	18	-	<3	9.5

[a] Minimum MPN g^{-1} or MPN mL-1 value; [b] Median MPN g^{-1} or MPN mL^{-1} value; [c] Maximum MPN g^{-1} or MPN mL^{-1} value (- = No minimum or maximum value)

in shrimp samples, *V. alginolyticus* was detected at a maximum concentration of 42 MPN g^{-1} in sampling one but subsequently decreased and remained low at <3 MPN g^{-1} until harvesting. Similarly, the concentration of *V. alginolyticus* in effluent samples in sampling one was relatively high at a maximum concentration of 1,000 MPN g^{-1}, even higher than both *V. parahaemolyticus* and *V. cholerae.* However, the concentration decreased and remained low at <3 MPN g^{-1} until the last sampling.

From the graphs comparing the concentration of *Vibrio* spp. in different samples (Figure 4), *V. alginolyticus* has the lowest overall concentration in all four samples towards harvesting. Although the concentration started high in shrimp and effluent samples, unlike *V. parahaemolyticus*, *V. alginolyticus* decreased towards the end of the production cycle.

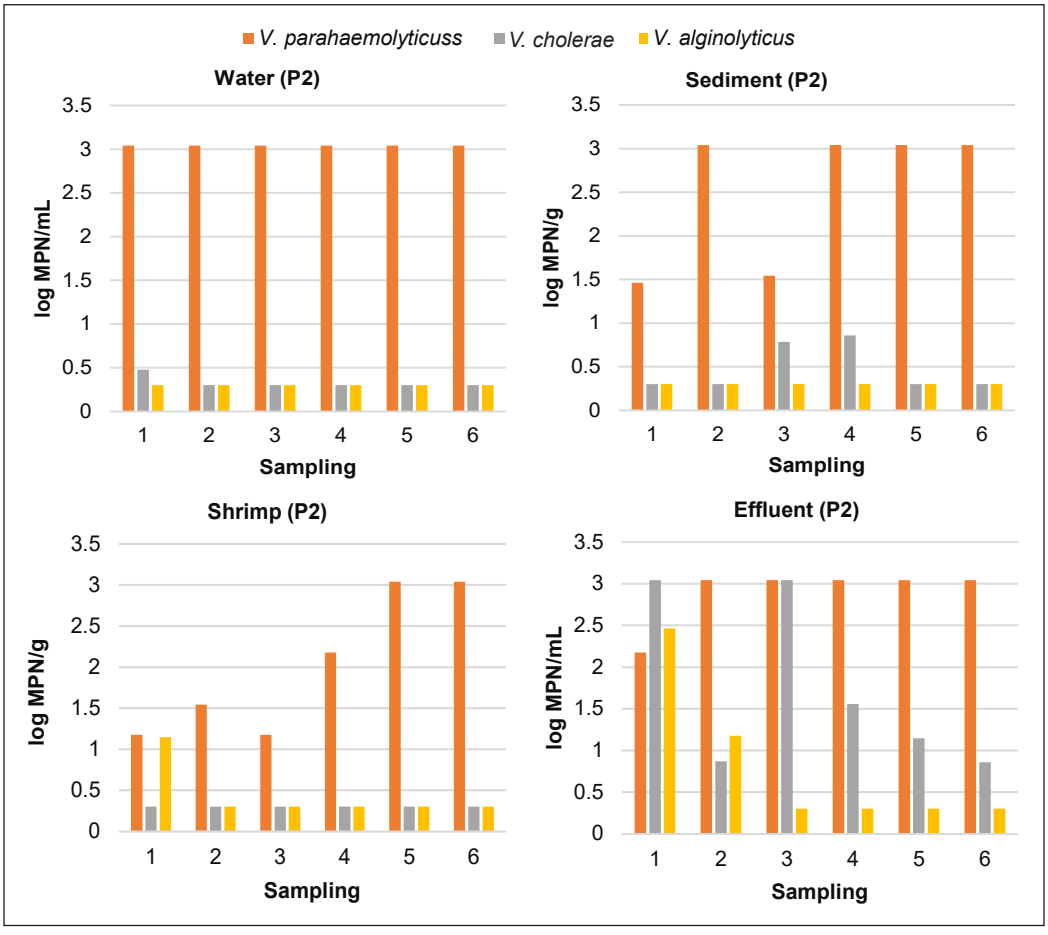

Figure 4. Estimated microbial load of Vibrio spp. in water, sediment, shrimp, and effluent samples collected from stocking (Sampling 1) to harvesting (Sampling 6) in P2. The concentrations are expressed in log median MPN mL^{-1} for water and effluent samples and log median MPN g^{-1} for sediment and shrimp samples. For values with no minimum or maximum limit, the log median is derived from the lowest or highest estimated number

Spearman's Correlation Analysis

Spearman's statistical analysis assessed the correlation between parameters and each Vibrio species. In P1, the temperature exhibited a weak negative correlation with the *V. parahaemolyticus* population, a positive correlation with the *V. cholerae* population, and no correlation with the *V. alginolyticus* population. Additionally, water salinity displayed a weak negative correlation with the *V. parahaemolyticus* population, a significant positive correlation with the *V. cholerae* population, and no correlation with the *V. alginolyticus* population in P1. Conversely, in P2, no correlation was observed between water salinity and all three Vibrio species. The pH of pond water in P1 was slightly negatively correlated with *V. parahaemolyticus* population, positively correlated with *V. cholerae*, and not correlated with *V. alginolyticus* population (Figure 5A).

In P2, the temperature did not correlate with the three Vibrio species. Similarly, no correlation was observed between water salinity and all three Vibrio species in P2. Moreover, there was no correlation between pH values and all three Vibrio spp. in P2 (Figure 5B).

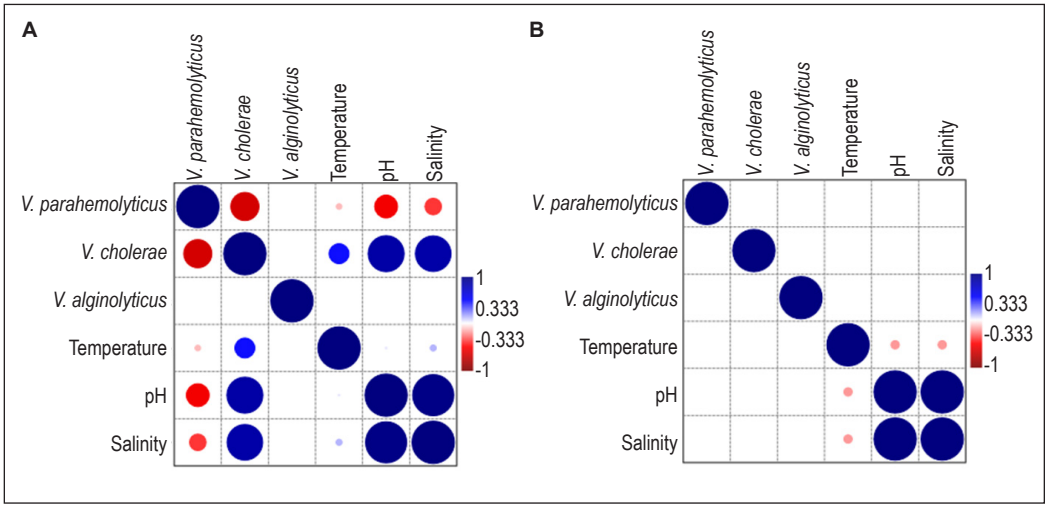

Figure 5. Spearman's rs analysis matrix shows the correlation between the three environmental and chemical parameters with each Vibrio spp. in (A) Pond 1 and (B) Pond 2. A rs of +1 (blue) represents a perfect association of ranks, a rs of zero (blank) shows no association between ranks, and a rs of -1 (red) indicates a perfect negative association of ranks. The closer rs is to zero, the weaker the association between the ranks

DISCUSSION

The vibriosis outbreaks in shrimp aquaculture can result in significant mortality rates and economic losses due to decreased production. The 2011 outbreak of Acute Hepatopancreatic Necrosis Diseases (AHPND) caused by *V. parahaemolyticus* in Peninsular Malaysia resulted in a mortality rate of 60%–90% in many shrimp farms and resulted in economic losses of approximately US$ 0.1 billion (Kua et al., 2016; NACA, 2012). These incidents raise serious concerns for the aquaculture sector as well as the economy at large. Therefore, early detection of *Vibrio* spp. in shrimp aquaculture can help to prevent significant damage at the early stage.

Many methods can be used to detect and enumerate microbial loads in a sample, such as viable plate counts, direct microscopic counts, metabolism-based detection, luminescence-based detection, and immunological/serological-based detection (Logue & Nde, 2017). In our study, the MPN-multiplex PCR (MPN-mPCR) method is used instead of the MPN-plate method because it allows for the enhanced detection and enumeration of *V. parahaemolyticus, V. cholerae,* and *V. alginolyticus* simultaneously. Compared to direct plating using selective media, MPN-mPCR allows for the rapid processing of many samples at the same time, which helps to reduce the processing time (Russo et al., 2014).

This technique also helps to reduce the number of Petri dishes and the cost of selective media used for the enumeration process. The MPN-mPCR technique also provides a more sensitive and rapid detection and enumeration than the conventional MPN-plate method. Without isolating pure isolates, the possibility of getting false negatives through the MPN-mPCR method is lower than the MPN-plate method (Miwa et al., 2003). Therefore, the MPN-mPCR method holds significant potential for further development into a rapid test kit. A comprehensive *Vibrio* detection kit could include pre-packaged enrichment media for sample processing, a simplified genomic DNA extraction kit, and a ready-to-use Polymerase Chain Reaction (PCR) mix with primers specific for the targeted *Vibrio* species. This kit would aim to streamline the detection process, making it accessible for shrimp farmers to monitor *Vibrio* populations in their farms with ease.

Water samples were collected from a few points in each pond to ensure random sampling of the targeted *Vibrio* spp., and the samples were taken at the surface level to maintain standardisation throughout the sampling period. Additionally, given that *Vibrio* spp. is reported to favour warmer temperatures and surface water is typically warmer than deeper depths due to direct sunlight exposure, it is anticipated that *Vibrio* spp. will be more abundant at the water surface (Brumfield et al., 2023).

Based on the findings of this study, *V. parahaemolyticus* is the most prevalent among the three *Vibrio* spp., with the highest load in all four samples exceeding 10^3 MPN g^{-1} or MPN mL^{-1}. Moreover, while *V. cholerae* and *V. alginolyticus* concentration decreased in most of the samples towards the harvesting period, *V. parahaemolyticus*, on the other hand, exhibited increments in all samples towards harvesting. The dominance of *V. parahaemolyticus* over *V. cholerae* and *V. alginolyticus* in all four samples may indicate that *V. parahaemolyticus* illustrates the "pathogen advantage theory". According to this theory, one pathogen species may have a competitive advantage over others in their natural ecosystem, commonly brought by temperature rise. This situation will eventually lead to the overabundance of that domineering species (Colwell, 2004; Thornstenson & Ullrich, 2021). In this case, *V. parahaemolyticus* was able to exploit available resources better than *V. cholerae* and *V. alginolyticus,* resulting in the positive growth of *V. parahaemolyticus* and reduced growth of *V. cholerae* and *V. alginolyticus* (McLaren & Callahan, 2020).

In shrimp samples, the overall load of the three *Vibrio* spp. was low during the stocking period compared to the other samples. However, as the production cycle progressed, exposure to environmental factors and contamination with *Vibrio* spp., which are already present in nature, caused the counts to increase towards harvesting (Kim & Lee, 2017). *V. cholerae*, while not considered a serious pathogen to shrimps, with only a few studies reporting their ability to cause vibriosis in shrimps, can still cause infection in humans through consumption of contaminated products (Cao et al., 2015; Gopal et al., 2005; Haldar et al., 2007). It has been reported that about 42% of *V. cholerae* infections in humans are

caused by consuming seafood products (Chen et al., 2022). Meanwhile, *V. alginolyticus* is an opportunistic pathogen, indicating that once contamination in the host crosses a certain threshold, vibriosis can occur, and mortality is a possible outcome (Brown et al., 2012). The minimum bacterial load of *Vibrio* spp. that can cause vibriosis varies depending on several factors, including the specific *Vibrio* spp., the health and immune status of the host, and the route of exposure. Additionally, individual susceptibility to vibriosis can differ. In our study, the incidence of *V. alginolyticus* contamination in all samples, particularly in shrimp samples when harvested, is still low, making it less likely to cause vibriosis.

Despite the low counts of both *V. cholerae* and *V. alginolyticus*, the concentration of *V. parahaemolyticus* in shrimp samples during the harvesting is concerningly high. At the same time, it is impossible to get shrimps that are entirely free of *Vibrio* spp. contamination due to their ubiquitousness in the marine environment, there are standard acceptable contamination levels set by every country for export purposes. For example, Crustacean seafood products for export to the United States must have less than 10^4 MPN g^{-1} of *V. parahaemolyticus* contamination. In Australia and New Zealand, the contamination level of *V. parahaemolyticus* in their imported crustacean products must be less than 10^3 MPN g^{-1} (Lokkhumlue & Prakitcaiwattana, 2014). In raw or improperly cooked seafood products, pathogenic bacteria such as *Vibrio* spp. can become highly accumulated, rendering the seafood unsafe for consumption (Harrison et al., 2022).

One of the factors that may contribute to the increase in the microbial load of Vibrio spp. in the ponds is the high temperature, typically prevalent between August and December. Malaysia has a tropical climate with a fairly slight variation in the average monthly temperature. With a mean annual temperature of 26.4°C, Malaysia experiences high temperatures all year round (https://climateknowledgeportal.worldbank.org/country/malaysia/climate-data-historical). The relatively high temperature in the shrimp farm can explain the high occurrence of *Vibrio* spp. in P1 and P2, with average temperatures in the range of 29°C to 34°C throughout the sampling period. Our results show that temperatures in this range correlate with culturable *Vibrio* spp. at high concentration, particularly *V. parahaemolyticus*.

However, according to Spearman's statistical analysis, temperature exhibits varying correlations with different *Vibrio* spp. populations. The temperature in P1 has a weak negative correlation with the *V. parahaemolyticus* population, a positive correlation with the *V. cholerae* population, and no correlation with the *V. alginolyticus* population. Meanwhile, the temperature in P2 does not correlate with all three *Vibrio* spp. These statistical results are in contrast with the findings in our study, as well as in many reported studies. This observation may be due to only slight changes in the temperature throughout the sampling, and other factors can affect the growth of the *Vibrio* spp. Many studies reported the correlation between temperature and growth patterns of *V. parahaemolyticus, V. cholerae,*

and *V. alginolyticus* in coastal environments (Thornstenson & Ullrich, 2021; Vezzulli et al., 2016; Williams et al., 2022). It has been shown that temperatures of 20°C and above are optimum for the growth of *Vibrio* spp. in marine water. Many studies have shown that *V. parahaemolyticus, V. cholerae,* and *V. alginolyticus* have optimum growth at 37°C and can still grow at a wide temperature range between 20°C–42°C (Gu et al., 2016; Ravel et al., 1995; Sheikh et al., 2022). In the case of *V. parahaemolyticus,* it has been observed that the increase in temperature leads directly to an increase in growth (Kim et al., 2012).

This observation supports our finding, where the concentration of *V. parahaemolyticus* in all samples, especially in water, increased and remained high at $>10^3$ MPN mL^{-1} when the temperature in P1 and P2 remained in the high range of >30°C. Meanwhile, temperatures less than 15°C have been reported to induce a viable but non-culturable (VBNC) state in *Vibrio* spp. (Baker-Austin et al., 2018). Although this condition is not a concern for coastal farming since the temperature in Malaysia remains relatively high throughout the year, it may raise concern during post-harvesting and importing, when the seafood needs to be kept on ice or frozen to prevent spoilage. Although bacteria in the VBNC state become dormant and are not detectable using conventional culture methods, they remain viable. In addition, functions such as metabolic activity, antibiotic resistance, specific gene expression, virulence, and pathogenicity are sustained (Fernández-Delgado et al., 2015).

In addition to temperature, salinity is another factor that can affect the growth patterns of *Vibrio* spp. in marine ecosystems (Sampaio et al., 2022). The salinity of water samples taken from P1 and P2 fell in the 20–25 ppt range throughout the sampling period. This range of salinity is favourable for the growth of shrimps in the coastal shrimp farm (Chu & Brown, 2021). Unfortunately, this range is also optimum for the growth of *V. parahaemolyticus. V. parahaemolyticus* is reported to have rapid growth in coastal water with salinity in the range of 15 to 25 ppt (Givens et al., 2014). The results also indicate that as the pond water salinity in P1 and P2 decreased slightly towards harvesting, the overall load of the three *Vibrio* spp., notably *V. parahaemolyticus,* increased. It has been reported that a decrease in salinity, especially in brackish water, encourages the growth and spread of *Vibrio* spp. (Baker-Austin et al., 2018). However, statistical analysis showed a weak negative correlation between water salinity and the *V. parahaemolyticus* population, a significant positive correlation with the *V. cholerae* population, and no correlation with the *V. alginolyticus* population in P1. In P2, no correlation was observed between water salinity with all three *Vibrio* spp. The result of this statistical analysis may be due again to the only slight changes in the salinity and the microbial population. The consistency in water salinity in both ponds can also be attributed to the farm's adherence to recommended physio-chemical standards that create an environment conducive to the healthy growth of the shrimp.

Another factor that can significantly affect the growth and survival of *Vibrio* spp. is the pH of water. Most *Vibrio* spp. prefer slightly acidic to neutral pH levels (pH 6.5–7.5) (Ross

& Schreiber, 1998). This information supported our observation that the overall growth of the three *Vibrio* spp., especially *V. parahaemolyticus*, is rapid from stocking to harvesting. At the same time, the pH of water in both ponds remained in the range of pH 5.9–7.9. While this pH range is considered optimum for their growth, some *Vibrio* spp. such as *V. cholerae,* was reported to tolerate a wide range of pH, including alkaline pH as high as pH 9.0 (Nhu et al., 2021). However, according to statistical analysis, the pond water pH is slightly negatively correlated to *V. parahaemolyticus* population, positively correlated to *V. cholerae,* and no correlation with *V. alginolyticus* population in P1. No correlation was observed again between pH values with all three *Vibrio* spp. in P2.

In general, pH levels below pH 6.5 might inhibit the growth of *Vibrio* spp. since acidic conditions can denature proteins and disrupt cellular processes (Kayser & Kayser, 2007). On the other hand, pH levels above pH 7.5 were reported to affect the growth of *Vibrio* spp. by interfering with the bacterial enzymes and other proteins required for their cellular processes, thus preventing their proper functioning (Kieliszek & Błażejowski, 2005). For *V. parahaemolyticus* studies, they can grow at a wide range of pH values, with optimal growth occurring at a pH of 7.0–8.0 (Suzuki et al., 2014). However, *V. parahaemolyticus* can also survive and grow at lower pH values, as low as pH 4.0 (Suzuki et al., 2014).

In addition to affecting the growth and survival of *Vibrio* spp., the pH of water can also influence the expression of certain virulence factors, such as toxins and enzymes, which can contribute to the pathogenicity of these bacteria (Kim et al., 2008). For example, *V. cholerae* has been shown to produce higher levels of cholerae toxin (CTX) and toxin-coregulated pilus (TCP), two of the main virulence factors, at low pH levels (Faruque & Nair, 2010; Weber & Klose, 2011)). In a study conducted by Whitaker et al. (2010), it was observed that acidic conditions induced the expression of lysine decarboxylase, an enzyme responsible for regulating the bacterial acid stress response system in *V. parahaemolyticus* O3:K6. This mechanism enables the bacteria to survive in acidic conditions, enhancing their virulence potential (Whitaker et al., 2010). Overall, the pH of water can significantly impact the presence and activity of *Vibrio* spp. in aquatic environments, with optimal pH levels being critical for their growth and survival.

The overall growth of *V. cholerae* and *V. alginolyticus* decreased towards the end of sampling, while *V. parahaemolyticus* showed increment or constant high concentration throughout the sampling. *V. cholerae* and *V. alginolyticus* might have been outcompeted by the rapidly growing *V. parahaemolyticus*, which could utilise the resources better (Thornstenson & Ullrich, 2021). This statement agrees with Caburlotto et al. (2010), who stated that another reason for the different growth patterns is the difference in the sensitivity of the different *Vibrio* spp., towards environmental conditions, including temperature, salinity, and pH. In this case, *V. parahaemolyticus* may be more sensitive to environmental factors than *V. cholerae* and *V. alginolyticus.*

In addition to being sensitive to environmental and chemical parameters, overfeeding has been identified as a potential factor contributing to the abundance of *Vibrio* spp. in shrimp ponds. Overfeeding leads to the formation of nutrient-rich sludge, creating conditions favourable for the proliferation of harmful microorganisms, including *Vibrio* species (Horowitz & Horowitz, 2020). However, in the Telaga Air shrimp farm, no sludge formation was observed throughout the sampling period. Additionally, information from the manager revealed the implementation of a well-timed feeding schedule using automatic timers to prevent shrimp from being overfed. Further studies are necessary to thoroughly investigate the feeding conditions and their relationship with Vibrio spp. in the farm. In environmental samples, *V. parahaemolyticus* is often reported to be the dominant species isolated, especially in brackish waters. Such is the case that the number of other *Vibrio* spp. isolated in a sample is used to evaluate the presence and risk of *V. parahaemolyticus.*

A study by Ruiz-Cayuso et al. (2021) found that for each *Vibrio* spp. detected, the odds of isolating *V. parahaemolyticus* increased by 2.84-fold. This study proved and supported the findings by Blackwell and Oliver (2008), who also reported the correlation between *V. parahaemolyticus* with the total *Vibrio* spp. and other coliforms such as *Escherichia coli* isolated from an environmental sample (Ruiz-Cayuso et al., 2021). The dominance of *V. parahaemolyticus* against other *Vibrio* spp. is due to their ability to withstand a wide range of temperatures and salinities, as well as to adapt and thrive in various environments (Janda & Abbott, 2010). This characteristic allows *V. parahaemolyticus* to outcompete other *Vibrio* spp. in marine and freshwater environments.

According to the project manager of PNK Satang Biru shrimp farm, a complete harvest of adult *P. vannamei* from each pond can yield approximately seven to eight metric tons of shrimp in one production cycle. During the last sampling (the sixth sampling), conducted at the time of harvesting adult shrimps, the average size of the harvested shrimp was approximately 130 mm long. Overall, it took approximately 90 to 100 days for the post-larvae shrimp to grow to a harvestable size. While the direct effect of the high presence of *Vibrio* spp. was not observed on the overall growth and production of the shrimp in the shrimp farm, the risk for infection is still present. Without stressors, *Vibrio* spp., as an opportunistic pathogen, can reach significant concentration without triggering the onset of vibriosis (Newman, 2022). However, the ability of *Vibrio* spp. to cause disease should not be overlooked, considering the relationship between the shrimp and the stressors (Valente & Wan, 2021). In addition, the accumulation of *Vibrio* spp. in shrimp products can still pose health risks to consumers when not processed or cooked appropriately (Kaysner et al., 2004).

The findings in our study raise concern since the microbial load in the harvested shrimp product exceeds the standards acceptable in most importing countries. However,

early detection of *Vibrio* spp. in shrimp farms, especially during stocking, is crucial because it helps to prevent the spread of the bacteria to other shrimps and can lead to a more effective treatment plan. If *Vibrio* spp. is not detected until it has spread throughout the farm, it might lead to a significant loss of shrimp and finances for the farm. Additionally, *Vibrio* spp. can cause serious health problems for humans who consume contaminated shrimp, so early detection and treatment can protect public health. Early detection also allows for more targeted and potentially less disruptive treatment methods rather than implementing broad and potentially harmful measures if the infection has spread widely.

Shrimp farmers should pay attention to their management practices to ensure the quality of their products and to prevent the spread of diseases. Good practices include controlling water quality, preventing overcrowding, and maintaining good hygiene. Continuous real-time monitoring of the critical control points (HACCP) in shrimp aquaculture is also essential to ensure food safety and prevent disease outbreaks. Overall, good farm management is the key to preventing vibriosis and protecting the health and well-being of both the shrimps and the seafood consumers.

CONCLUSION

Vibrio parahaemolyticus, V. cholerae, and *V. alginolyticus* were successfully detected and enumerated in all four sample types in both ponds, from stocking until harvesting. This study has shown that *V. parahaemolyticus* is the most dominant species compared to *V. cholerae* and *V. alginolyticus* in the Telaga Air shrimp farm, suggesting that the ability of *V. parahemolyticus* to exploit available resources more effectively than *V. cholerae* and *V. alginolyticus* contributes to the elevated *V. parahaemolyticus* load in the shrimp farm. It becomes a concern because *V. parahaemolyticus* is a well-recognised human pathogen associated with seafood-related infections, and its prevalence in shrimp farms can have implications for food safety. In the future, prevalence studies can be extended to encompass more shrimp farms across Sarawak, providing a more comprehensive dataset on the prevalence and distribution of *Vibrio* species.

The study's outcomes, serving as a valuable model, highlight the need for ongoing monitoring of *Vibrio* spp. prevalence in shrimp farms across Sarawak, addressing the current lack of comprehensive data on the subject in the region. This study also undescores the importance of employing the MPN-multiplex PCR technique to surveillance *Vibrio* spp. in shrimp farms. The method offers several advantages, including enhanced sensitivity and rapid simultaneous processing of multiple samples, which reduces processing time. The efficiency of this technique positions it as a promising tool for ongoing monitoring efforts, providing critical data for the prevention and control of vibriosis outbreaks in shrimp farms, thereby safeguarding public health and the sustainability of the shrimp industry.

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