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RESEARCH ARTICLE

Assessing the roles of environmental factors in shaping *Vibrio*-phytoplankton associations in Northern Sarawak, Malaysia

Marshiella Lahai Mering[1], Lesley Maurice Bilung[1*], Teng Sing Tung[1], Ho Ai Chia[2], Ahmad Syatir Tahar[3], Muhammad Khairil Syamri Bakeri[1], Kasing Apun[1]

[1]Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia
[2]Jabatan Kesihatan Negeri Sarawak, 93050, Kuching, Sarawak.
[3]Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia.

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☒ * **CORRESPONDING AUTHOR**
Lesley Maurice Bilung
Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia
Email: mblesley@unimas.my

ABSTRACT

The genus *Vibrio* forms associations with phytoplankton, using algal cells as vectors for proliferation and survival in aquatic environments. These interactions serve as a reservoir for *Vibrio* species, influencing the ecology of estuarine and potentially affecting the concentration and distribution of pathogenic *Vibrio*. Despite extensive research on *Vibrio* and phytoplankton interactions, little is known about their associations with key environmental factors such as temperature, pH, and salinity. This study aimed to determine the concentrations of *Vibrio* species, environmental factors influencing their proliferation, and analyse *Vibrio*-phytoplankton interactions. Water samples were collected from two estuaries, with physicochemical parameters recorded *in-situ*. Phytoplankton abundance was determined microscopically, while *Vibrio* isolation was conducted using TCBS agar. Molecular identification of *Vibrio* species was performed using PCR amplification of species-specific genes (*OmpW* for *V. cholerae* and *ToxR* for *V. parahaemolyticus*). The results showed that 36.7% (n=22/60) of the samples positive for *V. cholerae* and 31.7% (n=19/60) were positive for *V. parahaemolyticus*. None of the isolates tested positive for the *ctxA* and *tdh* virulence genes. Statistical analysis revealed significant differences ($p < 0.05$) between *Vibrio* concentrations, water temperature and salinity, but pH showed no significant effect. Pearson's correlation analysis revealed positive correlations between *Vibrio* concentrations with high temperature (31.2 – 34.5 ºC) and salinity (28.7 - 31.0 ppt), while exhibiting a negative correlation with phytoplankton concentration. Phytoplankton was influenced by temperature and salinity, with high salinity reducing abundance. The results of this study may provide insights into potential public health risks posed by *Vibrio* species in estuarine ecosystems and contribute to understanding their ecological dynamics in Northern Sarawak, Malaysia.

1. INTRODUCTION

The genus *Vibrio* is a Gram-negative bacteria belonging to the family *Vibrio*naceae, a group of aquatic microorganisms capable of tolerating a range of salinity levels and typically inhabiting warm waters (Sampaio et al., 2022). Morphologically, *Vibrio* species are characterized as straight or curved rod-shaped bacteria, measuring approximately 0.5 and 0.8 µm in width and 2 to 3 µm in length. Curved rods resemble comma-shaped cells and appear either as thin, elongated, delicate structures or as shorter, thicker forms. These cells commonly occur in pairs and occasionally assemble into short chains (Monsreal et al., 2021). *Vibrio* cells possess a single polar flagellum, which facilitates motility, and exhibit both fermentative and respiratory metabolic pathways (Caburlotto et al., 2016).

The *Vibrio* genus has more than 35 species, with at least 12 species associated with food-borne diseases (Pruzzo et al., 2005; Gomathi et al., 2013). These bacteria are natural inhabitants of river mouths, brackish waters, estuaries and marine waters (Vincent et al., 2014; Marques et al., 2022). *Vibrio* species populations proliferate in warm water with a moderate salinity range and decrease in cooler water (Dickinson et al., 2013; Brumfield et al., 2023). In countries with cold and temperate climates, as observed in the North Sea, Germany, the density of *Vibrio* species in estuarine and seawater is higher in summer compared to winter season. In recent years, the emergence of *Vibrio* species as major

microbiological agents has drawn research interest due to their role in producing a variety of ailments ranging from mild gastroenteritis to serious systemic infections.

Among other bacteria, *Vibrio* species exhibit the most rapid growth rates, proliferating immediately in response to favourable environmental conditions, such as high temperature, dissolved oxygen and salinity (Sampaio et al., 2022). The pathogenic group, comprising *Vibrio* cholerae, *Vibrio* parahaemolyticus, and *Vibrio* vulnificus is commonly encountered in estuarine and coastal environments worldwide, presenting significant implications for human health. *Vibrio* cholerae is the causative agent of cholera outbreaks globally. The estuarine ecosystem, serving as its reservoir, potentially contributes to the persistence and transport of pathogenic strains in the environment (Hsieh et al., 2007). *Vibrio* parahaemolyticus is associated with food-borne diseases, which cause gastroenteritis from consuming contaminated seafood (Tanil et al., 2005). Illness caused by *Vibrio* vulnificus can lead to septicemia and serious wound infection, resulting in a high mortality rate among susceptible individuals (Wong et al., 2012).

Previous research demonstrated a strong association between *Vibrio* abundance and physicochemical parameters, such as temperature, salinity, and attachment to planktonic organisms (Hsieh et al., 2007). The elevated temperature and salinity contribute to the proliferation of *Vibrio* species in aquatic ecosystems. Studies have also indicated that the presence of plankton, particularly algal cells, plays a crucial role in enhancing the proliferation and persistence of *Vibrio* species, especially during algal blooms. *Vibrio* species exhibit a tendency to attach to algal cells and detritus, suggesting a preference for these surfaces over other particles in the aquatic environment (Main et al., 2015). This attachment can occur either as free-living single cells or in biofilms, where large numbers of *Vibrio* can persist on abiotic substrates such as suspended slit particles, faecal matter, and sediment (Brumfield et al., 2023).

During phytoplankton blooms, a substantial concentration of nutrients is released in dissolved and particulate forms, supporting high *Vibrio* concentrations in the aquatic environment. Furthermore, the aggregation of microalgae during these blooms provides an extensive surface area for *Vibrio* attachment, contributing to the formation of diverse biological niches. The increase in harmful algal blooms (HABs) in recent decades has frequently coincided with an increase in *Vibrio* species populations, underscoring the close relationship between these phenomena (Sampaio et al., 2022).

The northern region of Sarawak, which encompasses cities such as Miri and Limbang, is characterized by its diverse aquatic ecosystems, including coastal areas, rivers, and estuaries. Miri, the second largest city in Sarawak, serves as a major urban centre in the northern part of the state. Limbang, a smaller municipality, is situated near the border of Brunei. Local communities rely on aquatic habitats for essential resources, such as fisheries, which contribute significantly to the region's economy. The dynamic environmental conditions and distinctive habitats in northern Sarawak provide an ideal setting for investigating the abundance and diversity of *Vibrio* species. According to the Borneo Post in 2011, 111 cases of gastroenteritis related to *Vibrio* were reported in Limbang after 6th March, with two cases of particular concern, as one patient had a pre-existing medical condition and the other was pregnant (Veno, 2011; Elexson et al., 2023). The presence of *Vibrio* species in these environments raises concerns regarding the safety of consuming seafood-related products and the potential for *Vibrio*-associated diseases.

Despite numerous studies worldwide demonstrating that *Vibrio* species are strongly influenced by physicochemical parameters and their association with planktonic organisms, especially phytoplankton, such interactions remain poorly understood in the context of Sarawak estuarine ecosystems. To date, environmental factors such as temperature, pH, and salinity jointly influence both *Vibrio* distribution and phytoplankton composition remains unclear. The lack of ecological data hinders a comprehensive understanding of *Vibrio* persistence and potential health risks in northern Sarawak estuaries.

Therefore, this study aims to address this knowledge gap by (i) investigating the distribution and concentration of *Vibrio* species in Northern Sarawak estuarines; (ii) analysing the influence of physicochemical parameters on the presence of *Vibrio* species and phytoplankton; and (iii) determining the phytoplankton species that influence the concentration of *Vibrio* species in estuarine waters. By integrating environmental and microbiological data, this study provides baseline information on *Vibrio*-phytoplankton associations in Sarawak estuaries, contributing to improved risk assessment and management of *Vibrio*-related health concerns in the region.

2. MATERIALS AND METHODS

2.1 Background of the study

This study focused on two estuaries in northern Sarawak, mainly Coco Cabana, Miri (4º23'07.4" N 113º58'14.0" E) and Kampung Limpaku Pinang, Limbang (4º52'47.1" N 115º01'13.9" E). These locations are significant to the local community, whose primary activities include tourism, fishing, and fisheries-related industries. Ten sampling

points were established at each site for comprehensive data collection and analysis. Figure 1 illustrates the map of the study sites, indicating the sampling points used for water sample collection. This study did not require ethical approval.

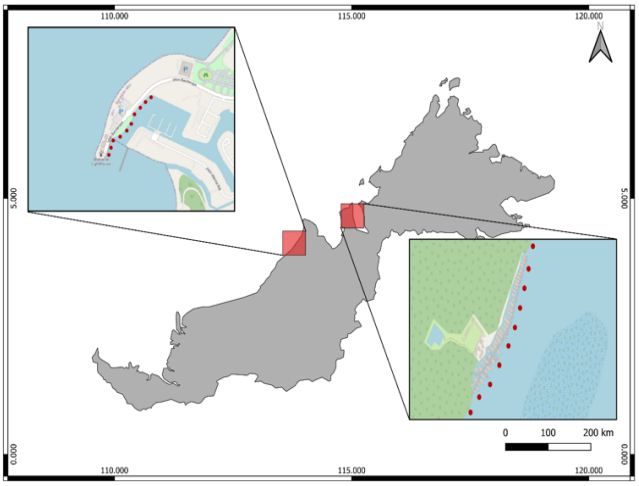

Figure 1: The map of study sites and 10 sampling points deployed for water sample collection. (Google, 2024).

Coco Cabana was selected as a representative estuarine environment, where freshwater from the river mixes with seawater from the South China Sea. Estuaries are dynamic ecosystems that function as natural reservoirs for *Vibrio* species due to fluctuating salinity gradients and nutrient availability (Morrison et al., 2024). The brackish water facilitates the proliferation and survival of *Vibrio*, including those of public health significance.

Kampung Limpaku Pinang was selected due to its documented history of cholera outbreaks in Sarawak (Benjamin et al., 2005; Nillian et al., 2018), indicating the potential presence of *V. cholerae* in the area. Similar to other estuarine environments, the persistence of *V. cholerae* in this site is influenced by physicochemical conditions, including pH and salinity variations, as well as anthropogenic activities. These characteristics make Kampung Limpaku Pinang a relevant site for assessing the environmental factors associated with *Vibrio* occurrence in estuarine systems.

2.2 Water sample collection and physicochemical parameters assessment

Water samples were collected as described by Huq et al. (2012). Sampling was conducted in May 2022 and October 2023. Surface water samples were collected using horizontal hauls and filtered through a 20µm mesh-size plankton net. Water samples were collected in triplicate at each sampling point (100 mL each). The collected samples were stored in an isothermal box containing ice packs and transported to the Molecular Microbiology 1 laboratory at Universiti Malaysia Sarawak within 24 h for further analysis.

Physicochemical parameters were measured in-situ during the sampling trip. A temperature datalogger (Extech SDL 100) was used to measure the water temperature and pH. Consequently, the salinity was measured using a hand refractometer (ATAGO S/MILL).

2.3 Sample processing

The collected water samples were processed by procedure described by Bilung et al. (2019). All water samples were concentrated through filtration using a sterile mixed cellulose membrane filter (0.45 µm pore size; 47 mm diameter) (WhatmanTM). The phytoplankton cells trapped on the membrane filter were washed with 25 mL of sterile 1× phosphate buffer solution (PBS) before vortexing. Subsequently, 1 mL of the sample was transferred into 25 mL of alkaline peptone water (APW) (1% peptone, 1% NaCl; pH 8.5) for enrichment and incubated for 16-24 hours at 35 °C.

2.4 Bacterial enumeration

A six-fold serial dilution was performed in a dilution tube containing 9 mL of sterile PBS. Next, 100 µL of each dilution solution was spread plated on thiosulfate citrate bile-salts sucrose (TCBS) agar and incubated at 37ºC for 24 h. The colonies grown on TCBS were enumerated expressed in colony-forming units (CFU mL^{-1}).

2.5 Molecular identification of *Vibrio* species

For the molecular identification of *Vibrio* species, representative green and yellow colonies were selected from TCBS agar and subjected to DNA extraction using the Bacterial Genomic DNA Isolation Kit (Norgen Biotek Corp., Canada), following the manufacturer's protocol. Polymerase chain reaction (PCR) was employed to detect specific target genes associated with *Vibrio* species, namely the outer membrane protein W gene (*OmpW*) of *V. cholerae* and the regulatory gene (ToxR) of V. parahaemolyticus, and the respective virulence genes, *ctxA* for *V. cholerae* and *tdh* for V. parahaemolyticus.

Single-plex PCR was performed using each primer pair. The PCR reaction was conducted in a final volume of 15 µL, comprising 7.5 µL 2× GoTaq® Green Master Mix (Promega, US), 0.6 µL of each primer (10 µM), 3.3 µL sterile distilled water, and 3 µL of DNA template. DNA of *V. cholerae* (KCDC 13589) and *V. parahaemolyticus* (ATCC 27969) were used as positive controls, and nuclease-free water served as a negative control. The following conditions were used in the PCR reactions: initial denaturation at 94ºC for 5 min, 30 cycles each of denaturation at 94ºC for 30 sec, annealing for 30 sec at: 52ºC for *OmpW* gene; 53ºC for *ToxR* gene; 54 ºC for *ctxA* gene; and 50 ºC for *tdh* gene, and extension at 72ºC for 30 sec, with a final extension at 72ºC for 5 min. The primers and

their corresponding annealing temperatures used for PCR amplification are listed in Table 1. Gel electrophoresis on 1.5% agarose gel was conducted at 75 V; 400 A for one hour. The gel was stained with ethidium bromide for an hour and visualized using blue light transilluminator (Cleaver Scientific, UK).

Table 1: List of primers and their corresponding annealing temperatures used in this study.

Target gene	Primer name	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (ºC)	Reference
OmpW	OmpW-F	CAC CAA GAA GGT GAC TTT ATT GTG	588	52	(Nandi et al., 2000)
	OmpW-R	GAA CTT ATA ACC ACC CGC G			
ToxR	ToxR-F	GTC TTC TGA CGC AAT CGT TG	368	53	(Chakraborty & Surendran, 2008)
	ToxR-R	ATA CGA GTG GTT GCT GTC ATG			
ctxA	ctxA-F	CTC AGA CGG GAT TTG TTA GGC AGT	302	54	(Bilung et al., 2019)
	ctxA-R	TCT ATC TCT GTA GCC CCT ATT ACG			
tdh	tdh-D3	CCA CTA CCA CTC TCA TAT GC	251	50	(Tunung et al., 2011)
	tdh-D5	GGT ACT AAA TGG CTG ACA TC			

2.6 Phytoplankton identification

Phytoplankton morphology-based analysis was conducted based on the method described by Chong et al. (2022). One litre of water samples was collected with a clean bucket, filtered through a 20 µm mesh plankton net, and concentrated to a final volume of 100 mL. Acidic Lugol's solution was subsequently added for preservation. Phytoplankton were quantified by pipetting 1 mL of water samples into Sedgewick-Rafter counting chambers, while ensuring the absence of air bubbles. The water samples were then allowed to settle for 5-10 min before being counted grid-by-grid using a compound microscope at 600× magnification. This process was repeated thrice to obtain the average count of the results (Awg Baki et al., 2024). Phytoplankton identification based on morphology was carried out by following phytoplankton identification keys (Lim et al., 2014; Tan et al., 2016; Hii et al., 2019). A drop of the water sample from each sampling point was transferred to a glass slide, and the observed cells were documented using an inverted microscope (Olympus FluoView 300) (Olympus Corporation, Japan). The camera installed on the inverted microscope used to capture the images was Infinity 3 (Teledyne Lumenera, Ottawa, Canada), and the software utilized for image analysis was Infinity Analyze (Teledyne Lumenera, Ottawa, Canada).

2.7 Statistical analysis

Vibrio abundance was log-transformed to obtain a normal distribution. For statistical analysis, *Vibrio* numbers below the detection limit were assigned a zero value. The significant effect of the sampling sites on the *Vibrio* concentration and physicochemical parameters, such as temperature, pH, and salinity were analysed by using a one-way analysis of variance (ANOVA). Pearson's correlation analysis was performed to investigate the correlation between *Vibrio* concentration, physicochemical parameters and phytoplankton. All analyses were performed using GraphPad Prism version 10.3.0 for Windows.

3. RESULT AND DISCUSSION

3.1 Concentration of *Vibrio* species in Northern Sarawak

Based on the results, the total mean concentration of *Vibrio* species in water samples from Kampung Limpaku Pinang (9.1×10^9 CFU mL^{-1}) was significantly higher than Coco Cabana (6.3×10^9 CFU mL^{-1}). The concentration of *Vibrio* in water samples collected across the sampling points at Coco Cabana are shown in Figure 2. The lowest concentration was observed at C4 (2.4×10^9 CFU mL^{-1}), whereas the highest was found at C3 and C10 (7.1×10^9 CFU mL^{-1} each). At Kampung Limpaku Pinang, the *Vibrio* species concentration ranged from 8.7×10^9 to 9.4×10^9 CFU mL^{-1} (Figure 3). The lowest concentration was found in sample L1 (8.7×10^9 CFU mL^{-1}), whereas the highest was observed at L7 (9.4×10^9 CFU mL^{-1}).

Comparing the two sampling sites, Kampung Limpaku Pinang consistently showed a higher abundance of *Vibrio* species across all samples than Coco Cabana. The lowest *Vibrio* concentration at Kampung Limpaku Pinang (8.7×10^9 CFU mL^{-1}) exceeded the highest *Vibrio* concentration observed at Coco Cabana (7.1×10^9 CFU mL^{-1}). This shows that water samples collected at Kampung Limpaku Pinang were more favourable for *Vibrio* growth and were subject to higher levels of contamination.

3.2 Molecular detection of *Vibrio* species using polymerase chain reaction (PCR)

The *OmpW* and *ToxR* genes were targeted for PCR amplification to identify *V. cholerae* and V. parahaemolyticus, respectively. The anticipated size of the PCR products was 588 bp for *OmpW* and 368 bp for ToxR. Gel electrophoresis of the PCR products revealed bands consistent with the

expected sizes. Species identification using PCR revealed that *V. cholerae* was 6.67% (2/30) from Coco Cabana and 76.7% (23/30) from Kampung Limpaku Pinang (Figure 4). Whilst *V. parahaemolyticus* was 50% (15/30) and 13.3% (4/30) from Coco Cabana and Kampung Limpaku Pinang (Figure 5).

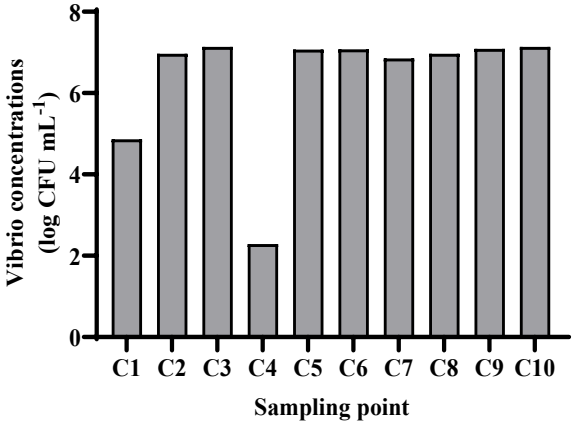

Figure 2: The concentration of *Vibrio* species at Coco Cabana.

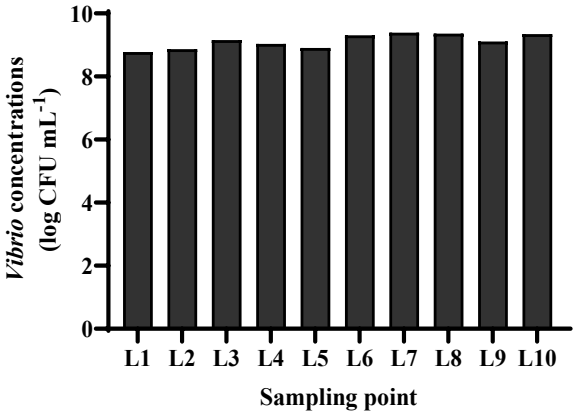

Figure 3: The concentration of *Vibrio* species at Kampung Limpaku Pinang.

3.3 Detection of virulence genes (*ctxA* and ToxR) in *V. cholerae* and V. parahaemolyticus

The successful amplification of the *OmpW* and *ToxR* genes confirmed the presence of *V. cholerae* and *V. parahaemolyticus* in the water samples. Subsequently, a PCR assay targeting the cholera toxin (CT) coding gene *ctxA* for *V. cholerae* and the toxin coding gene *tdh* for *V. parahaemolyticus* was performed to assess their pathogenicity. Despite the successful amplification of *OmpW* and *ToxR* genes, none of the representative samples produced bands for *ctxA* or *tdh* after PCR as shown in Figure 6 and Figure 7. This indicates that the *V. cholerae* and *V. parahaemolyticus* tested were likely non-toxigenic environmental isolates.

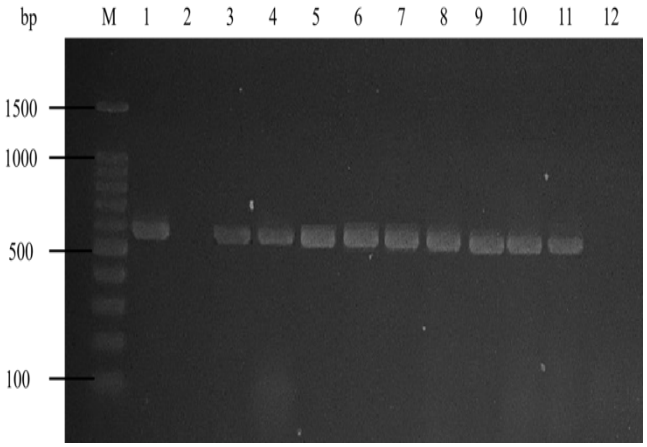

Figure 4: Detection of *V. cholerae* through PCR amplification of *OmpW* gene (588 bp). Lane M: 100 bp DNA ladder; Lane 1: positive control (*V. cholerae* DNA); Lane 2: negative control; Lane 3 – 12: representative positive samples.

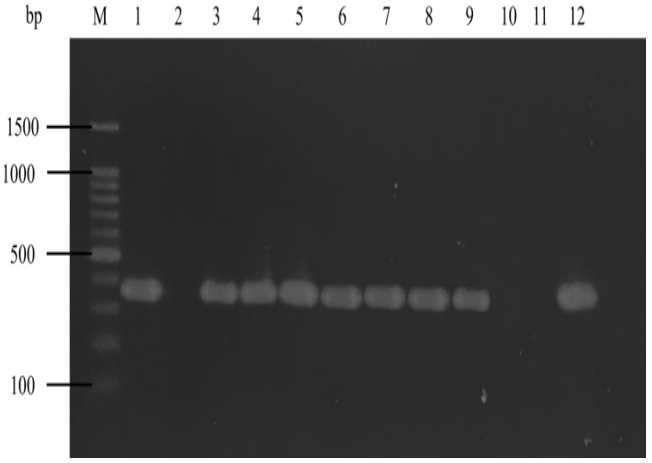

Figure 5: Detection of *V. parahaemolyticus* through PCR amplification of *ToxR* gene (368 bp). Lane M: 100 bp DNA ladder; Lane 1: positive control (*V. parahaemolyticus* DNA); Lane 2: negative control; Lane 3 – 12: representative positive samples.

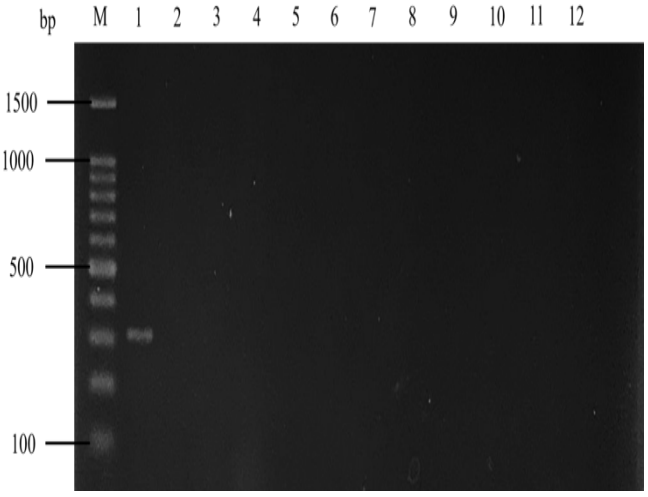

Figure 6: Detection of cholera toxin gene *ctxA* (302 bp) in *V. cholerae* isolates. Lane M: 100 bp DNA ladder; Lane 1: positive control (*V. cholerae* DNA); Lane 2: negative control; Lane 3 – 12: representative *V. cholerae* samples.

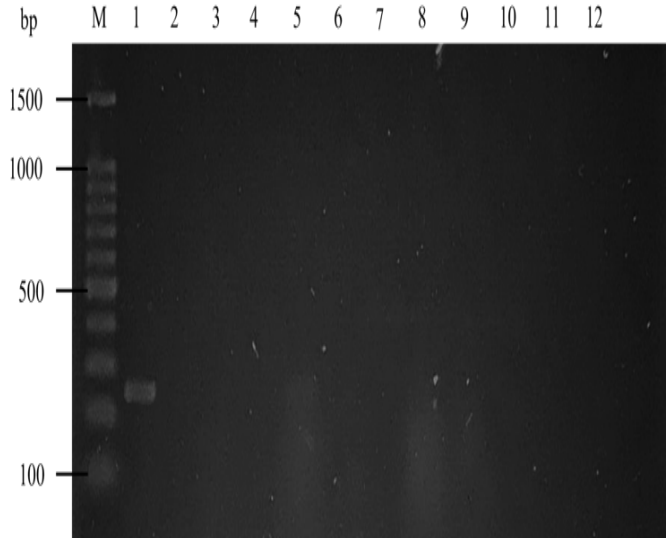

Figure 7: Detection of toxin coding gene (*tdh*) in *V. parahaemolyticus* isolates. Lane M: 100 bp DNA ladder; Lane 1: positive control (*V. parahaemolyticus* DNA); Lane 2: negative control; Lane 3 – 12: representative *V. parahaemolyticus* samples.

3.4 Physicochemical parameters (temperature, pH, and salinity)

The results of environmental parameters such as temperature, pH, and salinity recorded at Coco Cabana and Kampung Limpaku Pinang are shown in Figure 8. The water temperature, pH, and salinity from Coco Cabana ranged from 30.1 - 31.3 ºC, 8.5 - 9.1, and 28.7 - 31.0 ppt, respectively. At Kampung Limpaku Pinang, the temperatures observed ranged from 31.2 – 34.5 ºC, pH ranged from 6.3 – 6.6, and salinity ranged from 1.7 – 4.7 ppt.

3.5 Phytoplankton genera in Northern Sarawak

In this study, phytoplankton in water samples from Coco Cabana and Kampung Limpaku Pinang were analysed, identifying a total of 23 genera. The most abundant group of organisms was diatoms followed by dinoflagellates. Cyanobacteria were the least abundant group recorded. All phytoplankton genera observed are shown in Figure 9.

At Coco Cabana, diatoms were represented by 15 genera, including the centric forms: *Biddulphia* spp., *Chaetoceros* spp., *Coscinodiscus* spp., *Cyclotella* spp., *Melosira* spp., *Odontella* spp., *Proboscia* spp., and the pennate forms: *Amphiprora* spp., *Navicula* spp., *Nitzschia* spp., *Pleurosigma* spp., *Rhoicosphenia* spp., *Surirella* spp., *Synedra* spp., and *Thalassionema* spp. Dinoflagellates contributed six genera: *Ceratium breve*, *Ceratium furca*, *Ceratium fusus*, *Dinophysis caudata*, *Prorocentrum* spp., and *Protoperidinium* spp., whereas cyanobacteria were represented by *Oscillatoria* spp., and *Planktothrix* spp. (Figure 10). Among the phytoplankton, *Chaetoceros* spp. had the highest number of cells recorded, constituting 42.65% of the total cells observed, followed by *Melosira* spp. (21.56%) and *Nitzschia* spp. (16.81%). *Proboscia* spp. and *Ceratium furca* were the least abundant species recorded, each contributing only 0.04% to the total phytoplankton population.

Seven phytoplankton genera were identified in water samples collected from Kampung Limpaku Pinang (Figure 11). Notably, only diatoms were recorded, comprising of Amphipropra spp., Coscinodiscus spp., Navicula spp., Nitzschia spp., Pleurosigma spp., Surirella spp., and Synedra spp. The phytoplankton population was dominated by Pleurosigma spp. (45.62%), and Surirella spp. (2.14%) represented the least abundant species. No dinoflagellates or cyanobacteria were present in the water samples.

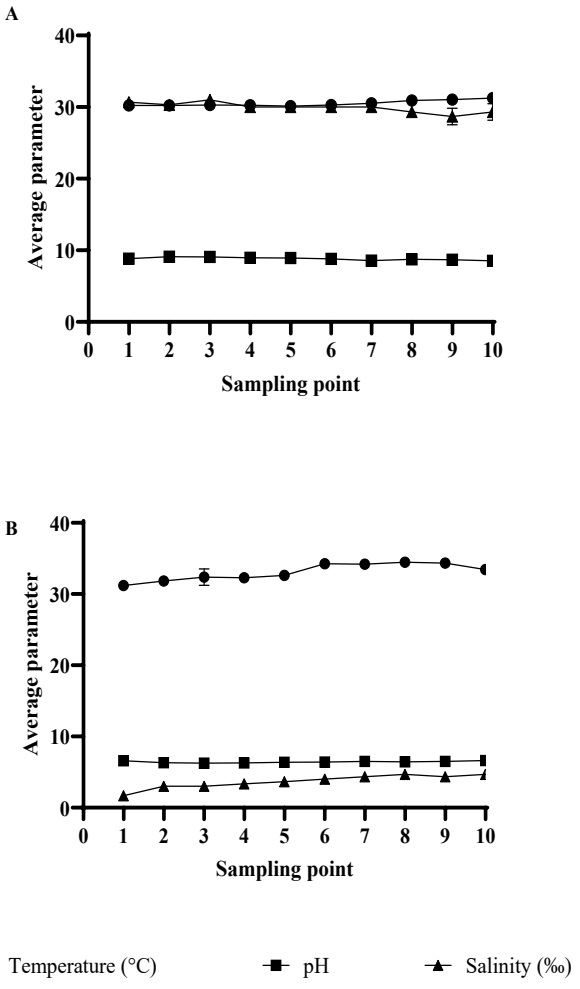

Figure 8: Physicochemical parameters at (A) Coco Cabana and (B) Kampung Limpaku Pinang.

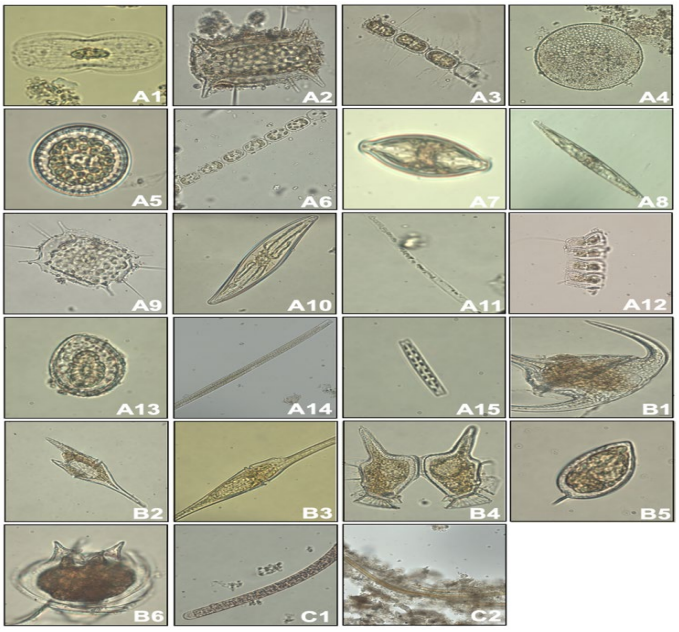

Figure 9: Phytoplankton genera observed in water samples collected from Coco Cabana and Kampung Limpaku Pinang. Diatom: (A1) *Amphiprora* spp.., (A2) *Biddulphia* spp.., (A3) *Chaetoceros* spp.., (A4) *Coscinodiscus* spp.., (A5) *Cyclotella* spp.., (A6) *Melosira* spp.., (A7) *Navicula* spp.., (A8) *Nitszchia* spp.., (A9) *Odontella* spp.., (A10) *Pleurosigma* spp.., (A11) *Proboscia* spp.., (A12) *Rhoicosphenia* spp.., (A13) *Surirella* spp.., (A14) *Synedra* spp.., (A15) *Thalassionema* spp..; Dinoflagellate: (B1) *Ceratium breve*, (B2) *Ceratium furca*, (B3) *Ceratium fusus*, (B4) *Dinophysis caudata*, (B5) *Prorocentrum* spp.., (B6) *Protoperidinium* spp..; *Cyanobacteria*: (C1) *Oscillatoria* spp.., (C2) *Planktothrix* spp.. Magnification: 600x.

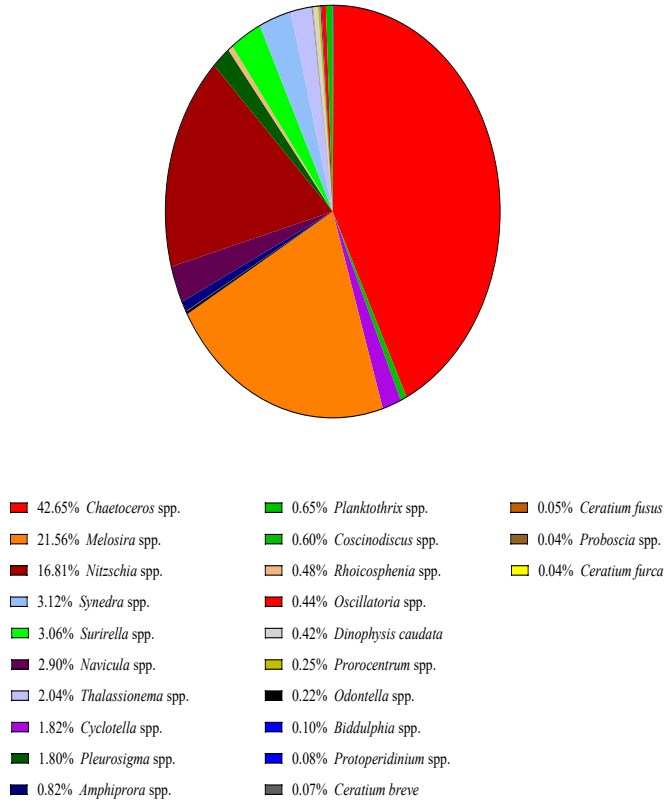

Figure 10: Phytoplankton genera observed at Coco Cabana.

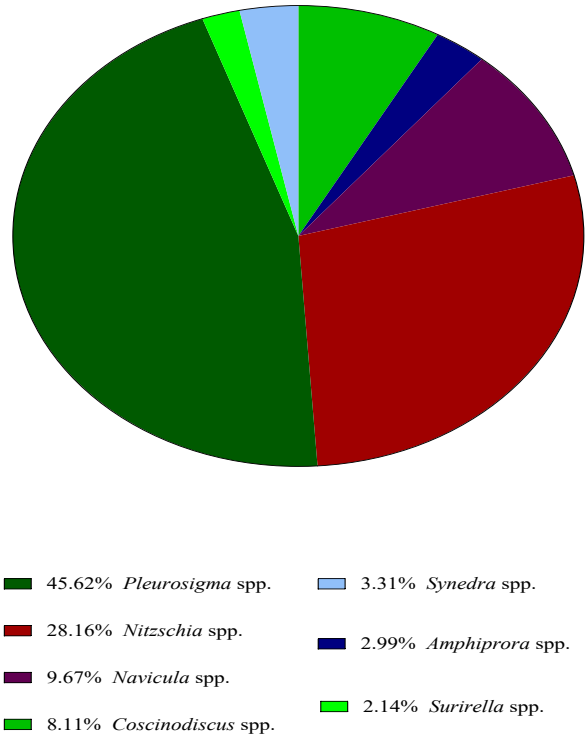

Figure 11: Phytoplankton genera observed at Kampung Limpaku Pinang.

3.6 Correlation between physicochemical parameters, *Vibrio* species and phytoplankton abundance

Overall, the mean of *Vibrio* species concentrations and temperature at Kampung Limpaku Pinang were slightly higher (mean: 9.13 ± 0.22 log CFU mL^{-1}, 33.10 ± 1.19°C) than at Coco Cabana (mean: 6.34 ± 1.58 log CFU mL^{-1} and 30.51 ± 0.41°C) (Table 2). However, the pH and salinity were substantially higher at Coco Cabana (pH 8.82 ± 0.20, 29.99 ± 0.58 ppt) than Kampung Limpaku Pinang (mean: pH 6.45 ± 0.11, 3.61 ± 0.96 ppt). A significant effect of sampling sites on *Vibrio* species, temperature, and salinity ($p<0.05$). The Tukey pairwise comparison test based on 95% confidence intervals showed that the *Vibrio* species and temperature were significantly higher at Kampung Limpaku Pinang, but pH and salinity were higher at Coco Cabana. However, weak correlation was observed on *Vibrio* abundance and salinity measured at both sampling locations.

Pearson's correlation analysis showed *Vibrio* concentrations at Coco Cabana were less correlated with temperature ($r = 0.30$, $p<0.05$), but weakly correlated with pH ($r = -0.27$, $p<0.05$) and salinity ($r = -0.21$, $p<0.05$) (Figure 12). However, at Kampung Limpaku Pinang, the concentration of *Vibrio* species was strongly correlated with temperature ($r = 0.85$, $p < 0.05$) and salinity ($r = 0.82$, $p<0.05$), but less correlated with pH ($r = 0.13$, $p<0.05$). A weak correlation was found between *Vibrio* concentrations and phytoplankton (r = -

0.45, p<0.05) at Coco Cabana and a stronger negative correlation in Kampung Limpaku Pinang (r = -0.73, p<0.05). Additionally, phytoplankton cells abundance was inversely correlated with water temperature, as shown at Coco Cabana (r = -0.45, p<0.05) and Kampung Limpaku Pinang (r = -0.73, p<0.05). The highest correlation at Coco Cabana was observed between phytoplankton and pH (r = 0.80, p<0.05)), while a moderate correlation was found at Kampung Limpaku Pinang (r = 0.38, p<0.05). Salinity was weakly correlated with phytoplankton at Coco Cabana (r = 0.38, p<0.05) and inversely correlated at Kampung Limpaku Pinang (r = -0.80, p<0.05).

Table 2: Means and standard deviations of Vibrio concentrations, physicochemical parameters, and phytoplankton abundance.

Sampling site	Parameter	Mean ± Standard deviation (SE)
Coco Cabana, Miri	Vibrio concentrations (log CFU mL^{-1})	6.34 ± 1.58
	Temperature (°C)	30.51 ± 0.41
	pH	8.82 ± 0.20
	Salinity (ppt)	29.99 ± 0.58
	Phytoplankton abundance (cells mL^{-1})	14198 ± 10686
Kampung Limpaku Pinang, Limbang	Vibrio concentrations (log CFU mL^{-1})	9.13 ± 0.22
	Temperature (°C)	33.10 ± 1.19
	pH	6.45 ± 0.11
	Salinity (ppt)	3.61 ± 0.96
	Phytoplankton abundance (cells mL^{-1})	242.2 ± 64.86

4. DISCUSSION

The concentration of *Vibrio* species was significantly higher in Kampung Limpaku Pinang compared to Coco Cabana. This difference is likely influenced by environmental and anthropogenic factors, such as the proximity to river mouths (Gangwar et al., 2023), the level of human activity, and varying hydrodynamic conditions (Kopprio et al., 2020). These findings are particularly significant given the site's history of cholera outbreaks (Benjamin et al., 2005; Nillian et al., 2018), suggesting that environmental persistence, rather than episodic contamination, may underpin long-term public health risk. Specifically, *V. cholerae* accounted for 76.7% of the isolates, suggesting a persistent presence of this pathogen in the region. Environmental factors such as high temperatures (31.2 – 34.5 °C) and low salinity (1.7 – 4.7 ppt) provide favourable conditions for *V. cholerae* proliferation (Rahman et al., 2018). Additionally, inadequate sanitation, poor waste disposal and insufficient water treatment exacerbate microbial hazards in estuarine waters used for domestic purposes and fisheries (Maheshwari et al., 2011; Patrick et al., 2012; Bilung et al., 2014; Pande et al., 2018). The increase in *Vibrio* concentrations could also be attributed to elevated nutrient levels, distinct microbiome composition, or increased and heavy rainfall (Jutla et al., 2013; Lutz et al., 2013).

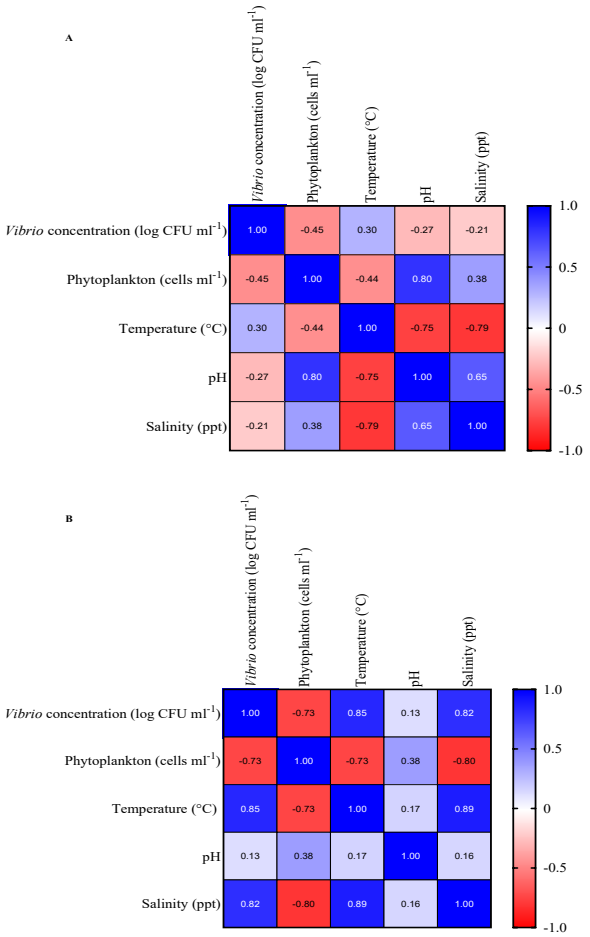

Figure 12: Pearson's correlation analysis of Vibrio concentrations, physicochemical parameters and phytoplankton at (A) Coco Cabana and (B) Kampung Limpaku Pinang.

Temperature emerged as a key driver of *Vibrio* abundance in the estuarine environment (Deeb et al., 2018). The results revealed a strong positive correlation between *Vibrio* concentration and temperature in Kampung Limpaku Pinang, where the temperature ranged from 31.2 to 34.5 °C, falling within the optimal range for *Vibrio* growth (Abdalla et al., 2022; Sheikh et al., 2022). Given Malaysia's consistently high temperatures throughout the year, with a mean annual temperature of 26.4 °C and minimal monthly variation, this finding highlights the role of warm temperatures in promoting *Vibrio* growth in tropical estuaries (León Robles et al., 2013; Canellas et al., 2021; Brumfield et al., 2023). The weaker temperature relationship observed at Coco Cabana suggests that effective environmental management and stable hydrodynamic conditions may partially buffer temperature-driven microbial risks.

Salinity played a crucial role in the prevalence of *Vibrio* species in the estuarine environment. Salinity in estuaries typically ranges from 0.5 to 35 ppt, influenced by

freshwater inflows, tidal movements, and geographic (Mahamood et al., 2024). At Coco Cabana, salinity levels ranged from 28.7 - 31.0 ppt, creating favourable conditions for V. parahaemolyticus, which thrives in high salinity environments (Johnson, 2015; Fleischmann et al., 2022). In contrast, the lower salinity levels at Kampung Limpaku Pinang (1.7 – 4.7 ppt) supported higher concentrations of *V. cholerae*, consistent with studies indicating *V. cholerae* flourishes in low salinity environments (Grant et al., 2015; Racault et al., 2019). The occurrence of *V. cholerae* and *V. parahaemolyticus* at different salinities is further supported by research that stated salinity acts as an environmental filter, selecting for specific *Vibrio* species based on their halophilic characteristics (Posada-Izquierdo et al., 2021). Changes in freshwater inflow due to climate variability may shift *Vibrio* community composition and associated health risks rather than increasing or decreasing overall abundance.

Vibrio species can survive across a wide pH range, from acidic to alkaline conditions, typically between 5 and 10 (Velez et al., 2023), and Costa et al. (2010) stated that the optimal growth of *Vibrio* falls between pH 8.4 and 8.6. In this study, the mean pH observed at Coco Cabana and Kampung Limpaku Pinang was 8.82 and 6.45, respectively. However, no significant correlation was observed between *Vibrio* concentrations and pH, possibly due to the relatively stable pH across the sampling points, given their proximity. Similarly, Smalls et al. (2024) found no significant correlation between pH and *Vibrio* concentrations due to limited pH variation across sampling sites, with other environmental factors such as temperature and salinity playing a more prominent role in influencing *Vibrio* populations at Maryland Coastal Bays.

Seasonal factors may also influence the distribution and concentration of *Vibrio* species. Although this study was conducted during the dry season, other research in similar environments have shown that *Vibrio* concentrations generally increase during warmer months, coinciding with elevated water temperatures and decreased salinity (Gonzalez et al., 2014; Di et al., 2017; Diner et al., 2021). This seasonal variation is further supported by studies in North Carolina estuaries, which reported higher *Vibrio* prevalence following seasonal changes that correlated with temperature and salinity shift (Pfeffer et al., 2003). While seasonal data were not collected in this study, these findings suggest that future investigations in Northern Sarawak estuaries should account for seasonal variability to gain a broader understanding of *Vibrio* dynamics.

Phytoplankton and *Vibrio* interactions further revealed non-linear and site-specific patterns. The pronounced negative relationship between phytoplankton and *Vibrio* concentrations, particularly at Kampung Limpaku Pinang, indicates competitive or inhibitory mechanisms that regulate microbial populations in tropical estuaries (Sharifah & Eguchi, 2011). This contrasts with the commonly reported positive association between *Vibrio* and phytoplankton in shaping microbial dynamics. The dominance of Chaetoceros spp. at Coco Cabana and a negative association with *Vibrio* abundance provides evidence that certain diatom assemblages exert a suppressive effect on *Vibrio* in estuarine systems (Rehnstam-Holm et al., 2010).

The detection of Cyanobacteria at Coco Cabana at low concentrations underscores the influence of water clarity and sediment dynamics on microbial and phytoplankton interactions. Given the role of Cyanobacteria as reservoirs of *Vibrio* species (Jesser & Noble, 2018), the absence at Kampung Limpaku Pinang partly explains the contrasting relationship between *Vibrio* and phytoplankton observed between sites. These findings highlight the importance of turbidity and sediment resuspension as indirect regulators of microbial risk in tropical estuaries (Bargu et al., 2023).

The absence of virulence genes (*ctxA* and *tdh*) suggests that the *Vibrio* isolates may be non-toxigenic strains, as not all *V. cholerae* and *V. parahaemolyticus* strains carry the genes responsible for producing the toxins (Castillo et al., 2018; Meyer et al., 2024). Moreover, the targeted *ctxA* and *tdh* genes may be absent in the *Vibrio* strains due to genetic variations or deletions (Jiang et al., 2003; Zhang et al., 2018). These results suggest that while *V. cholerae* and *V. parahaemolyticus* were present in the environment, they did not possess the specific virulence factors associated with severe disease, as determined by the absence of *ctxA* and *tdh* genes. The absence of these genes implies that the isolates were less likely to cause severe diarrheal diseases typically associated with pathogenic *Vibrio* species (Raghunath, 2014; Gobarah et al., 2022). Therefore, continuous environmental surveillance, particularly in estuaries supporting fisheries, recreation, and coastal livelihood remains essential for minimizing potential public health risks.

5. CONCLUSION

This study provides baseline evidence that *Vibrio* is widespread in northern Sarawak estuaries, with consistently higher concentrations in Kampung Limpaku Pinang than in Coco Cabana. PCR confirmed the presence of *V. cholerae* and V. parahaemolyticus, but no *ctxA* and *tdh* genes were detected, suggesting the isolates examined were non-toxigenic. Temperature emerged as the strongest predictor of *Vibrio* abundance, particularly in Kampung Limpaku Pinang, while salinity acted as an important environmental filter

shaping species patterns, with higher salinity favouring *V. parahaemolyticus* at Coco Cabana and low salinity supporting *V. cholerae* dominance at Kampung Limpaku Pinang. Although pH showed no direct relationship with *Vibrio*, it was positively associated with phytoplankton abundance. Across both sites, *Vibrio* concentrations were inversely related to phytoplankton, indicating potential inhibitory or competitive interactions, with diatom dominance (notably Chaetoceros spp.) potentially contributing to this pattern. Overall, these findings highlight how physicochemical conditions and plankton community structure jointly regulate *Vibrio* dynamics in tropical estuaries, providing a foundation for future monitoring and risk assessment under climate-driven shifts in temperature, salinity, and phytoplankton abundance.

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