Faculty of Resource Science and Technology

Environmental Factors Influencing the Association of *Vibrio* Species and Phytoplankton in Northern Sarawak

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

The genus *Vibrio* is commonly found in aquatic environments such as rivers, estuaries and coastal areas, where they thrive under favorable conditions like warm temperatures, moderate salinity and their attachment to planktonic organisms, for example, phytoplankton during algal bloom. Pathogenic strains such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, pose significant health risks, leading to illnesses ranging from gastroenteritis to septicemia. Phytoplankton blooms create nutrient-rich conditions that enhance *Vibrio* growth and persistence in the aquatic ecosystems. The estuary in northern Sarawak offers an ideal environment for studying the interaction between *Vibrio* species and phytoplankton. Despite numerous reports of *Vibrio*-related outbreaks globally, limited studies have been conducted on the occurrence and association of *Vibrio* species with phytoplankton in this region. The aim of this study is to examine the relationship between environmental parameters and the occurrence of *Vibrio* species and phytoplankton in the estuarine of northern Sarawak. Water samples were collected from two estuaries, Coco Cabana, Miri and Kampung Limpaku Pinang, and environmental parameters were recorded *in-situ* at the study sites. Phytoplankton abundance was measured microscopically, while bacterial isolation was performed using selective media (TCBS agar). Molecular identification of *Vibrio* species was conducted using PCR amplification of species-specific genes (*OmpW* for *V. cholerae* and *ToxR* for *V. parahaemolyticus*). In this study, PCR analysis revealed that 36.7% (22/60) samples were tested positive for *V. cholerae*, as evidenced by the detection of *OmpW* gene (588 bp). The *OmpW* gene encodes an outer membrane protein that plays a crucial role in the structure integrity and environmental adaptability of *V. cholerae*. Furthermore, 31.7% (19/60) of the samples collected from both estuaries were positive for *V. parahaemolyticus*, confirmed by the presence of the *ToxR* gene

(368 bp) that encodes the regulatory protein that governs the expression of virulence factors essential for the pathogenicity in aquatic environments and during human infection. Statistical analysis one-way ANOVA revealed significant correlation ($p < 0.05$) between *Vibrio* abundance, water temperature and salinity, but not pH. Pearson's correlation analysis showed a negative relationship ($r = -0.45$ and -0.73) between the concentration of *Vibrio* species and phytoplankton in both sampling sites. This suggests that the increase in *Vibrio* species abundance heightens the competition for resources, resulting in a reduction in phytoplankton density. The analysis also revealed that phytoplankton was influenced by temperature and salinity. Elevated temperatures led to a decrease in phytoplankton count, indicating that high temperature can negatively affect their growth and abundance. Furthermore, the measured high salinity reduced phytoplankton abundance, possibly due to osmotic stress, thereby further influencing interactions with *Vibrio* species. This result will offer understandings to the potential public health risks posed by *Vibrio* species in estuarine ecosystems and contribute to the understanding of their ecological dynamics in estuarine environments in northern Sarawak, Malaysia.

Keywords: Association, environmental parameters, phytoplankton, *Vibrio cholerae*, *Vibrio parahaemolyticus*

Faktor Persekitaran yang Mempengaruhi Hubungan Spesies Vibrio dan Fitoplankton di Sarawak Utara

ABSTRAK

Genus Vibrio *terdapat di persekitaran akuatik, seperti sungai, muara sungai, dan persisiran pantai, di mana ia membiak dalam persekitaran yang sesuai seperti suhu suam, kemasinan sederhana, dan penggantungan pada organisma planktonic, contohnya, fitoplankton semasa blum alga. Strain yang patogenik seperti* Vibrio cholerae*,* Vibrio parahaemolyticus*, dan* Vibrio vulnificus *boleh menyebabkan masalah kesihatan yang serius, seperti penyakit gastroenteritis dan sepsis. Blum fitoplankton meningkatkan kadar nutrien dalam air sekaligus meningkatkan pembiakan dan ketahanan* Vibrio *dalam ekosistem akuatik. Muara sungai di utara Sarawak memberikan persekitaran yang ideal untuk mengkaji interaksi antara spesies* Vibrio *dan fitoplankton. Walaupun terdapat banyak laporan mengenai wabak berkaitan Vibrio di peringkat global, kajian mengenai kewujudan dan hubungan spesies* Vibrio *dengan fitoplankton di rantau ini adalah terhad. Kajian ini bertujuan untuk menyelidiki hubungan antara parameter persekitaran seperti suhu, pH, dan saliniti dengan kehadiran spesies* Vibrio *serta fitoplankton di muara sungai utara Sarawak. Sampel air diambil dari dua muara sungai iaitu Coco Cabana, Miri dan Kampung Limpaku Pinang, Limbang dan parameter fizikokimia diambil secara* in-situ *di tempat kajian.Ketumpatan fitoplankton dikira secara mikroskopik, manakala pengasingan bakteria dilakukan dengan menggunakan media selektif (agar TCBS). Pengenalpastian molekul spesies* Vibrio *dijalankan melalui kaedah PCR dengan menggunakan gen spesifik spesies (OmpW untuk* V. cholerae *dan ToxR untuk* V. parahaemolyticus*). Dalam kajian ini, analisis PCR mendedahkan bahawa sebanyak 36.7% (22/60) sampel diuji positif untuk* V. cholerae *melalui pengesanan gen OmpW (588 bp). Gen OmpW mengekod protein membran luar yang*

memainkan peranan penting dalam intergiti struktur dan adaptasi persekitaran V. Cholerae*. Selain itu, 31.7% (19/60) sampel yang dikumpul dari kedua-dua muara adalah positif untuk* V. parahaemolyticus*, disahkan melalui kehadiran gen ToxR (368 bp) yang mengekod protin yang mengawal faktor-faktor virulen penting bagi patogenisiti dalam persekitaran akuatik dan semasa jangkitan terhadap manusia. Analisis statistik ANOVA sehala menunjukkan hubungan yang signifikan (p < 0.05) antara kuantiti* Vibrio*, suhu air, dan saliniti, tetapi tiada kolerasi dengan pH. Analisis "*Pearson's correlation*" menunjukkan hubungan negatif (r = -0.45 dan -0.73) antara kuantiti Vibrio dan fitoplankton di kedua-dua lokasi pensampelan. Ini mencadangkankan bahawa peningkatan dalam kuantiti* Vibrio *meningkatkan persaingan untuk sumber keperluan, yang membawa kepada pengurangan ketumpatan fitoplankton. Analisis ini juga menunjukkan bahawa ketumpatan fitoplankton dipengaruhi oleh suhu dan saliniti. Suhu yang lebih tinggi menyebabkan penurunan bilangan fitoplankton, menunjukkan bahawa suhu yang tinggi boleh memberi kesan negatif kepada pertumbuhan dan kelimpahan fitoplankton. Selain itu, saliniti yang tinggi turut mengurangkan kepadatan fitoplankton, disebabkan oleh tekanan osmosis, yang seterusnya menpengaruhi interaksi dengan spesies* Vibrio*. Penemuan ini memberikan pemahaman mengenai potensi risiko kepada kesihatan orang awam yang ditimbulkan oleh spesies* Vibrio *dan menyumbang kepada pemahaman tentang dinamik ekologi spesies* Vibrio *dalam persekitaran muara sungai di utara Sarawak, Malaysia.*

Kata kunci: *Fitoplankton, hubungan, parameter persekitaran,* Vibrio cholerae, Vibrio parahaemolyticus

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LIST OF ABBREVIATIONS

HABs	Harmful algal blooms
WHO	World Health Organization
CO_2	Carbon dioxide
DOC	Dissolved organic carbon
PBS	Phosphate buffer solution
APW	Alkaline peptone water
NaCl	Sodium chloride
TCBS	Thiosulphate-citrate-bile salts-sucrose
CFU	Colony forming unit
PCR	Polymerase chain reaction
AGE	Agarose gel electrophoresis
DNA	Deoxyribonucleic acid

CHAPTER 1

INTRODUCTION

1.1 Study Background

The genus *Vibrio* is a Gram-negative bacteria that belong to the family *Vibrionaceae*, 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, with a size between 0.5 and 0.8 μm in width and 2 to 3 μm in length. Cells are comma-shaped, varying from thin and elongated structures to 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, where at least 12 species have been associated with food borne diseases and are harmful to humans (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 population proliferates in warm water with moderate range of salinity and decreased 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 (Di et al., 2017). In recent years, the emergence of *Vibrio* species as major microbiological agents has drawn curiosity due to their role in causing a variety of ailments, ranging from mild gastroenteritis to serious systemic infections.

Among other bacteria, *Vibrio* species exhibit the most rapid growth rates, with proliferation strongly influenced by 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 outbreak globally. The estuarine ecosystem, serving as its reservoir, potentially contributes to the persistence and transport of pathogenic strains in the environment (Ford et al., 2020). *Vibrio parahaemolyticus* is associated with food-borne diseases, which cause gastroenteritis from consuming contaminated seafood (Venggadasamy et al., 2021). Illness caused by *Vibrio vulnificus* can lead to septicemia and serious wound infection, resulting in a high mortality rate among susceptible individuals (Coerdt & Khachemoune, 2021).

Previous studies have shown that the abundance of *Vibrio* species is influenced by temperature, salinity, phytoplankton biomass and eutrophication (Fernández-Juárez et al., 2024). Elevated temperatures and salinity levels facilitate *Vibrio* growth in aquatic environments, while algal cells enhance their persistence during bloom events. *Vibrio* often attach to algal cells and detritus, showing a preference for these surfaces over other suspended particles (Main et al., 2015). This attachment occurs either as free-living cells or as biofilms, where large populations persist on substrates such as slit, faecal matter, and sediment (Brumfield et al., 2023). Phytoplankton blooms release dissolved and particulate nutrients that support high *Vibrio* concentrations, while aggregated microalgae provide extensive surfaces colonization. The global increase in harmful algal blooms (HABs) has coincided with rising *Vibrio* populations, underscoring their close ecological relationship

(Sampaio et al., 2022). Understanding these global patterns is essential for assessing the ecological and health risks associated with *Vibrio* in Sarawak's aquatic environments.

These interactions are particularly relevant to northern Sarawak, where diverse aquatic ecosystems include coastal waters, rivers, and estuaries. Miri, the state's second largest city, and Limbang, near the Brunei border, rely on these habitats for fisheries and other resources. The region's dynamics conditions provide a suitable setting to investigate *Vibrio* diversity and abundance. However, the presence of *Vibrio* also poses health risks. In March 2011, Limbang reported 111 gastroenteritis cases linked to Vibrio, including two severe cases involving vulnerable patients (Veno, 2011; Elexson et al., 2023). Such outbreaks highlight the potential threat of seafood-related infections in northern Sarawak.

Despite frequent reports on the *Vibrio* species and their outbreaks, there are limited studies on the presence of *Vibrio* species in phytoplankton in Sarawak. Hence, the aim of this study is to determine the association of environmental parameters with the presence of *Vibrio* species and phytoplankton in estuarine environment in northern Sarawak.

1.2 Problem Statement

Vibrio species, known for their fast proliferation and association with variety range of environmental conditions, have been identified as significant contributors to foodborne diseases and serious infection worldwide. While extensive research has demonstrated the relationship between *Vibrio* species and environmental parameters such as temperature, pH, and salinity, there is limited information regarding their association with phytoplankton in estuaries environments of Sarawak, Malaysia. Northern Sarawak's aquatic ecosystems, which support local fisheries and are vital to the region's economy, face potential risks from

the occurrence of *Vibrio* species, especially during phytoplankton blooms that may promote *Vibrio* growth.

Despite the global concern about the infection of *Vibrio* species in human health and disease, there is notable gap in understanding how these bacteria interact with phytoplankton under local environmental conditions in this region. This study aims to address the gap of knowledge by investigating the association between *Vibrio* species, phytoplankton, and environmental factors in the estuarine environment of northern Sarawak, providing insights into potential public health.

1.3 Objectives

This study aims to provide a comprehensive evaluation of the association between *Vibrio* species and phytoplankton in northern Sarawak. To achieve these goals, the following objectives have been established:

i. To investigate the distribution and concentration of *Vibrio* species presence in northern Sarawak, mainly in Miri and Limbang.

ii. To analyse the physicochemical parameters that influence the presence of *Vibrio* species and phytoplankton.

iii. To identify phytoplankton species that are associated with variations of *Vibrio* species concentration in estuarine waters.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

The association between pathogenic *Vibrio* species and phytoplankton is a subject of increasing interest due to their implications for marine ecology, public health, and environmental management. Pathogenic *Vibrio* species, for instance, *V. cholerae*, and *V. parahaemolyticus*, are ubiquitous in aquatic environments that can pose serious health risks to humans and wildlife, especially estuarine and coastal areas where phytoplankton blooms frequently occur. Studies by Greenfield et al. (2017) and Qiao et al. (2020) showed that phytoplankton can promote the growth and survival of *Vibrio* bacteria, resulting in an increase of public health risks associated with seafood consumption and water exposure.

Understanding these associations is vital for developing effective monitoring and management strategies aimed at mitigating the risks posed by pathogenic *Vibrio* species and phytoplankton. This literature review explores the link between pathogenic *Vibrio* and phytoplankton in current research findings, focusing on environmental factors driving their association and the potential impacts on public health, thereby highlighting the need for integrated approaches to address these interconnected challenges in aquatic ecosystems.

2.2 Pathogenic *Vibrio* species

The genus *Vibrio* is a Gram-negative bacterium distinguished by the presence of lipopolysaccharide in its outer membrane and is classified under the phylum *Proteobacteria*. It belongs to the class *Gammaproteobacteria*, recognized as the most diverse class of Gram-negative bacteria (Soto, 2022). Within the order *Vibrionales*, the *Vibrionaceae* family

comprises aquatic bacteria that predominantly inhabit warm waters and can adapt to varying salinity levels, including freshwater, brackish, and marine environments (Banchi et al., 2022; Sampaio et al., 2022). Members of the *Vibrionaceae* family represent a phylogenetically, genomically, and functionally diverse group of curved rod-shaped motile bacteria, primarily exhibiting polar flagellation, which is essential for their motility and virulence (Echazarreta & Klose, 2019). *V. cholerae* possesses a single polar flagellum, whereas other species such as *V. parahaemolyticus* and *V. alginolyticus* display both a single polar flagellum and thin flagella extending in all directions (Khan et al., 2020). The genus *Vibrio* encompasses the largest number of species, with 147 species and 4 subspecies currently recognized, of which fewer than a dozen are pathogenic to humans (Sampaio et al., 2022). Pathogenic *Vibrio* species are associated with environmental niches primarily influenced by temperature and salinity (Diner et al., 2021). Previous studies have shown that *Vibrio* species are frequently associated with aquatic invertebrates such as crustaceans, plankton, and bivalve molluscs (Destoumieux-Garzón et al., 2020). They often accumulate in copepods and filter-feeding bivalves like oysters, thereby increasing the risk of pathogen transmission (Diner et al., 2021). Although certain species infect marine animals including shrimps, fishes, and corals, the majority are non-pathogenic and may function as symbionts (Soto, 2022). Various *Vibrio* species are pathogenic, leading to infections through food consumption or in soft tissues. Strains that cause illness are often associated with gastroenteritis but can also infect open wounds and lead to sepsis. Notable species include *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, with infections typically resulting from the consumption of inadequately cooked seafood. Their pathogenic potential is significantly influenced by virulence factors, environmental adaptation, and global climate change. Recent analyses underscore the

widespread prevalence of pathogenic *Vibrio* species globally, highlighting their significance in both clinical and environmental contexts (Engku Abd Rahman et al., 2024).

2.2.1 Vibrio cholerae

V. cholerae, a Gram-negative bacterium with a single polar flagellum, is a significant enteric pathogen responsible for the diarrheal disease cholera (Khan et al., 2020). Without immediate treatment, this disease can cause a public health threat, leading to dehydration and potentially fatal outcomes, particularly in regions characterized by inadequate water, sanitation, and hygiene, overcrowding, and poorly developed urban settlements (Asantewaa et al., 2024). The transmission of this bacterium occurs either directly through the faecal-oral route or indirectly via contaminated food and water (Montero et al., 2023). *Vibrio cholerae* is one of the few *Vibrio* species capable of surviving in the absence of salt. Cholera is transmitted through freshwater resources, but its occurrence is restricted to regions where virulent strains are endemic (Daboul et al., 2020). Once *V. cholerae* cells enter and survive through the stomach's acidic environment, they secrete a toxin that acts on the host's epithelium cells, leading to the substantial efflux of ions and water into the intestinal lumen. The resulting excessive secretory diarrhoea can cause severe fluid depletion, which leads to circulatory collapse and potentially be fatal (Kessel & Camilli, 2024).

V. cholerae is classified into over 200 serogroups based on the structure of the O-antigen of lipopolysaccharide (LPS) (Brumfield et al., 2017). Among these, the O1 and O139 serogroups are responsible for producing cholera toxin that cause severe diarrhoea in humans (Ramamurthy et al., 2020). The non-O1 and non-O139 serogroups do not produce cholera toxin and are associated with minor gastroenteritis outbreaks, wound infections, and bacteraemia, but not cholera. Cholera toxin (CTX) is the major virulence factor in toxigenic *V. cholerae* strains. CTX is composed of a catalytic A subunit (CTX-A) and a pentameric

receptor-binding B subunit (CTX-B). This toxin induces the severe secretory diarrhoea that is the characteristic of cholera (Montero et al., 2023).

Cholera has been endemic in Sarawak since 1873, with major epidemics occurring periodically during the dry months of May, June, and July. These outbreaks predominantly affect coastal populations and are associated with poor sanitation, inadequate water supply, and improper waste disposal practices. The most severe epidemic recorded occurred in 1902, resulting in a total of 1,500 deaths due to severe drought that affected water sources (Yadav & Meng Chee, 1990). In 1999, cholera had spread from the Limbang District to Lawas District, causing an explosive epidemic for short duration due to the rapid transmission and high morbidity in the affected populations (Benjamin et al., 2005). Climate variability such as temperature and precipitation significantly influences cholera cases in Malaysia. Recent studies showed that Sarawak is accounted for approximately 6.5% of total cholera cases in Malaysia from 2000 to 2010. The study found that a 1°C increase in temperature could lead to a significant rise in cholera cases (Hassan et al., 2020). Beyond climate factors, outbreaks in Sarawak were also linked to the presence of virulent *V. cholerae* strains. The detection of CTX genes in *V. cholerae* isolated from an outbreak in Bintulu demonstrates the pathogenic potential of these strains (Bilung et al., 2014). Moreover, spatiotemporal analyses revealed clustering patterns of cholera cases within geographic areas, identifying regions at higher risk of outbreaks (Maluda et al., 2024).

2.2.2 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus, a halophilic bacterium is found in estuarine and marine environments. They can thrive at temperatures ranging from 5 °C to 43 °C, with optimal growth at 37 °C (Kehinde & Nakaguchi, 2021). Infections caused by *V. parahaemolyticus* were historically concentrated in Japan, however after the 1960s, cases have been reported

globally, particularly in the United States (Food and Agriculture Organization of the United Nations, 2021). *V. parahaemolyticus* has at least 13 O serogroups and 71 K serogroups. The most notable serotype, O3:K6 is associated with clinical cases, which was responsible for significant outbreaks in India and subsequent occurrences worldwide (Chen et al., 2017). The specific virulence markers, including the Kanagawa phenomenon (KP), helps to distinguish between pathogenic and non-pathogenic *V. parahaemolyticus* strains (Wang et al., 2015).

The pathogenicity of *V. parahaemolyticus* is primarily associated with several virulence factors, notably two hemolysins such as thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH). These toxins induce cytotoxic effects and are useful in the bacterium's ability to invade the host cells (Letchumanan et al., 2014). *Vibrio parahaemolyticus* also possesses type 3 secretion systems (T3SS1 and T3SS2), which facilitate the translocation of effector proteins into host cells, further enhancing its pathogenic potential (Rezny & Evans, 2023).

Vibriosis cases caused by *V. parahaemolyticus* are primarily linked to seafood consumption. *V. parahaemolyticus* is responsible for the bacterial gastroenteritis associated with seafood in the United States (Koutsoumanis et al., 2024). Outbreaks often occur during warmer months when environmental conditions are in favour for bacterial proliferation. The symptoms caused by vibriosis include fever, chills, abdominal cramps, diarrhea, nausea, and vomiting (Rezny & Evans, 2023). In addition to gastroenteritis, *V. parahaemolyticus* can also cause wound infections and septicemia, particularly in individuals with underlying health conditions (Letchumanan et al., 2014).

Gastroenteritis cases caused by *V. parahaemolyticus* have been reported in Malaysia, often associated with the consumption of contaminated seafood. Recent studies show that *V. parahaemolyticus* is the predominant species among the non-cholera *Vibrio* infections. From 2017 to 2020, out of 270 non-cholera *Vibrio* species isolated from patients, 57.8% were identified as *V. parahaemolyticus* (Hassan et al., 2023). This bacterium was consistently isolated from various seafood sources, including fish and shellfish, underscoring its role as a leading cause of foodborne gastroenteritis in the region (Loo et al., 2023). Additionally, a study focusing on shrimp farms revealed that *V. parahaemolyticus* was dominant among the isolated *Vibrio* species in Sarawak, and many isolates exhibited multi antibiotic resistance (Elexson et al., 2023). The environmental conditions in these areas, such as temperature and salinity, are conducive to the proliferation of this pathogen, further increasing the risk of vibriosis outbreaks.

2.3 Ecological Dynamics of *Vibrio* Species

Vibrio species are heterotrophic bacteria ubiquitous in aquatic environments, often found in association with phytoplankton. This genus occurs both as free-living organisms and within planktonic aggregates, reflecting their ecological versatility. Within these ecosystems, *Vibrios* play a crucial role in organic matter mineralization and nutrient recycling. The broad enzymatic capabilities allow *Vibrio* to degrade a wide range of substrate, including carbohydrates, proteins, lipids, and complex polymers such as starch, chitin, lignin, hydrocarbons, collagen, gelatine and alginate. These metabolic processes significantly contribute to carbon and nutrient cycling, underscoring their importance in aquatic biogeochemical dynamics (Sampaio et al., 2022). Moreover, *Vibrios* are capable of synthesizing polyunsaturated fatty acids, which is essential compounds for many aquatic organisms that cannot produce them independently (Estupiñán et al., 2020). As a food source

for flagellates, *Vibrios* further facilitate organic matter recycling. The abundance and diversity of *Vibrio* species have been shown to correlate closely with phytoplankton community composition. For example, studies in coastal waters have demonstrated significant associations between *Vibrio* concentrations and specific plankton taxa, such as copepods and diatoms, suggesting that phytoplankton serve not only as habitat but also as a nutrient reservoir for *Vibrio* populations (Diner et al., 2021).

Furthermore, the relationship between *Vibrio* species and phytoplankton can be influenced by environmental factors such as temperature, salinity and pH (Brumfield et al., 2023; Velez et al., 2023). For example, *Vibrio* abundance was found to correlate positively with temperature and plankton composition in oyster farming environments, suggesting that these factors play a crucial role in modulating *Vibrio* dynamics (Xu et al., 2020). Seasonal variations also affect these associations where higher temperatures tend to enhance *Vibrio* abundance, particularly when certain phytoplankton taxa are prevalent (Cruz et al., 2020).

2.4 Association of pathogenic *Vibrio* with Environmental Factors

Vibriosis outbreaks are caused by the changes in the water physicochemical properties (Purgar et al., 2022). Environmental factors significantly influence the growth and distribution of *Vibrio* species in aquatic environment. These factors include temperature, salinity, pH and nutrient availability. Changes in these factors create conditions that can either promote or inhibit *Vibrio* proliferation in the environment.

2.4.1 Temperature

Temperature is widely recognized as a major determinant controlling the distribution and abundance of pathogenic *Vibrio* species. Numerous studies have demonstrated that high water temperatures enhance bacterial growth. For instance, research in Chesapeake Bay

revealed that *V. parahaemolyticus* and *V. vulnificus* showed increased numbers at temperatures above 15 °C, with maximum counts occurring at temperatures exceeding 25 °C (Brumfield et al., 2023). Similarly, temperature strongly influences the frequency of *Vibrio* isolation in freshwater and temperate environments (Abioye et al., 2021). In contrast, within equatorial and tropical aquatic ecosystems, where seasonal variation in temperature is minimal, the relationship between *Vibrio* abundance and temperature is often weak or absent. In such environments, salinity emerges as a primary factor shaping *Vibrio* distribution and population dynamics. In Hawaiian waters, where temperatures remain consistently warm, rainfall-driven salinity variation is the primary factor regulating *V. vulnificus* abundance, responsible for most of the observed monthly variation (Nigro et al., 2022). Nonetheless, warmer waters generally promote *Vibrio* persistence, thereby increasing bacterial concentrations and the risk of human exposure. This pattern is of particular concern under global climate change scenarios, which are projected to increase both the frequency and geographic range of *Vibrio* associated diseases.

2.4.2 pH

Among these factors, pH plays a critical role in the growth, survival, and pathogenicity of *Vibrio* species. Research indicates that non-cholera *Vibrio* species can thrive across a broad range of pH, typically from 5 to 10, with optimal growth conditions often found at neutral to slightly alkaline pH levels (7.5 to 8.5) (Gundogdu et al., 2023). For instance, a study examining the planktonic and biofilm growth of *V. parahaemolyticus* and *V. vulnificus* demonstrated significant variations in growth rates depending on pH levels, emphasizing the need to incorporate pH into predictive models for *Vibrio* risk assessment (Velez et al., 2023). Environmental changes, particularly related to climate change can lead to fluctuations in pH levels in coastal waters. Ocean acidification due to increased

atmospheric carbon dioxide (CO_2) is projected to lower oceanic pH significantly, potentially impacting *Vibrio* populations by shifting their optimal growth conditions (Gundogdu et al., 2023). Conversely, events such as harmful algal blooms (HABs) can elevate local pH levels due to photosynthetic activity, creating favorable conditions for *Vibrio* proliferation in the aquatic environment (Brumfield et al., 2023). As phytoplankton blooms increase pH through photosynthesis, they may simultaneously create an environment conducive for *Vibrio* growth (Velez et al., 2023).

2.4.3 Salinity

Most *Vibrio* species are halophilic, thriving best in brackish waters where freshwater and seawater mix. Estuarine environments, characterized by dynamic salinity gradients, provide favourable conditions for *Vibrio* growth. The optimal salinity ranges between 10 to 15 parts per thousand (ppt). *V. cholerae* is commonly found in low salinity waters with high temperatures and high concentrations of organic nutrients. Variations in salinity exert a strong influence on *Vibrio* populations, with higher salinities often supporting greater abundance (Baker-Austin et al., 2018; Brumfield et al., 2023). For example, in Vembanad Lake, India, *V. cholerae* was detected across a wide salinity range of 1-32 psu, although higher values exceeded the optimal growth conditions typically reported (Anas et al., 2021). Similarly, in British coastal waters, *V. cholerae* populations increased substantially during higher temperature conditions and at lower salinity sites (Ford et al., 2020).

Species-specific responses are also evident. *V. vulnificus* grows optimally at 10-15 ppt, with increased abundance reported at 20-25 ppt (Baker-Austin & Oliver, 2020). However, salinities above 10 ppt generally suppress growth, indicating an upper threshold for persistence. Salinity fluctuations are driven by freshwater inflow, precipitation, and anthropogenic inputs. Reduced rainfall and high evaporation during dry seasons elevate

salinity, often reducing *Vibrio* abundance, whereas monsoon events or riverine inputs lower salinity and enhance growth (Gangwar et al., 2023).

Salinity further interacts with temperature and nutrient availability. Higher temperatures combined with moderate salinity promote *v. parahaemolyticus* proliferation (Brumfield et al., 2023). Seasonal transitions highlight this interaction, as *Vibrio* populations typically increase when both salinity and temperature fall within optimal ranges.

2.5 Occurrence of *Vibrio* outbreaks in Malaysia

Cholera remains a significant public health challenge, with its distribution heavily influenced by socio-economic factors. In recent years, natural catastrophe, droughts, floods and conflicts have forced millions of people to relocate to areas with limited access to clean water, poor sewage management, and increasing disease risks, which has worsened the global impact of cholera. At present time, 1 billion people are at risk of contracting cholera, 24 countries with active outbreaks, and 28 countries with outbreaks in 2023 reported by the World Health Organization (WHO) (Memish et al., 2024). The countries in Sub-Saharan Africa have the highest incidence rates of cholera, though cases are also reported in Southeast Asia (World Health Organization, 2024). In Malaysia, the cholera outbreaks are mainly caused by *V. cholerae O1 El Tor* occurs periodically, with infections by O139 sporadically. In historical aspect, the outbreaks occurred in the states of Sarawak, Kelantan and Terengganu.

A significant outbreak of cholera occurred in Terengganu in November 2009. The outbreak started in the capital of Kuala Terengganu and spread to several districts within seven days. Several eateries such as street food vendors and restaurants, two fish cracker factories and five ice factories were ordered to halt their business operations because they

were suspected of being the possible sources of transmission and cholera infection. Two variants of *V. cholerae* resistant to common antimicrobial drugs were identified during the outbreak. This outbreak has resulted in 187 confirmed cases and one death reported by the local health authorities (Teh et al., 2012).

In Sabah, a total of 2,865 cases of cholera were confirmed from 2005 to 2020. In 2015, 705 symptomatic cholera cases were reported, with almost all *V. cholerae* detected belonged to serogroup O1 El Tor. The seasonal occurrence of cholera cases was highest between June and September, with a peak in July. Asymptomatic cholera data reported a total of 727 cases were detected from 2015 onwards. Majority of the cases were classified as Malaysian, followed by Filipinos, Indonesian and Sea Gypsies. The cases were concentrated in specific time periods and locations, with several districts experiencing repeat outbreaks, especially in rural coastal areas such as Kunak, Lahad Datu, Tawau and Semporna. These districts are higher in population movement leading to undocumented immigration, resulting to the persistence of cholera and difficulty in managing the outbreaks in the area (Maluda et al., 2024).

Research conducted between 2014 and 2020 revealed that non-cholera *Vibrio* species are prevalent in Malaysian waters, with *V. parahaemolyticus* being most frequently isolated species from clinical cases. The study reported a total of 270 non-cholera *Vibrio* isolates from patients, predominantly from stool samples, indicating a significant public health concern related to seafood consumption. Emerging species such as *V. fluvialis* have also been noted for their increasing prevalence in clinical settings.

The occurrence of *Vibrio* species is not limited to human infections; they also pose threats to aquaculture. For instance, studies on giant tiger shrimp have identified multiple virulent

strains of *Vibrio*, underscoring the need for monitoring these pathogens within aquaculture systems to prevent economic losses.

2.6 Phytoplankton Genera in the Environment

Phytoplankton are autotrophic, self-feeding components of plankton communities that play a key role in both freshwater and ocean ecosystems. The name "phytoplankton" comes from the Greek words *phyto* (plant) and *plankton* (made to drift or wander). They are microscopic organisms that live in freshwater, brackish and marine environments. Phytoplankton are the base of aquatic food webs, providing food for a wide range of sea creatures.

In estuarine ecosystems, phytoplankton exhibit rich taxonomic diversity, typically spanning diatoms, cyanobacteria, and dinoflagellates. These communities are shaped by the strong gradients in salinity, nutrients, and turbidity that characterize transitional waters. Recent studies of estuaries demonstrate high diversity, with diatoms frequently comprising majority of the taxa, followed by cyanobacteria and dinoflagellates, while chlorophytes and cryptophytes are more prominent in riverine or low salinity zones (Gong et al., 2020; Niveditha et al., 2022). Such variability reflects the capacity of different genera to adapt to fluctuating conditions at the freshwater-marine environment.

Environmental drivers, particularly nutrient availability, temperature, and hydrodynamics strongly influence the distribution and seasonal succession of phytoplankton genera. In tropical estuaries, chlorophytes and cyanobacteria dominate in turbid, nutrient-rich freshwater inputs, whereas diatoms and dinoflagellates increase in abundance under higher salinities and high temperatures (Elferink et al., 2020; Nwankwegu et al., 2020). Seasonal changes linked to monsoonal rainfall, river discharge, and tidal mixing further shape community composition by altering flushing rates, and nutrient regimes (Cereja et al.,

2021; Zainol et al., 2020). These dynamics highlight the close coupling between estuarine phytoplankton and physicochemical variability. Understanding the interactions between phytoplankton genera and estuarine processes is essential, as these organisms underpin primary production, nutrient cycling, and food-web stability. Against this backdrop, diatoms, dinoflagellates, and cyanobacteria are of particular importance due to their ecological dominance, bloom-forming potential, and associations with bacterial communities such as *Vibrio*.

2.6.1 Diatoms

Diatoms are unicellular or colonial photoautotrophic microalgae characterized by their silica-based cell wall, known as frustule. Their size can range from 5 µm to over 1 mm in diameter or length. While many species occur as solitary cells, others form chains or colonies. Diatoms occupy diverse environments, including freshwater, marine, and extreme habitats such as sea ice and deep sediments. In coastal and estuarine ecosystems, they often dominate other microalgal groups and cyanobacteria due to their adaptive strategies and rapid growth rates. They are also the dominant group in microphytobenthos, which are biofilm-forming communities of microalgae and cyanobacteria that inhabiting intertidal flats and shallow sediments (Serôdio & Lavaud, 2020).

The distribution and seasonal dynamics of diatoms in estuaries are regulated by silica and nitrogen inputs, light penetration, salinity gradients, and hydrodynamics mixing (Bharathi et al., 2022). In tropical estuaries, diatoms abundance typically peaks during dry monsoon seasons characterized when lower turbidity and higher light availability coincides with moderate nutrient enrichment. Genera such as *Cyclotella* spp. serving as useful

indicators of transitional dry conditions (Hilaluddin et al., 2020). Diatom blooms are coincided with pulses of upwelling, nutrient influx or transitional monsoonal phases, which significantly impacting water quality and ecosystem dynamics (Retnamma et al., 2021). In many estuaries, harmful diatom genera such as *Pseudo-nitzschia*, *Skeletonema*, and *Thalassiosira* have become dominant bloom-formers during eutrophication, with implications for biogeochemical cycling and bloom management (Huang et al., 2024). While most diatom blooms are ecologically beneficial, the decay of dense populations can lead to oxygen depletion and shifts in community composition.

2.6.2 Dinoflagellates

Dinoflagellates are unicellular protists characterized by the presence two flagella. Although they are primarily marine plankton, dinoflagellates can also be found in freshwater habitats. Their distribution is influenced by factors in environment such as temperature, pH, and depth. Dinoflagellates vary in size, ranging from approximately 5 to 2,000 μm (0.0002 to 0.08 inch). While most species are microscopic, some are capable of forming visible colonies.

The distribution of dinoflagellates is shaped by nutrient composition, temperature, and phosphate levels (Accoroni et al., 2020). Water stratification favours dinoflagellates proliferation, as it reduces turbulence and allows motile cells to exploit nutrient and light gradients (Tester et al., 2020). Nutrient pulses from freshwater inflow, upwelling events and anthropogenic inputs also stimulate dinoflagellate growth, often shifting community composition toward bloom-forming species (Fischer et al., 2020). Temperature fluctuations

further influence population dynamics, with warmer conditions generally promoting growth and expanding the temporal window for bloom development in estuarine and coastal waters.

Dinoflagellates are well known for their bloom-forming potential, including the occurrence of harmful algal blooms (HABs). Genera such as *Alexandrium*, *Prorocentrum*, and *Karenia* produce potent toxins that can accumulate in shellfish, posing risks to human health and fisheries (Rolton et al., 2022). When dinoflagellates bloom in concentrations of more than a million cells per milliliter, they can produce toxins known as dinotoxins (Cusick & Widder, 2020). These toxins are harmful to marine life, causing fish mortality and accumulating in filter feeders such as shellfish, which can then transfer the toxins to humans upon consumption. This phenomenon is known as a red tide, because of the reddish colouration imparted to the water by the bloom. Additionally, some dinoflagellates are bioluminescent and emit blue-green light (Park et al., 2021). They are among the most common sources of bioluminescence observed at the ocean's surface.

2.6.3 Cyanobacteria

Formerly known as blue-green algae, cyanobacteria are a diverse group of photosynthetic prokaryotes with the existence of approximately 3,500 million years on Earth (Allaf & Peerhossaini, 2022). They are photosynthetic autotrophs with chlorophyll pigments, similar to terrestrial plants. Cyanobacteria inhabit a wide array of environments. They can exist as single cells or in colonies, forming spheres or filaments.

Cyanobacterial blooms occur when cyanobacteria populations grow rapidly in stagnant, warm, nutrient-rich waters, forming visible blooms on the water's surface. These blooms are often referred to as harmful algal blooms (CyanoHABs) because many bloom-forming cyanobacteria produce cyanotoxins, secondary metabolites that are toxic to people, animals, and the environment (Khanna, 2021). Excessive abundance of nutrients, such as

nitrogen and phosphorus, in water bodies from agricultural, industrial, and domestic waste is a major contributor to cyanobacterial blooms (Zhang et al., 2022). Climate change, with rising temperatures and CO_2 levels, is expected to exacerbate the problem. The impacts are multifaceted, ranging from toxin production and alterations in water chemistry to negative effects on aquatic flora and fauna and risks to human health. Common toxin-producing genera include *Microcystis* species, *Dolichospermum* species, and *Planktothrix*, among others (US EPA, 2018). These toxins, released during cell death or, in some cases, actively secreted, can cause a range of health issues, from mild gastroenteritis to severe and potentially life-threatening conditions. Cyanobacterial blooms can change the physical and chemical properties of water and cause pollution. As cyanobacteria accumulate and decompose, they cause hypoxia (low oxygen) in the water. The decomposition of cyanobacteria releases organic substances and soluble nutrients, which can lower water transparency and exacerbate eutrophication.

2.7 Harmful Algal Blooms (HABs) and Occurrence in Malaysia

When too many nutrients are available, phytoplankton may grow out of control and form harmful algal blooms. In coastal regions, the occurrence of harmful algal blooms (HABs) has become more frequent and has expanded across wider geographical areas (Griffith & Gobler, 2020). These blooms are characterized by their rapid growth, often leading to the depletion of oxygen, production of toxins and reduced penetration of light in the water needed for other aquatic organisms (Hennessey et al., 2025). Algal blooms have been found to trigger bacteria-driven hypoxic or anoxic events, resulting in significant fish mortality, the release of toxic substances, and the production of off-flavour compounds, such as 2-methylisoborneol and geosmin, which impart undesirable taste and odour to drinking water and aquaculture-raised fish (Anantapantula et al., 2025). HABs are most frequently

observed in coastal marine ecosystems; however, they can also affect the open ocean, brackish or freshwater environments. The majority of HABs events are attributed to blooms of microscopic phytoplankton, including specific cyanobacteria (blue-green algae), though the term also encompasses blooms of macroalgae such as seaweed (Zaki et al., 2024).

The primary cause of HABs is nutrient pollution, especially from phosphorus and nitrogen. These nutrients often originate from agricultural runoff, industrial discharges and urban wastewater. The excessive proliferation of algae due to eutrophication can cause blooms that are harmful to aquatic life and human health. According to Renuka et al. (2021) different algal species have varying nutrient requirements. The specific ratios of nitrogen to phosphorus can influence which species dominate a bloom. For instance, nitrogen-fixing bacteria, such as *Microcystis* and *Anabaena*, often thrives in conditions where nitrogen is limited compared to phosphorus, leading to favourable bloom conditions in high phosphorus environments with insufficient nitrogen. This selective advantage can lead to shifts in community composition, increasing the likelihood of toxic blooms.

Harmful algal blooms (HABs), including phytoplankton blooms have been a significant ecological concern in Malaysian estuaries and coastal waterways. Environmental factors such as anthropogenic activities, climate conditions and nutrient enrichment all have an impact on these occurrences. There have been reports of phytoplankton blooms in several Malaysian locations, most notably the Johor and Malacca Straits, as well as the coastal waters of Sabah. For example, a study conducted in the Johor Strait found two notable blooms between February and June 2018 with high levels of chlorophyll-a (>10 $\mu g\ L^{-1}$). A strong correlation between nutrient enrichment and phytoplankton proliferation was demonstrated by the close association between these blooms and elevated DOC levels (Cheong et al., 2024).

Table 2.1 provides a summary of the occurrence and bloom characteristics of HABs in Malaysia, supporting the findings of these studies. Studies conducted in Malacca Strait and South China Sea demonstrated the geographical distribution of micro-plankton assemblages, showing that bloom-forming species including *Skeletonema* species, *Coscinodiscus* species, and *Chaetoceros* species were common throughout the sampling locations. The elevated levels of nitrogen and silicate, two essential nutrients for diatom development, were found to coincide with high biomass blooms (Mohd-Din et al., 2025).

Table 2.1: Summary of the occurrence of HABs in Malaysia

	Location	Type of phytoplankton	Year	Bloom characteristics
1.	Johor Strait	*Karenia* species, *Cochlodinium* species, *Akashiwo sanguinea*	2009, 2014	Massive fish kills; high biomass blooms (Mohd-Din et al., 2025).
2.	Kuantan, Pahang	*Alexandrium tamiyavanichii*	2013, 2014	Associated with paralytic shellfish poisoning events (Azanza et al., 2024).
3.	Sabah Coastal Waters	*Gonyaulax polygramma*, *Noctiluca scintillans*, *Cochlodinium polykridoides*, *Pyrodinium bahamense*	2014-2024	Water discoloration, occurrence of red tide events and paralytic shellfish poisoning (Jipanin et al., 2019; Azanza et al., 2024).
4.	Malacca Strait	*Chaetoceros* species, *Coscinodiscus* species, *Skeletonema* species	2022	High chlorophyll-a concentrations; diatom-dominated blooms (Mohd-Din et al., 2025).

2.8 Attachment of pathogenic *Vibrios* to algal cells

Vibrio species are widely recognized for their ecological associations with phytoplankton in estuarine and marine environments. Research indicates that *Vibrio* species utilize multiple strategies to attach to phytoplankton cells. One significant mechanism is biofilm formation, where *Vibrio* can adhere to algal surfaces using polysaccharides and pili, creating a microenvironment rich in nutrients (Teschler et al., 2022). This biofilm not only facilitates attachment but also enhances nutrients acquisition from the surrounding water column. Furthermore, the surface characteristics of different phytoplankton species play a crucial role in the attachment process. *Vibrio* species preferentially associate with certain algal types, such as diatoms and raphidophytes, which may offer more more favorable conditions compared to other substrates like copepods. For instance, *Vibrio* has been observed to form significant associations with diatom-dominated assemblages during algal blooms, highlighting the importance of specific phytoplankton species in promoting bacterial attachment (King et al., 2022).

The attachment of *Vibrio* species to phytoplankton not only facilitates their colonization but also significantly promotes their growth through enhanced nutrient availability. Phytoplankton released dissolved organic carbon (DOC) during senescence or decay, which serves as a vital nutrient source for attached *Vibrio*. This relationship has been quantitatively supported by studies showing that higher concentrations of DOC correlate with increased *Vibrio* abundance during algal blooms (King et al., 2022). Moreover, the presence of phytoplankton provides a refuge from predation by microzooplankton and bacterivorous protozoa. This protective mechanism allows *Vibrio* populations to thrive even under higher grazing pressure. Experimental evidence suggests that while grazing can reduce

overall *Vibrio* populations, the associated growth in biomass can offset these losses, leading to net increase in bacterial abundance during algal blooms.

There are species-specific dynamics that affect growth outcomes in the interactions between *Vibrio* species and various phytoplankton types. For instance, coculture experiments have demonstrated that different phytoplankton species can either enhance or inhibit the growth of *Vibrio* depending on their metabolic profiles and the nature of chemical exchanges between partners. Specifically, diatoms like *Actinocyclus curvatulus* significantly enhance *V. cholerae* growth by releasing high levels of DOC, while other species such as *Picochlorum* species can lead to reduced bacterial abundance due to potential release of antagonistic compounds. This research indicates that the presence of *V. cholerae* also promotes the growth of both *A. curvatulus* and *Picochlorum*, showcasing a complex interplay between these organisms.

In recent work, the association of *V. cholerae* with *Anabaena* sp. cyanobacteria follows the model of symbiosis relationship between nitrogen-fixing freshwater cyanobacteria and heterotrophic bacteria (Takemura et al., 2014). In such associations, heterotrophs use chemotaxis to find their hosts and benefit from the abundance of cyanobacterial exudates. In exchange, the oxidative metabolism relieves oxygen inhibition of nitrogen fixation while also producing carbon dioxide for the purpose of photosynthesis assimilation.

Bloom-associated increases in *Vibrio* populations elevate the risk of seafood contamination, particularly in filter-feeding shellfish such as oysters, mussels, and clams (Diner et al., 2023; Sampaio et al., 2022). Pathogenic species including *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* can accumulate in shellfish tissues and subsequently cause foodborne illness when consumed or undercooked (Castello et al., 2023). In addition

to ingestion, direct contact with bloom-affected waters increases the risk of wound infections and septicemia, particularly among vulnerable individuals with medical conditions such as liver disease or immunosuppression (Brumfield et al., 2021; Hooper, 2022). Seasonal peaks in *Vibrio* abundance during bloom events thus correspond with increased incidence of wound-related *Vibrio* infections. Climate change and eutrophication can alter precipitation regimes and intensify phytoplankton bloom frequency (Paerl & Barnard, 2020). This will create environmental conditions favourable for *Vibrio* proliferation. Predictive models integrating chlorophyll, temperature and salinity data demonstrate that climate-driven ocean warming is expanding the geographic range and seasonal duration of *Vibrio*-associated health risks (Trinanes & Martinez-Urtaza, 2021).

CHAPTER 3

MATERIALS AND METHODS

3.1 Background of the Study

Sarawak, located on the island of Borneo in Malaysia, and the northern region of Sarawak, which borders Sabah and Brunei, has estuarine ecosystems that play crucial role in supporting the local economy through fisheries and water-related activities. Miri and Limbang are located in the northern part of the state Sarawak, Malaysia. This study focused on two estuaries in northern Sarawak, specifically Coco Cabana in Miri (4°23'07.4"N 113°58'14.0"E) and Kampung Limpaku Pinang in Limbang (4°52'47.1"N 115°01'13.9"E). These locations are significant for the local community, whose primary activities include tourism, fishing, and fisheries-related industries. A total of 10 sampling points were established at each site, allowing for comprehensive data collection and analysis. Figure 3.1 illustrates the map of the study sites, indicating the sampling points used for water sample collection.

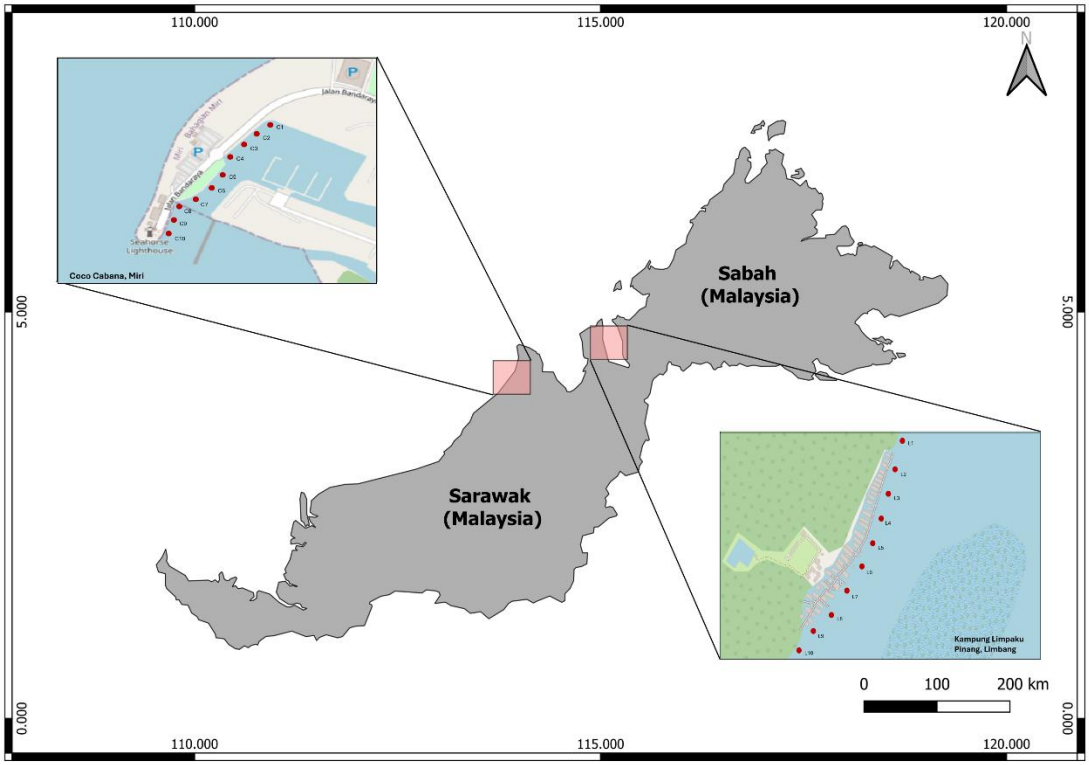

Figure 3.1: Map of study sites: Coco Cabana, Miri and Kampung Limpaku Pinang, Limbang (Google Maps, 2024).

The Coco Cabana Estuary was selected as a representative estuarine environment, where freshwater from the river mixes with saltwater 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). Brackish water conditions facilitate the survival and proliferation of various *Vibrio* species, including those of public health significance. Consequently, the Coco Cabana Estuary presents an optimal location for assessing the baseline *Vibrio* species abundance and distribution in a typical estuarine setting in Sarawak.

Kampung Limpaku Pinang was selected due to its documented history of cholera outbreaks in Sarawak (Benjamin et al., 2005; Nillian et al., 2018). The occurrence of cholera

indicates the potential presence of *V. cholerae* in the aquatic environment. Research has demonstrated that *V. cholerae* can persist in estuarine environments, particularly in association with copepods and zooplankton (Islam et al., 2020). The sampling at Kampung Limpaku Pinang aims to investigate the presence of pathogenic *Vibrio* species and environmental factors contributing to their proliferation and persistence in the environment.

3.2 Water Sample Collection and Physicochemical Parameter Assessment

Water sample collection was conducted following the method described by Huq et al. (2012). Sampling was carried out at two estuarine sites, Coco Cabana in May 2022 and Kampung Limpaku Pinang in November 2023.Each site was sampled once during the respective period of fieldwork. The difference in sampling times was due to both site-specific considerations and logistical constraints. Surface water samples were collected by horizontal hauls and filtered through a 20 µm 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 hr for further analysis.

Physicochemical parameters such as water temperature, pH and salinity were measured *in situ* during the sampling trip. Temperature datalogger (Extech SDL 100) was used to measure water temperature and pH. Meanwhile, the salinity was measured using hand refractometer (ATAGO S/MILL).

3.3 Sample processing

Collected water samples were processed by procedure described by Bilung et al. (2019). All water samples collected were concentrated through filtration by using sterile mixed cellulose membrane filter (0.45 µm pore size; 47 mm diameter) (Whatman^{TM}). The trapped phytoplankton cells on the membrane filter were washed with 25 mL of sterile 1×

phosphate buffer solution (PBS) (Himedia, India) before being vortexed. Next, 1 mL of the sample was transferred into 25 mL of alkaline peptone water (APW) (1% peptone, 1% NaCl; pH 8.5) (Himedia, India) for enrichment and was incubated for 16-24 hours at 35 °C.

3.4 Enumeration of *Vibrio* Species

A six-fold serial dilution was conducted 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 (Himedia, India) agar before being incubated at 37ºC for 24 h. The colonies grown were enumerated, and the *Vibrio* spp. abundance was expressed in colony-forming units (CFU mL^{-1}) of both green and yellow colonies grown on the agar. *Vibrio* colonies grown on TCBS agar were counted in colony forming unit (CFU mL^{-1}) using the Equation 3.1 below:

$$\text{CFU/mL} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of culture plate (mL)}} \qquad \text{Equation 3.1}$$

3.5 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, name 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 reaction mixture, 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 DNA template. DNA of *V. cholerae* (KCDC 13589) and *V. parahaemolyticus* (ATCC 27969) were used as positive controls, while nuclease-free water served as a negative control. The following conditions were used in the PCR reactions: a cycle of initial denaturation at 94ºC for 5 min, followed by 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 following table (Table 3.1) provides a list of primers and their corresponding annealing temperatures utilized in the PCR amplification process for this study.

Table 3.1: List of primers used in this study.

Target Gene	Primer name	Primer sequence (5' → 3')	Product size (bp)	Annealing temperature (°C)	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			

The PCR products were analysed via electrophoresis in 1.5% agarose gel. The gel electrophoresis was conducted with a constant voltage of 75 V, and current of 400 A for 60 min. The gel was stained with ethidium bromide for 60 min and visualised using blue light transilluminator (Cleaver Scientific, UK).

3.6 Phytoplankton Identification

The phytoplankton morphology-based analysis was conducted based on the study by (Chong et al., 2022). One litre of surface water samples was collected with a clean bucket, filtered through a 20 µm mesh size 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 sample was then allowed to settle for 5-10 min before being counted grid-by-grid using a compound microscope at 400 and 600 magnifications. This process was repeated thrice to obtain the average count of the results (Awg Baki et al., 2024). Phytoplankton identification based on morphology were carried out by following phytoplankton identification keys (Lim et al., 2014; Tan et al., 2016; Hii et al., 2019). A drop of 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), while the software utilized to for image analysis was Inifinity Analyze (Teledyne Lumenera, Ottawa, Canada).

3.7 Statistical Analyses

Vibrio concentrations was log-transformed to a normal distribution. For statistical analysis, *Vibrio* numbers below the detection limit were assigned zero value. A one-way

analysis of variance (ANOVA) was performed to determine the statistically significant different in the physicochemical parameters such as temperature, pH, and salinity and *Vibrio* species concentration across the sampling points at Coco Cabana and Kampung Limpaku Pinang. Pearson's correlation analysis was conducted to investigate the relationships between temperature, pH, and salinity with *Vibrio* species concentrations and phytoplankton abundance. The analysis was used to analyse whether variations in the physicochemical parameters significantly influence the concentration of *Vibrio* species in the estuarine environment. Furthermore, the analysis was also performed to observe the correlation between concentration of *Vibrio* species and phytoplankton abundance at the study site. All analyses were carried out using GraphPad Prism version 10.3.0 for Windows.

CHAPTER 4

RESULTS

This chapter presents the results of the study, which investigated the concentration of *Vibrio* species in northern Sarawak. The results also examined the association of selected environmental parameters such as temperature, pH, and salinity with *Vibrio* species concentration and phytoplankton cell density, as well as the correlation between *Vibrio* species and phytoplankton species in the environment.

4.1 *Vibrio* species concentrations in Northern Sarawak

Enumeration of *Vibrio* species was performed on TCBS agar, a selective medium commonly used for *Vibrio* isolation. On TCBS agar, green colonies were observed in water samples collected from Coco Cabana, indicated sucrose-fermenting species such as *V. parahaemolyticus* (Figure 4.1). Meanwhile, yellow colonies were observed in water sample collected from Kampung Limpaku Pinang, indicated non-sucrose fermenting species such as *V. cholerae* (Figure 4.2).

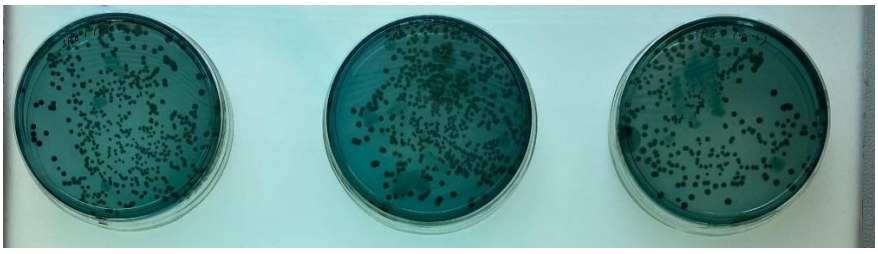

Figure 4.1: Representative TCBS agar plates showing *Vibrio* colonies isolated from Coco Cabana water samples. The green colonies indicate *V. parahaemolyticus*.

Figure 4.2: Representative TCBS agar plates showing *Vibrio* colonoies isolated from Kampung Limpaku Pinang water samples. The yellow colonies indicate *V. cholerae*.

Based on the results, the total mean concentration *Vibrio* species in water samples collected at Kampung Limpaku Pinang (9.1×10^9 CFU mL^{-1}) was significantly higher than Coco Cabana (6.3×10^9 CFU mL^{-1}) as shown in Figure 4.1.

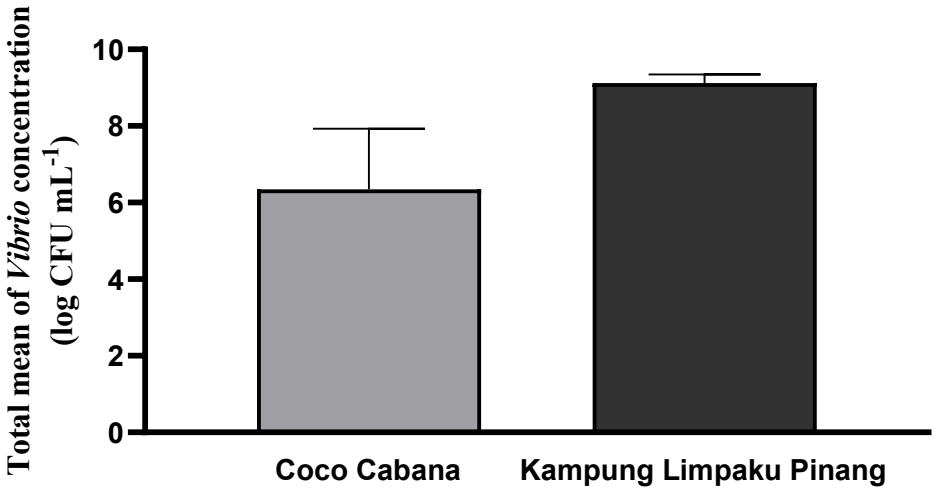

Figure 4.3: Total mean concentration of *Vibrio* species in collected water samples from Coco Cabana and Kampung Limpaku Pinang.

The concentration of *Vibrio* in water samples collected across the sampling points at Coco Cabana were shown in Figure 4.2 below, which were ranged from 2.4×10^9 to 7.1×10^9 CFU mL^{-1}. The lowest concentration was observed at C4 (2.4×10^9 CFU mL^{-1}), while the highest was found at C3 and C10 (7.1×10^9 CFU mL^{-1} each).

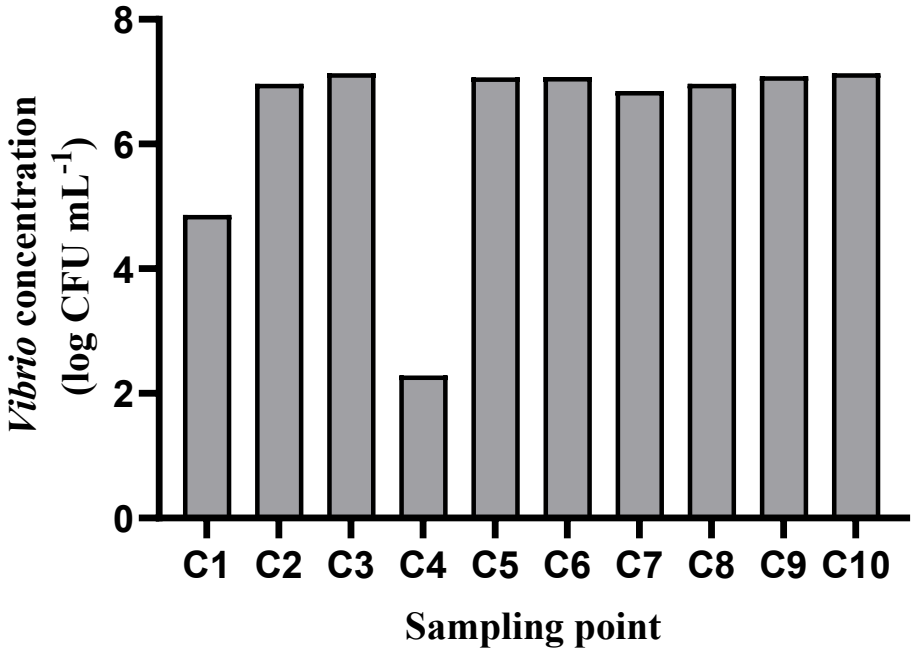

Figure 4.4: Concentration of *Vibrio* species across 10 sampling points at Coco Cabana.

At Kampung Limpaku Pinang, the *Vibrio* species concentration was ranged from 8.7 × 10^9 to 9.4 × 10^9 CFU mL^{-1} (Figure 4.3). The lowest concentration was found in sample L1 (8.7 × 10^9 CFU mL^{-1}), while the highest was observed at L7 (9.4 × 10^9 CFU mL^{-1}).

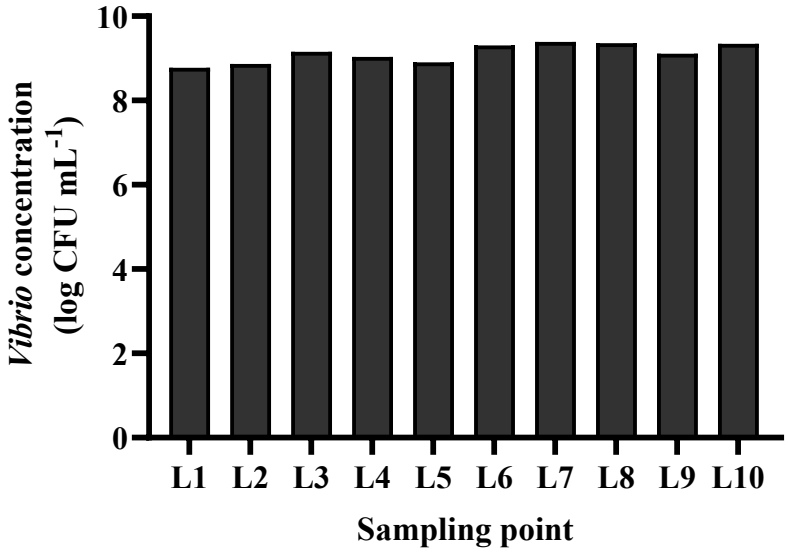

Figure 4.5: Concentration of *Vibrio* species across 10 sampling points at Kampung Limpaku Pinang.

Comparing the two sampling sites, Kampung Limpaku Pinang consistently showed higher *Vibrio* species abundances across all samples compared to 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 are subject to higher levels of contamination.

4.2 Molecular detection of *Vibrio* species using Polymerase Chain Reaction (PCR)

The *OmpW* gene and *ToxR* genes were targeted for PCR amplification to identify *V. cholerae* and *V. parahaemolyticus*. The anticipated sizes of the PCR products were 588 bp for *OmpW* and 368 bp for *ToxR*. Agarose gel electrophoresis (AGE) of the PCR products revealed bands consistent with the expected sizes (Figure 4.4 and 4.5). 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. Whilst *V. parahaemolyticus* was 50% (15/30) and 13.3% (4/30) from Coco Cabana and Kampung Limpaku Pinang.

One of the critical factors in PCR that affects the specificity and efficiency of the primer binding is the annealing temperature. To ensure the success of the PCR, a temperature gradient was applied to optimize the annealing temperature for the *OmpW* and *ToxR* genes. Based on the results, the optimum annealing temperature for the *OmpW* gene was determined to be 52 ºC, while the optimum annealing temperature for the *ToxR* gene was 53 ºC. It was found that these temperatures helped the efficient and specific amplification of the target DNA fragments, as shown by the distinct bands observed on the agarose gel.

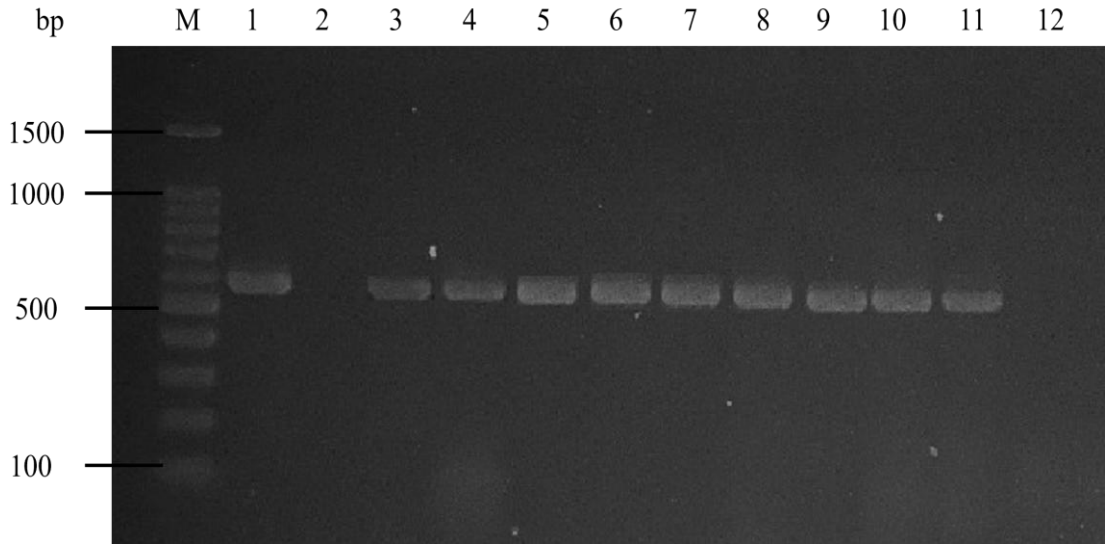

Figure 4.6: Gel electrophoresis of PCR products of *Vibrio* species through amplification of *OmpW* gene for identification of *V. cholerae* (588 bp). Lane M: 100 bp DNA ladder; Lane 1: positive control (*V. cholerae* KCDC 13589); Lane 2: negative control; Lane 3 – 12: representative positive samples.

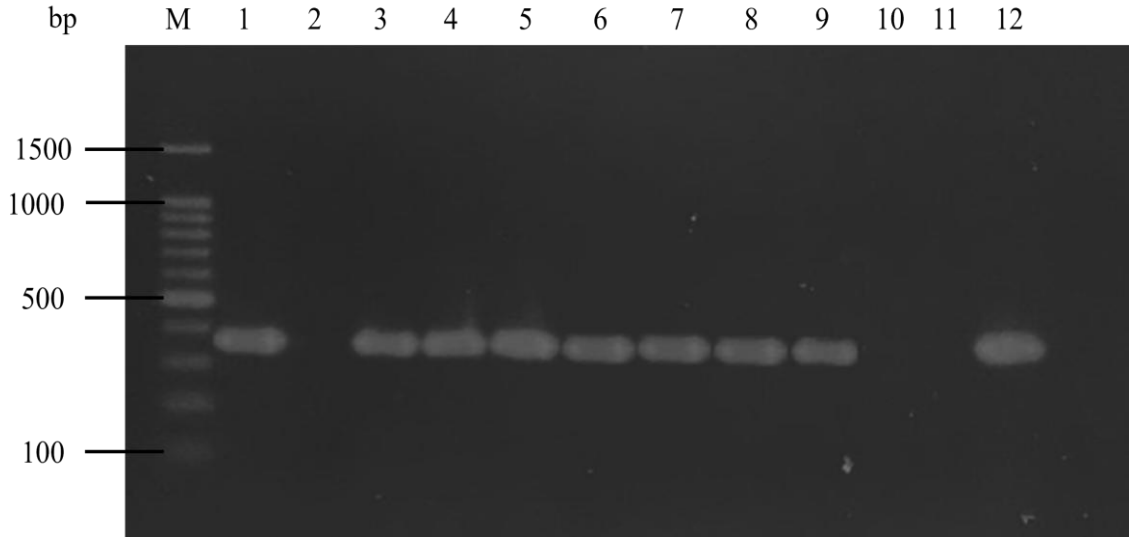

Figure 4.7: Gel electrophoresis of PCR products of *Vibrio* species through amplification of *ToxR* gene for identification of *V. parahaemolyticus* (368 bp). Lane M: 100 bp DNA ladder; Lane 1: positive control (*V. parahaemolyticus* ATCC 27969); Lane 2: negative control; Lane 3 – 12: representative positive samples.

4.3 Detection of Pathogenic Gene of *ctxA* in *V. cholerae* and *tdh* in *V. parahaemolyticus*

The successful amplification of the *OmpW* and *ToxR* genes confirmed the presence of *V. cholerae* and *V. parahaemolyticus* in the water samples collected. Subsequently, PCR assay targeted the cholera toxin (CT) coding gene *ctxA* for *V. cholerae* and the toxin coding gene *tdh* for *V. parahaemolyticus* 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 (Figure 4.6 and 4.7). This indicates that the tested *V. cholerae* and *V. parahaemolyticus* were non-pathogenic.

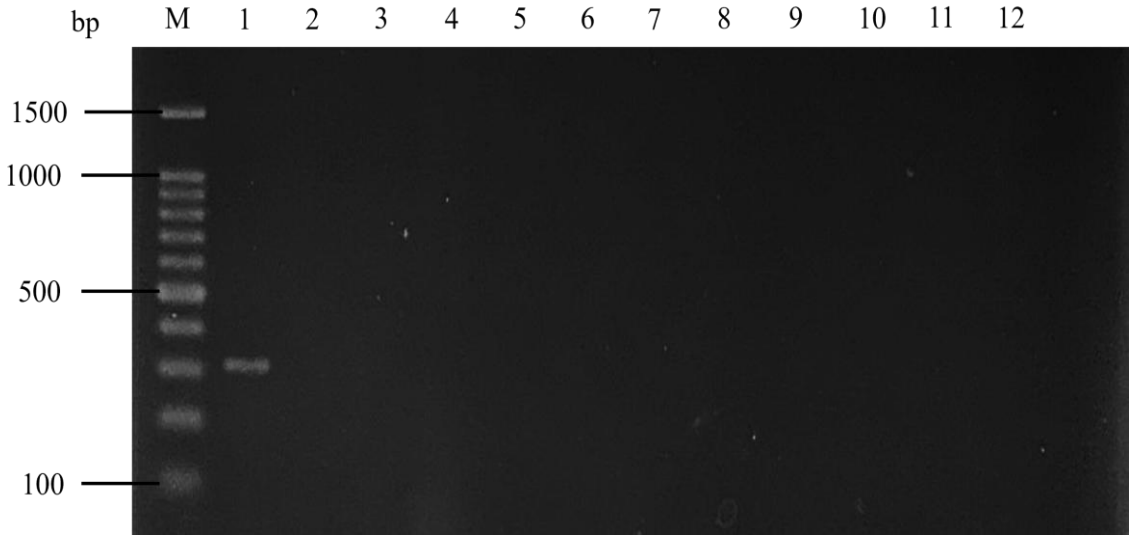

Figure 4.8: Gel electrophoresis of PCR of *V. cholerae* representatives through amplification of *ctxA gene* (302 bp). Lane M: 100 bp DNA ladder; Lane 1: positive control (*V. cholerae* KCDC 13589); Lane 2: negative control; Lane 3 – 12: representative *V. cholerae* samples.

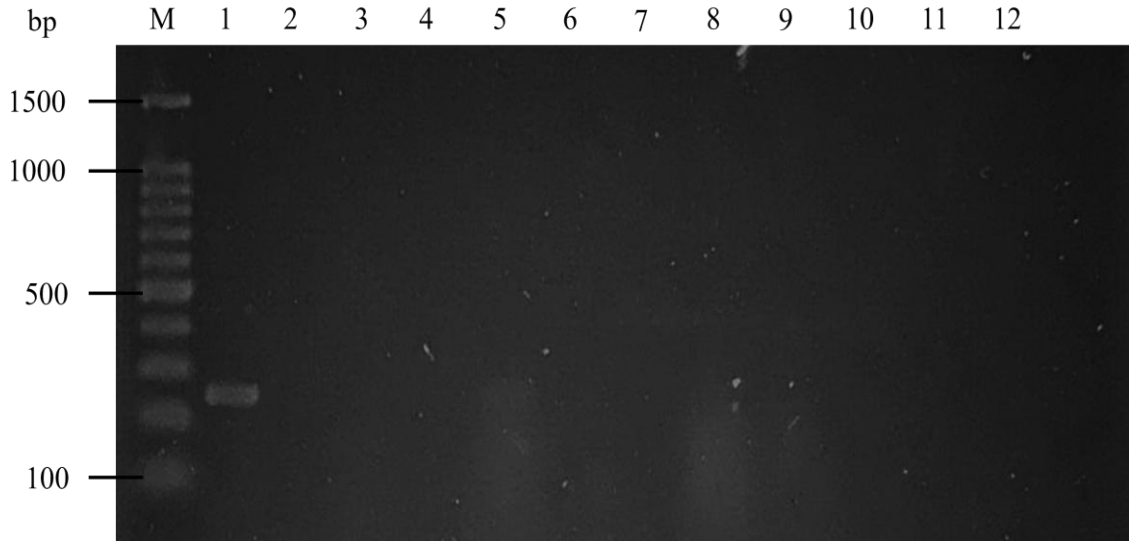

Figure 4.9: Gel electrophoresis of PCR of *V. parahaemolyticus* representatives through amplification of *tdh* gene (251 bp). Lane M: 100 bp DNA ladder; Lane 1: positive control (*V. parahaemolyticus* ATCC 27969); Lane 2: negative control; Lane 3 – 12: representative *V. parahaemolyticus* samples.

4.4 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 were shown in Figure 4.8 below. Water temperature, pH, and salinity from Coco Cabana were 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 were ranged from 31.2 – 34.5 ºC, pH ranged from 6.3 – 6.6, and salinity ranged from 1.7 – 4.7 ppt.

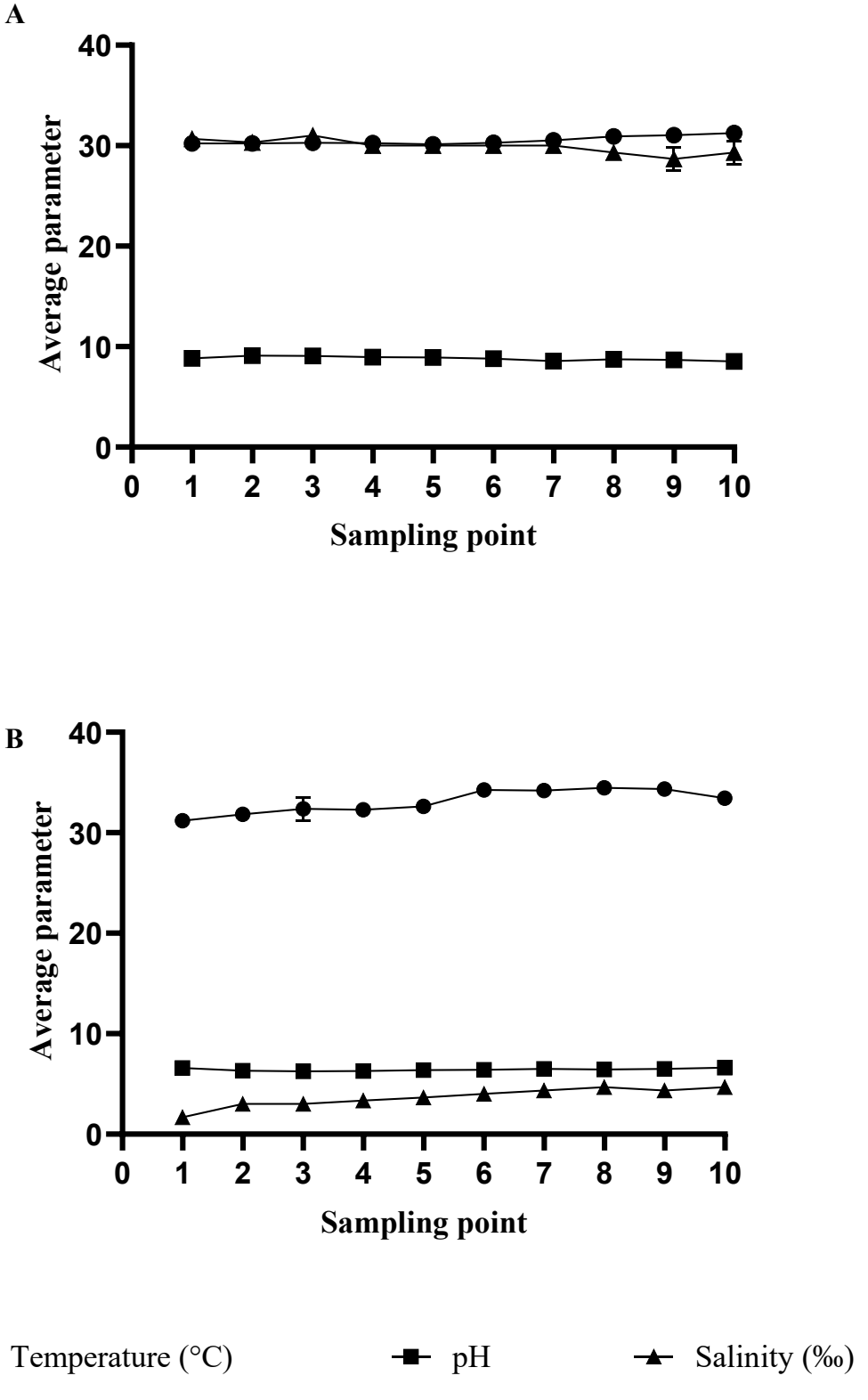

Figure 4.10: Average temperatures, pH, and salinity in (A) Coco Cabana and (B) Kampung Limpaku Pinang

4.5 Phytoplankton Genera in Northern Sarawak

In this study, phytoplankton in water samples collected from Coco Cabana and Kampung Limpaku Pinang were analysed, identifying a total of 23 genera. The results were tabulated and classified into three categories, comprising of diatoms, dinoflagellates, and cyanobacteria (Appendix B). The most abundant group of organisms was diatoms followed by dinoflagellates. Cyanobacteria was the least abundant group recorded in this study. As shown by Figure 4.9, 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., while cyanobacteria were represented by *Oscillatoria* spp,, and *Planktothrix* spp. Among the phytoplankton, *Chaetoceros* spp. was found to be 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 populations.

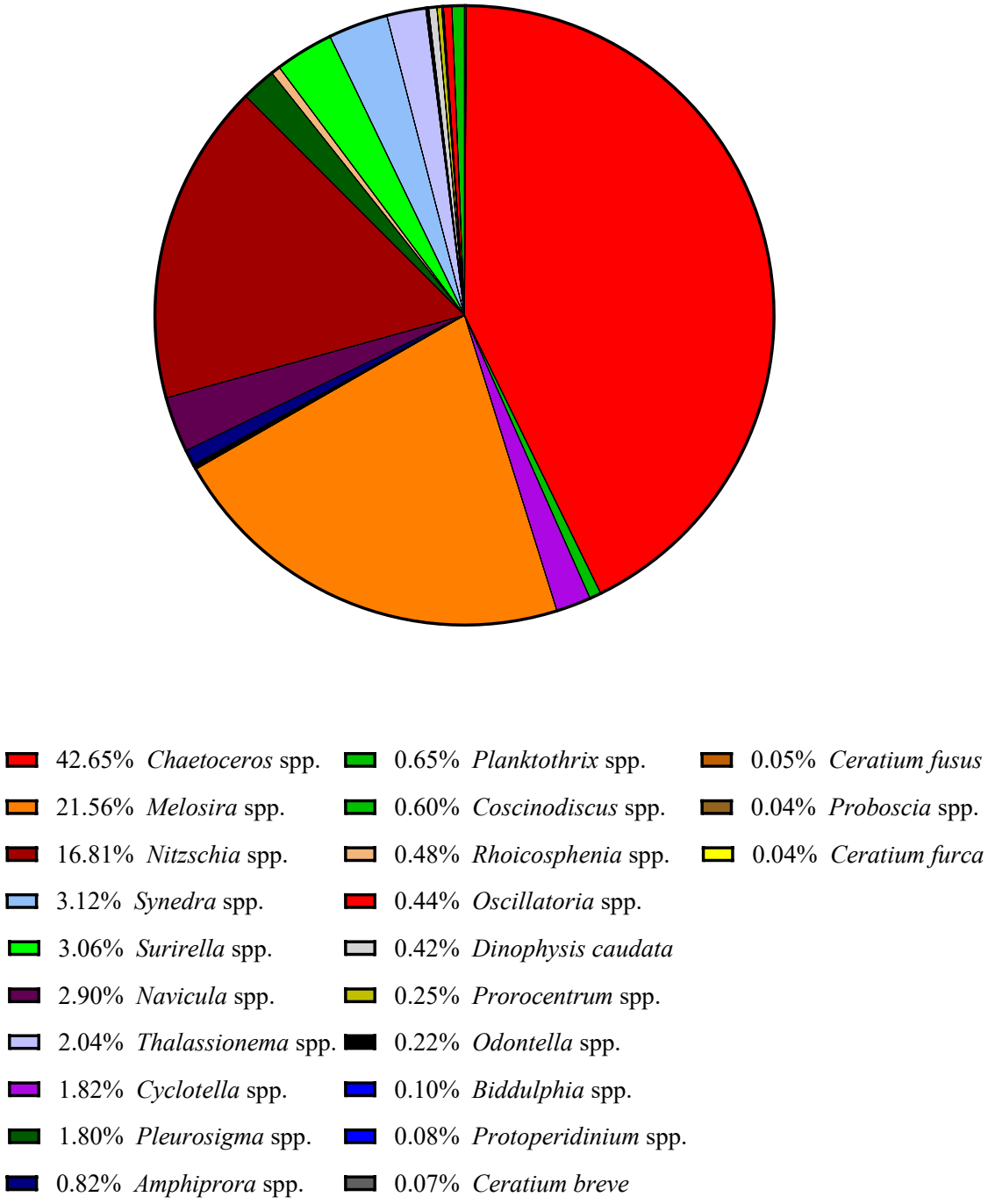

Figure 4.11: Percentage of phytoplankton genera observed in water samples collected from Coco Cabana.

A total of seven phytoplankton genera were identified in water samples collected from Kampung Limpaku Pinang (Figure 4.10). Notably, only diatoms were recorded,

comprising of *Amphipropra* spp., *Coscinodiscus* spp., *Navicula* spp., *Nitzschia* spp., *Pleurosigma* spp., *Surirella* spp., and *Synedra* spp. The phytoplankton populations were dominated by *Pleurosigma* spp. (45.62%), while *Surirella* spp. (2.14%) represented the least abundant species. There were no dinoflagellates or cyanobacteria present in the water samples. The number of phytoplankton cells observed in this study were compiled in a table provided in Appendix C.

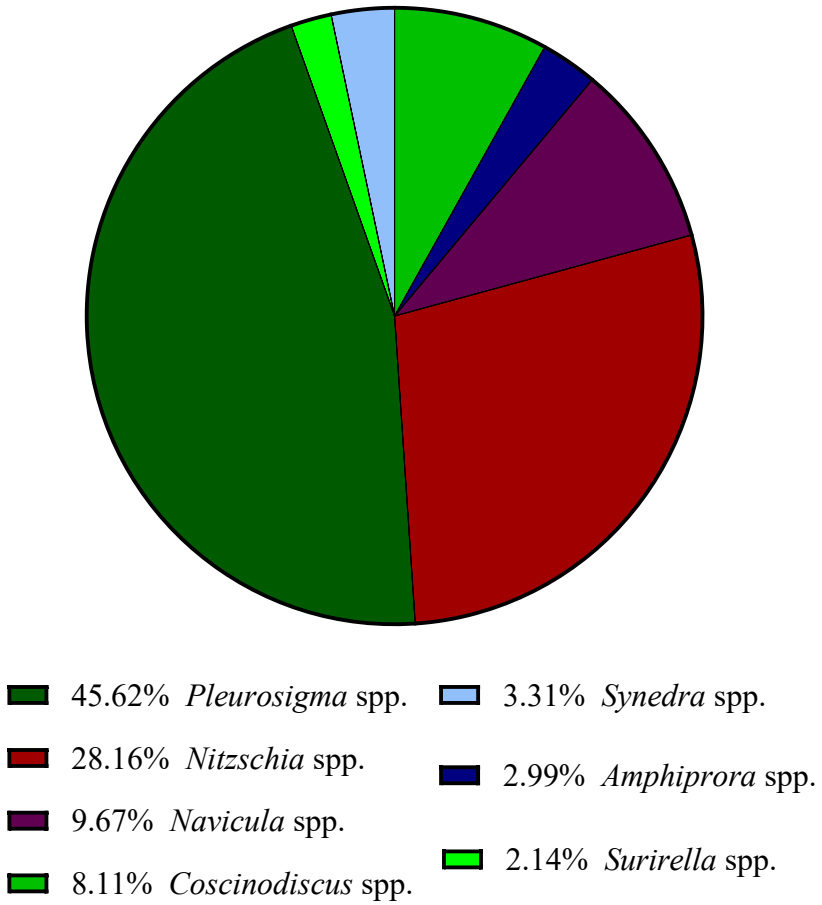

Figure 4.12: Percentage of phytoplankton genera observed in water samples collected from Kampung Limpaku Pinang.

4.6 Correlation between Physicochemical Parameters, *Vibrio* species and Phytoplankton Abundance

The results of one-way ANOVA were tabulated in Table 4.1 below. Overall, the mean of *Vibrio* abundance and temperature at Kampung Limpaku Pinang were slightly higher (mean: 9.13 ± 0.22 log CFU mL^{-1}, 33.10 ± 1.19°C) than Coco Cabana (mean: 6.34 ± 1.58 log CFU mL^{-1} and 30.51 ± 0.41°C). However, the pH and salinity were substantially higher at Coco Cabana (pH 8.82 ± 0.20, 29.99 ± 0.58‰) than Kampung Limpaku Pinang (mean: pH 6.45 ± 0.11, 3.61 ± 0.96‰). A significant effect of sampling sites on the *Vibrio* abundance, temperature, and salinity ($p<0.05$). The Tukey pairwise comparison test based on 95% confidence intervals showed that the *Vibrio* abundance 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.

Figure 4.11 below summarizes the results of the Pearson's correlation analysis. The findings showed *Vibrio* concentration at Coco Cabana was less correlated with temperature ($r = 0.30$, $p<0.05$), but weakly correlated with pH ($r = -0.27$, $p<0.05$) and salinity ($r = -0.21$, $p<0.05$). 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 ($r = 0.13$, $p<0.05$). The Pearson's correlation showed a weak correlation between *Vibrio* concentration 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 cell density 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).

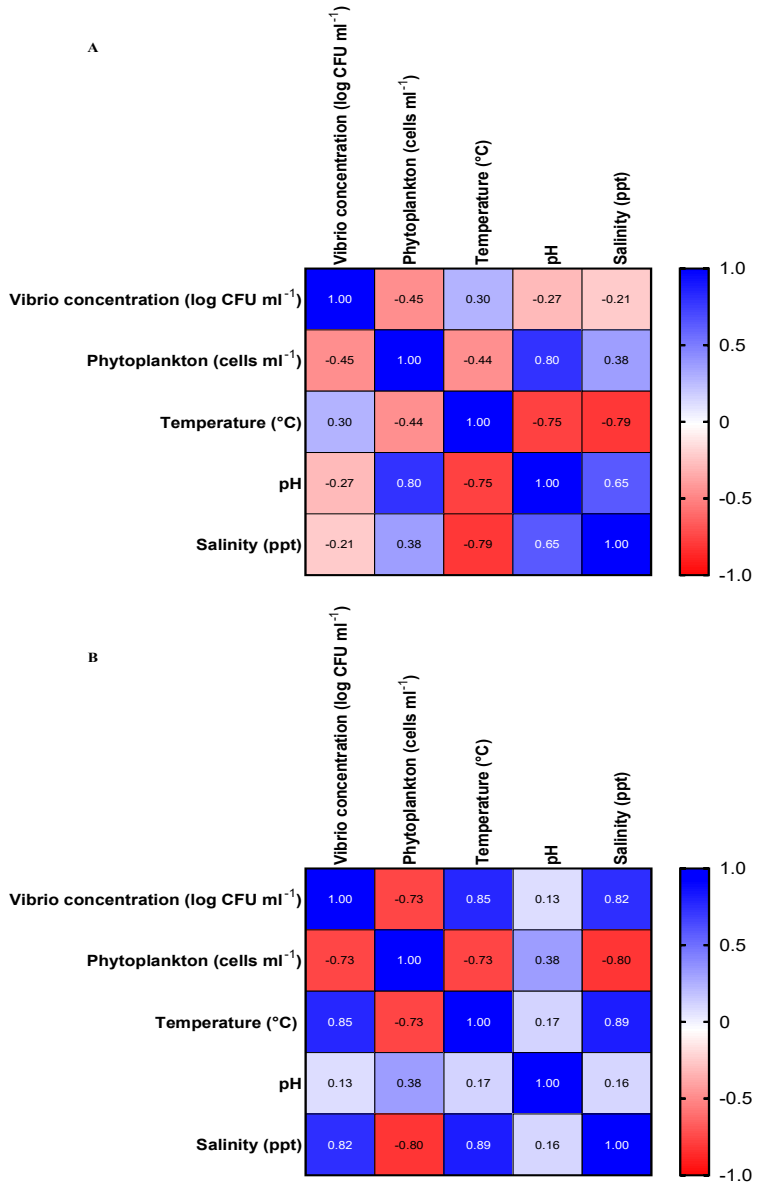

Figure 4.13: Heat map of the correlation between *Vibrio* species concentrations with environmental parameters and phytoplankton abundance.

Table 4.1: One-way ANOVA results for physicochemical parameters across sampling sites

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

CHAPTER 5

CHAPTER 6 DISCUSSION

Based on the findings, the concentration of *Vibrio* species was significantly higher in Kampung Limpaku Pinang compared to Coco Cabana. This is likely due to environmental and anthropogenic factors, such as the proximity to river mouths (Gangwar et al., 2023), the level of human activity, and differences in hydrodynamic conditions (Kopprio et al., 2020). Kampung Limpaku Pinang consistently exhibited higher *Vibrio* concentrations across 10 sampling points compared to Coco Cabana indicating a concerning trend given the area's history of cholera outbreaks (Benjamin et al., 2005; Nillian et al., 2018). The higher concentrations of *Vibrio* species, particularly *V. cholerae* (76.7%), in Kampung Limpaku Pinang, suggested this pathogen remains prevalent in the region. The local environment characteristics such as the low salinity (1.7 – 4.7 ppt) and high temperatures (31.2 – 34.5 ºC) favours the proliferation and survival of *V. cholerae* (Rahman et al., 2018). In addition, previous studies found that inadequate sanitation infrastructures, waste disposal and improper treatment of water supply contribute to the persistence of *Vibrio* species in the environment. (Maheshwari et al., 2011; Patrick et al., 2012; Bilung et al., 2014; Pande et al., 2018). The increased in *Vibrio* concentrations is also likely attributed to elevated nutrient levels, distinct microbiome composition, or increased and heavy rainfall (Bullington et al., 2022).

In contrast, the lower *Vibrio* concentrations observed at Coco Cabana could be attributed to the recreational nature, where water quality is actively monitored and maintained. Recreational waters typically undergo frequent inspections and management, which helps to limit bacterial growth, including *Vibrio* species. Studies in various regions,

such as the Southern California, have shown that *Vibrio* species are commonly present in tropical recreational estuaries during warmer months, highlighting the need for effective management practices (Sampaio et al., 2022). While specific studies on *Vibrio* concentrations in estuarine and coastal environments in Miri are limited, existing research on environmental management in Miri underscores the importance of reducing pollution to support lower microbial loads. For instance, Arumugam (2016) demonstrated that ecological risk assessment in Miri highlight the importance of efficient environmental practices in reducing pollution and maintaining water quality. These initiatives are particularly beneficial for recreational areas like Coco Cabana, where clean water is essential for public health and tourism. Moreover, *Vibrio* species, especially the pathogenic strains, are sensitive to environmental changes such as pollution levels, water temperature, and nutrient availability (Canellas et al., 2021; Brumfield et al., 2023). Effective environmental management at Coco Cabana likely reduces nutrient inputs and controls pollution sources, helping to maintain lower *Vibrio* concentrations in the water.

The presence of *V. cholerae* and *V. parahaemolyticus* through PCR amplification of *OmpW* and *ToxR* genes aligns with previous studies that accentuate the ubiquity of these species in estuarine environments (Sedas, 2007; Fleischmann et al., 2022). González-Escalona et al. (2015) also affirmed that both *V. cholerae* and *V. parahaemolyticus* are prevalent in estuarine and marine environments. *Vibrio parahaemolyticus*, in particular, is commonly found in estuarine environments with moderate salinity and warm temperatures, similar to environmental characteristics observed at Coco Cabana where salinity ranged from 28.7 to 34.5 ppt and temperatures between 30.1 to 31.3 ºC, which were conducive to the growth and survival of the bacteria (Menezes et al., 2017). The detection of *V. cholerae* and

V. parahaemolyticus at both sampling sites is consistent with previous studies indicating that estuarine environments serve as reservoirs for these bacteria.

The absence of *ctxA* and *tdh* genes revealed that the *Vibrio* isolates may be non-toxigenic strains, as not all *V. cholerae* and *V. parahaemolyticus* strains carry the genes responsible for producing 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 generic variations or deletions (Jiang et al., 2003; Y. 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).

In terms of environmental drivers, temperature emerged as a significant factor influencing *Vibrio* concentrations 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 ºC, within the optimal range for *Vibrio* growth (Abdalla et al., 2022; Sheikh et al., 2022). Malaysia experiences high temperatures all year round, with a mean annual temperature of 26.4 ºC and slight variation in average monthly temperature. This finding underscores the importance of warm temperatures in promoting *Vibrio* growth in tropical estuaries, where water temperatures remain consistently high throughout the year (León Robles et al., 2013; Canellas et al., 2021; Brumfield et al., 2023). A similar result observed at Coco Cabana, albeit weaker, as the temperature variation across the 10 sampling points was minimal (30.1

– 31.3 ºC), suggesting that other factors, such as salinity or nutrient availability, may play a more dominant role in regulating *Vibrio* populations at this site.

Salinity also played a crucial role in the prevalence of *Vibrio* species in the estuarine environment. The salinity of water in estuaries can range from 0.5 to 35 ppt and varies based on freshwater inflows, tidal movement, and location (Mahamood et al., 2024). At Coco Cabana, salinity levels ranged from 28.7 - 31.0 ppt, creating favourable conditions for *V. parahaemolyticus* (50%), which thrives in high salinity environments (Johnson, 2015; Fleischmann et al., 2022). Conversely, the lower salinity levels at Kampung Limpaku Pinang (1.7 – 4.7 ‰) supported higher concentrations of *V. cholerae* (76.7%), consistent with previous studies that highlight the species thrives in low salinity conditions (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).

Vibrio species are capable of surviving across a wide pH range, from acidic to alkaline conditions, typically between 5 to10 (Velez et al., 2023) and Costa et al. (2010) stated that the optimal growth of *Vibrio* falls between pH 8.4 to 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 relative stable in 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 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 which reported higher *Vibrio* prevalence following seasonal changes that correlated with temperature and salinity shift (Brumfield et al., 2023). While seasonal data was 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.

The statistical analysis revealed a significant negative relationship between phytoplankton and temperature in this study. The negative correlation at Coco Cabana and Kampung Limpaku Pinang indicates that higher temperatures tend to reduce phytoplankton abundance (Schabhüttl et al., 2013). Elevated mean temperatures observed at Coco Cabana (30.51 ºC) and Kampung Limpaku Pinang (33.10 ºC) can lead to an increase in metabolic demands, reduced nutrient availability, and altered stratification patterns in the water column, which collectively contribute to the decline in phytoplankton abundance (Eker-Develi et al., 2022). The observed correlation underscores the sensitivity of phytoplankton to temperature fluctuations, suggesting temperature influence the distribution and abundance of phytoplankton in the environment.

Furthermore, the relationship between pH and phytoplankton abundance showed a positive correlation. At Coco Cabana, a strong positive correlation was found, indicating that higher pH levels (8.5 – 9.1) were associated with an increase in phytoplankton abundance. This finding is consistent with previous research, which suggests that higher pH levels can enhance phytoplankton growth (Ariffian & Mohamed, 2024). Similarly, Kampung Limpaku Pinang demonstrated a moderate correlation positive correlation with pH (6.3 – 6.6). The variations in strength of the correlation may occur depending on local nutrient dynamics and other environmental factors such as temperature and salinity.

For salinity, the statistical analysis showed mixed influence on phytoplankton abundance, with both positive and negative correlations observed. At Coco Cabana, a weak positive correlation was observed in salinity ranged from 28.7 to 31.0 ppt. In contrast, Kampung Limpaku Pinang showed a significant inverse correlation between phytoplankton and salinity (1.7 to 4.7 ppt). Estuaries have a natural salinity gradient due to the freshwater input from river and saltwater intrusion at the river mouth (Sew & Todd, 2020). Increased salinity can impose osmotic stress on phytoplankton, which affects their growth and reproduction in high salinity lakes and coastal regions (Sun et al., 2023). Higher salinity levels may alter the availability and solubility of essential nutrients, such as nitrogen and phosphorus, which are needed for phytoplankton growth.

Phytoplankton dynamics were analyzed in relation to *Vibrio* concentrations at both sampling sites, revealing a pronounced negative correlation, particularly at Kampung Limpaku Pinang than Coco Cabana. In this study, sampling point L7 recorded the highest concentration of *Vibrio* species, yet it also exhibited the lowest phytoplankton abundance (189 cells mL^{-1}), suggesting that the increased in *Vibrio* species concentrations may either

compete with phytoplankton for nutrients or phytoplankton produce inhibitory compounds that suppress *Vibrio* growth (Sharifah & Eguchi, 2011). A contrasting observation was found at sampling point C4, where the lowest concentration of *Vibrio* species coincided with the highest phytoplankton abundance, further supporting the inverse relationship. These results were aligned with studies conducted by King et al. (2022), which reported similar competitive interactions between phytoplankton and *Vibrio* species. Furthermore, the observed patterns may reflect the ecological shift that occurs when phytoplankton populations decline, leading to the release of dissolved organic carbon (DOC) and subsequent *Vibrio* proliferation during senescence (Asplund et al., 2011).

Among the 23 genera of phytoplankton recorded in this study, diatoms were the most dominant group, with *Chaetoceros* spp. being the most abundant genus representing 42.6% of the total phytoplankton populations This genus is often associated with nutrient-rich waters (Malviya et al., 2016), and the absence of this genus in Kampung Limpaku Pinang indicates less ideal environmental conditions for their proliferation. While many microalgae exhibit reduced growth at 33 º C, but *Chaetoceros* spp. has been reported to tolerate higher temperature and salinities, which is consistent with the temperature range (30.1 - 31.3 ºC) and salinity levels (28.7 - 31.0 ppt) observed in this study (Minggat et al., 2020). Interestingly, previous studies have demonstrated a positive correlation between *Chaetoceros* spp. and *V. parahaemolyticus* (Diner et al., 2021). This study found a negative correlation between *Chaetoceros* spp. abundance and *Vibrio* species concentrations. These findings are consistent with study conducted by (Soto-Rodriguez et al., 2022), which reported an inverse relationship between *Chaetoceros* spp. and vibrios, suggesting that some species within the genus may exert inhibitory effects on *Vibrio* populations.

The presence of pennate diatoms such as *Nitzschia* spp. and *Navicula* spp. in both estuaries highlight their adaptability to diverse environmental conditions, as indicated by the notable difference in mean salinity between Coco Cabana (29.99 ppt) and Kampung Limpaku Pinang (3.61 ppt). The occurrence of *Nitzschia* spp. and *Navicula* spp. in both high salinity and low-salinity waters is consistent with the findings reported by Chintapenta et al. (2018). These genera are commonly associated with warmer waters that exhibit higher levels of phosphorus and nitrogen as reported by (Heramza et al., 2021). Such conditions not only promote the growth of these diatoms but may also indirectly support the proliferation of *Vibrio* species. This result aligns with studies indicating that *Vibrio* populations show a positive correlation with phosphate and ammonium (Rosales et al., 2022).

Cyanobacteria were detected only in Coco Cabana, with *Oscillatoria* spp. and *Planktothrix* spp present at relatively low concentrations (5,980 cells mL^{-1} and 8,960 cells mL^{-1}, respectively). In contrast, no cyanobacteria were detected in Kampung Limpaku Pinang, which may be attributed to the sediment resuspension and increased turbidity, both which inhibit growth of cyanobacteria (Bargu et al., 2023). Cyanobacteria are known to contribute to the persistence of *Vibrio* species in the aquatic ironment (Jesser & Noble, 2018). Similarly, Diner et al. (2021), reported positive relationship between *Vibrio* species and cyanobacteria, showing that vibrios thrive in environments where cyanobacteria are present. The occurrence of cyanobacteria, albeit at low concentrations in Coco Cabana, may enhance the growth and survival of *Vibrio* species in the estuary.

Some harmful phytoplankton genera comprise of dinoflagellates were observed in this study such as *Ceratium breve*, *Ceratium furca*¸ *Ceratium fusus*, *Dinophysis caudata*, and *Prorocentrum* spp. These species were known for their adverse impacts on aquatic

ecosystems. For instance, *Ceratium* spp. has been associated with harmful algal blooms that caused water discolouration, oxygen depletion and in some cases, toxin release. These blooms can result in hypoxic and anoxic conditions, creating “dead zones” where aquatic life, such as fish, cannot survive due to insufficicent oxygen levels (Shen et al., 2012). Simirlary, *Dinophysis caudata* and *Prorocentrum* spp. were linked to the production of diarrhetic shellfish toxins, posing significant risk to both marine ecosystems and human health (Reguera et al., 2014; Arteaga-Sogamoso et al., 2022). Interestingly, *V. parahaemolyticus* has been reporat higher concenat higher concenat higher concentrations (Seong & Jeong, 2011)eat higher concentrations (Seong & Jeong, 2011). The presence of these dinoflagellates in the environment may provide potential food source for *V. parahaemolyticus*, thus contributing to the persistence and proliferation of *Vibrio* in estuarine environment. These interactions may serve as a natural control mechanism, influencing the abundance of *Vibrio* species and phytoplankton in the estuaries of Northern Sarawak. Further study is needed to explore the predator-prey interactions between *Vibrio* species and phytoplankton to better understand the ecological interactions between these species in the estuarine environment.

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

This study reported the association between *Vibrio* species concentration and environmental parameters (temperature, pH, and salinity), and their association with phytoplankton in the estuarine ecosystems in Northern Sarawak. The results demonstrate a strong positive correlation between *Vibrio* concentration and temperature ($r = 0.85$), suggesting temperature is a key driver of *Vibrio* proliferation in estuaries. This finding aligns with previous studies showing high water temperature increased *Vibrio* growth, highlighting the potential impact of rising global temperatures on *Vibrio* populations in estuarine environment.

While pH and salinity generally showed weaker correlations with *Vibrio* abundance, Kampung Limpaku Pinang exhibited a significant positive correlation between *Vibrio* and salinity ($r = 0.82$). This suggests that the fluctuations of salinity were likely influenced by freshwater inputs or tidal movements, which may affect *Vibrio* distribution in specific estuarine conditions. Although pH did not emerge as a significant predictor of *Vibrio* species concentration overall, its potential role in shaping microbial communities should not be dismissed and warrants further investigation.

Furthermore, the study also showed a negative correlation between phytoplankton density and *Vibrio* species at both sampling locations. The Pearson's correlation analysis at Coco Cabana ($r = -0.45$) and Kampung Limpaku Pinang ($r = -0.73$) (Kampung Limpaku Pinang) suggests a potential competitive interaction between phytoplankton and *Vibrio* populations in aquatic environment. Phytoplankton dynamics, particularly the dominance of

Chaetoceros spp., futher elucidated the ecological interplay between microbial communities and environmental factors. The detection of cyanobacteria only in Coco Cabana also points to potential nutrient enrichment, which may indirectly support *Vibrio* species in this estuary. These findings are important for understanding how *Vibrio* bacteria thrive in estuaries, particularly during climate change, and emphasize the need for more research on the interactions between *Vibrio* and phytoplankton in these complex environments.

7.2 Recommendations

From this study, it has been highlighted that future studies should incorporate a broader range of environmental parameters, including nutrient concentrations such as nitrogen and phosphorus and dissolved oxygen levels. This will provide a more comprehensive understanding of the factors influencing *Vibrio* species in estuarine ecosystems. These broader environmental variables may provide valuable insights into other mechanisms contributing to *Vibrio* abundance in the environment.

Since temperature was found to be a key driver for *Vibrio* proliferation, assessing the potential impacts of climate change on *Vibrio* species is crucial, especially in the area with the history of cholera. Rising sea temperatures, a result of climate change, could trigger *Vibrio* outbreaks, with significant implications for both human health and ecosystems. Therefore, further studies should model climate change scenarios to predict how shifts in temperature could affect *Vibrio* populations in estuaries, helping to inform management strategies for mitigating these risks.

Lastly, the negative correlation observed between phytoplankton and *Vibrio* concentration suggested that future studies should focus on species-specific interactions between these groups. Experimental research aimed at investigating whether particular species of phytoplankton inhibit *Vibrio* growth through chemical inhibition or resource

competition could enhance our understanding of the competitive dynamics in estuarine ecosystems. Understanding these interactions at a species level will contribute to improved ecological management practices.

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APPENDICES

Appendix A: Journal Publications (This paper has been submitted to a peer-reviewed journal and is currently waiting for a response)

1. "Spatiotemporal variability of *Vibrio* spp. in relation to phytoplankton and water physiochemical parameters in Northen Sarawak, Malaysia"

   Authors: **Marshiella Lahai Mering**, Lesley Maurice Bilung, Sing Tung Teng, Ai Chia Ho, Ahmad Syatir Tahar, Kasing Apun

APPENDIX B: Total number of phytoplankton observed in water samples collected from Coco Cabana and Kampung Limpaku Pinang.

Genera	Family	Cells mL^{-1}	
		Coco Cabana	**Kampung Limpaku Pinang**
Centric diatoms:			
1. *Biddulphia* spp.	Biddulphiaceae	1330	-
2. *Chaetoceros* spp.	Chaetocerotaceae	585290	-
3. *Coscinodiscus* spp.	Coscinodiscaceae	8300	1250
4. *Cyclotella* spp.	Stephanodiscaceae	24980	-
5. *Melosira* spp.	Melosiraceae	295830	-
6. *Odontella* spp.	Eupodiscaceae	2980	-
7. *Proboscia* spp.	Rhizosoleniaceae	500	-
Pennate diatoms:			
8. *Amphiprora* spp.	Amphipleuraceae	11300	460
9. *Navicula* spp.	Naviculaceae	39780	1490
10. *Nitzschia* spp.	Bacillariaceae	230640	4340
11. *Pleurosigma* spp.	Pleurosigmataceae	24640	7030
12. *Rhoicosphenia* spp.	Rhicospheniaceae	6630	-
13. *Surirella* spp.	Surirellaceae	41960	330
14. *Synedra* spp.	Bacillariophyceae	42790	510
15. *Thalassionema* spp.	Thalassionemaceae	27950	-

Dinoflagellate:			
16. *Ceratium furca*	Ceratiaceae	980	-
17. *Ceratium fusus*	Ceratiaceae	490	-
18. *Ceratium breve*	Ceratiaceae	650	-
19. *Dinophysis caudata*	Dinophysaceae	5830	-
20. *Prorocentrum* spp.	Prorocentraceae	3460	-
21. *Protoperidinium* spp.	Protoperidiniaceae	1150	-
Cyanobacteria:			
22. *Oscillatoria* spp.	Oscillatoriaceae	5980	-
23. *Planktothrix* spp.	Phormidiaceae	8960	-

APPENDIX C: Phytoplankton observed in water samples collected.

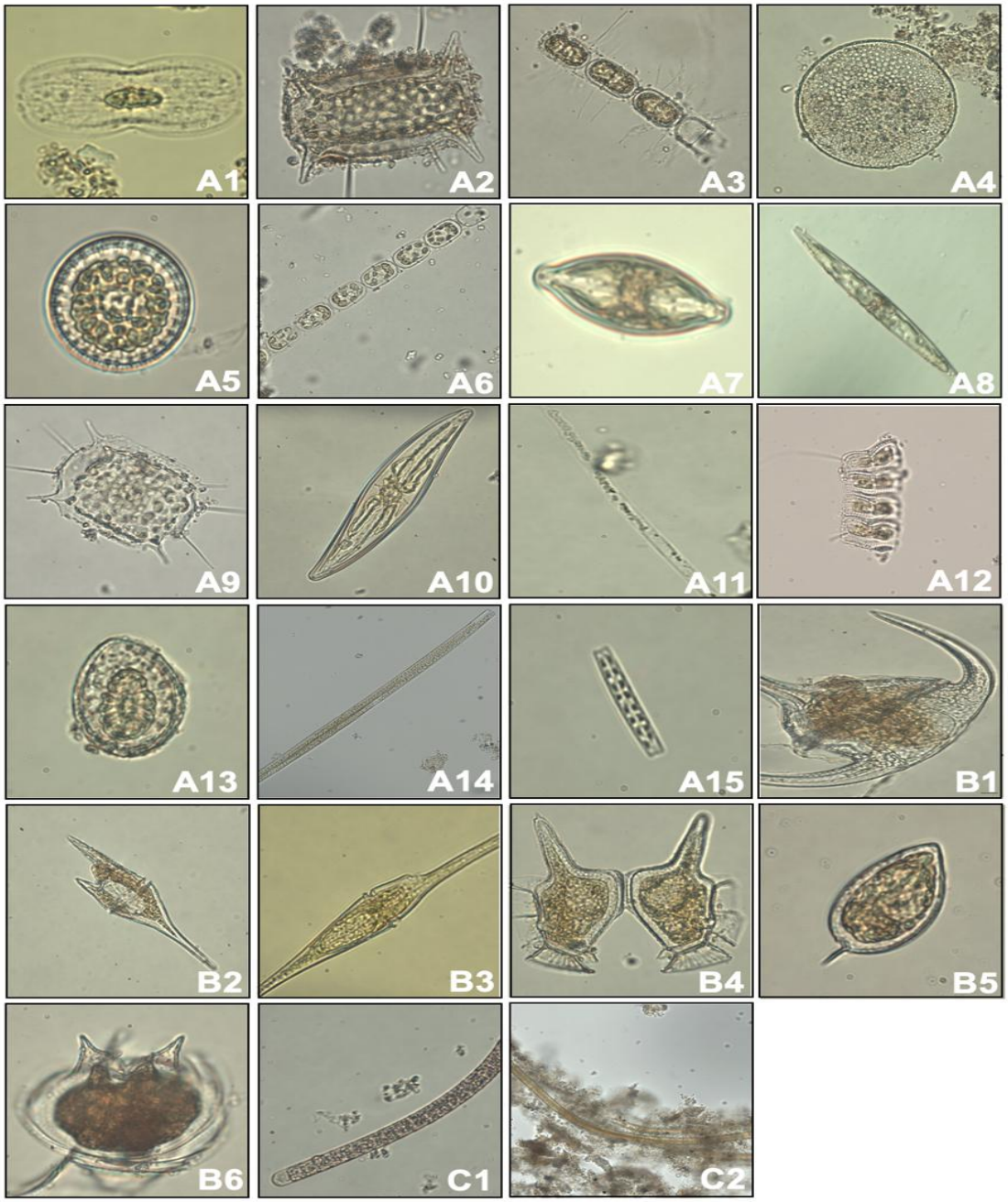

Figure 4.9: Phytoplankton in collected water samples from Coco Cabana, Miri and Kampung Limpaku Pinang, Limbang. Diatom: (A1) *Amphiprora* spp.., (A2) *Biddulphia* spp.., (A3) *Chaetoceros* spp.., (A4) *Coscinodiscus* spp.., (A5) *Cyclotella* spp.., (A6) *Melosira* spp.., (A7) *Navicula* spp.., (A8) *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) P*rorocentrum* spp.., (B6) *Protoperidinium* spp..; Cyanobacteria: (C1) *Oscillatoria* spp.., (C2) *Planktothrix* spp.. Scale bar = 10 µm