



Faculty of Resource Science and Technology

The Association of *Vibrio* species and Cyanobacteria in Selected Local Shrimp Farm

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The Association of *Vibrio* species and Cyanobacteria in Selected Local
Shrimp Farm

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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 discovery of cyanobacteria supporting the persistence of *Vibrio* species in the environment have bridged the gap for the discovery of *Vibrio* related association, however, the existing evidence are mixed. Some studies showed that cyanobacteria may be the side-kick to *Vibrio* species in marine environments, while others have found little or no association between the two groups. In this research, the association between *Vibrio* species and cyanobacteria in a selected local shrimp farm in Sarawak was examined. Over the course of 5 months starting from August 2021 to December 2021 with 2 weeks intervals between each sampling starting from the stocking of shrimp larvae up to the harvesting time, thirty-two ($n=32$) samples were collected from two shrimp ponds labelled as Pond A and Pond B, the effluent, and influent water of the shrimp farm from Persatuan Nelayan Kawasan Satang Biru, Telaga Air. *Vibrio* species and cyanobacteria population density were observed, and canonical Correspondence Analysis (CCA) was used to evaluate their correlation. Molecular method called duplex-Polymerase Chain Reaction (duplex-PCR) was used to identify *Vibrio parahaemolyticus* and *Vibrio cholerae* at molecular levels. An inverted microscope with a DIC filter that can produce pseudo-3D images was used for the morphological identification of cyanobacteria. As a result, *V. parahaemolyticus* showed 78%, 100%, 90%, and 90% prevalence while *V. cholerae* showed 72%, 61%, 90% and 70% prevalence from Pond A, Pond B, effluent, and influent, respectively. The morphological identification of cyanobacterial species revealed the presence of *Pseudanabaena*, *Chroococcus*, *Phormidium*, *Oscillatoria* and *Lyngbya* genus in our local shrimp farm. Results from CCA showed an insignificant correlation between *Vibrio* species and cyanobacteria with p -values of 0.847, 0.255, 0.288, and 0.304 for Pond A, Pond B, effluent, and influent water, respectively. This lack of association suggests that other factors such as species-specific association, regional

factors, and other multidimensional landscapes may play a role in the persistence of *Vibrio* species in shrimp farms. Additionally, this research also examined the virulence genes and antibiotic susceptibility of *V. cholerae* and *V. parahaemolyticus*. Ten ($n=10$) *V. cholerae* and ten ($n=10$) *V. parahaemolyticus* isolates were selected from the same study. The primers used were *tdh*, *trh* for *V. parahaemolyticus* isolates and *ctxB*, *rtxA* and *rtxC* for *V. cholerae* isolates. 70% of *rtxA* and 90% of *rtxC* virulence genes were present in *V. cholerae* isolates while *tdh* and *trh* were absent in *V. parahaemolyticus* isolates. Antibiotic susceptibility testing showed that all *V. cholerae* and *V. parahaemolyticus* isolates were resistant to at least one antibiotic with the mean MAR indices of 0.34 for *V. cholerae* and 0.24 for *V. parahaemolyticus*. The MAR index of 0.20 and greater indicates that antibiotics are heavily contaminating the shrimp farm water. *V. cholerae* isolates showed resistance towards 25 µg sulfamethoxazole (100%), 30 µg cephalothin (80%), 30 µg neomycin (70%), 10 µg streptomycin (70%) and 30 µg ceftriaxone (60%). On the other hand, *V. parahaemolyticus* showed resistance towards is 30 µg cephalothin (100%), 10 µg streptomycin (100%), 25 µg sulfamethoxazole (100%) and 300 µg compound sulphonamides (80%). With no evidence of resistance from *V. cholerae* isolates, 10 µg imipenem, 30 µg tetracycline, 10 µg norfloxacin appeared to be the most potent antibiotics. All of *V. parahaemolyticus* isolates were susceptible with 30 µg ceftazidime, 30 µg ceftriaxone, 30 µg amikacin, 30 µg neomycin, 30 µg nalidixic acid, 5 µg ciprofloxacin, 25 µg sulfamethoxazole-trimethoprim and 30 µg chloramphenicol. For the treatment against *V. cholerae* and *V. parahaemolyticus*, 5 µg ciprofloxacin was found to be the best with the exception of banned antibiotics in food animals. This study contributes to the better understanding of *Vibrio* species' distribution level in shrimp farm, and the possibility of them in causing diseases. The need for proper antibiotic administration and water treatment in shrimp farming environments is

recommended to reduce the risk of antibiotic-resistant infections caused by *V. cholerae* and *V. parahaemolyticus* and minimize negative impact on the environment.

Keywords: Antibiotics, cyanobacteria, shrimp farm, *Vibrio cholerae*, *Vibrio parahaemolyticus*

Hubungan antara Spesies Vibrio dan Cyanobacteria di Ladang Udang Tempatan

ABSTRAK

Penemuan cyanobacteria dalam menyokong pembiakan spesies Vibrio terhadap alam sekitar telah ditemui, namun begitu, bukti yang sedia ada didapati pelbagai. Sesetengah kajian menunjukkan spesies Vibrio boleh ditemui bersama dengan cyanobacteria dalam habitat marin, manakala dapatan lain menemui sedikit atau tiada kaitan antara kedua-dua bakteria. Dalam penyelidikan ini, perkaitan antara spesies Vibrio dan cyanobacteria di dua kolam udang tempatan di Sarawak telah dikaji. Sepanjang tempoh 5 bulan bermula daripada bulan Ogos 2021 sehingga Disember 2021 dengan selang 2 minggu antara setiap persampelan bermula dari penyimpanan larva udang sehingga masa penuaian, tiga puluh dua (n=32) sampel telahpun dikumpul. Sampel telah dilabel sebagai Kolam A dan Kolam B, efluen, dan influen daripada Persatuan Nelayan Kawasan Satang Biru, Telaga Air. Analisis Koresponden Kanonikal (AKK) telah digunakan untuk menilai perkaitan antara spesies Vibrio dan cyanobacteria. Kaedah molekular yang dipanggil tindak balas rantai polimerase dupleks (dupleks-PCR) telah digunakan untuk mengenal pasti spesies Vibrio parahaemolyticus dan Vibrio cholerae dalam kolam-kolam tersebut. Pengenalpastian morfologi cyanobacteria pula dikaji menggunakan mikroskop dengan filter DIC untuk menghasilkan imej pseudo-3D. Hasilnya, V. parahaemolyticus menunjukkan kelaziman 78%, 100%, 90%, dan 90% manakala V. cholerae menunjukkan kelaziman 72%, 61%, 90% dan 70% daripada Kolam A, Kolam B, efluen, dan influen masing-masing. Pengenalpastian morfologi spesies cyanobacteria telah membuktikan kewujudan genus Pseudanabaena, Chroococcus, Geitlerinema, Phormidium, Oscillatoria dan Lyngbya dalam kolam-kolam tersebut. AKK menunjukkan korelasi yang tidak signifikan antara spesies Vibrio dan cyanobacteria dengan nilai p ialah 0.847, 0.255, 0.288, dan 0.304 untuk Kolam A, Kolam

B, efluen, dan air influen masing-masing. Kekurangan perkaitan ini menunjukkan bahawa faktor lain seperti perkaitan khusus spesies, faktor serantau dan landskap multidimensi lain mungkin memainkan peranan dalam pertumbuhan spesies Vibrio dalam kolam udang. Selain itu, penyelidikan ini juga mengkaji gen virulensi dan ketahanan terhadap antibiotik daripada V. cholerae dan V. parahaemolyticus. Sepuluh koloni (n=10) V. cholerae dan sepuluh (n=10) koloni V. parahaemolyticus telah dipilih daripada kajian yang sama. Primer yang digunakan ialah *tdh*, *trh* untuk koloni V. parahaemolyticus dan *ctxB*, *rtxA* dan *rtxC* untuk koloni V. cholerae. 70% daripada *rtxA* dan 90% daripada gen virulens *rtxC* telah ditemui dalam koloni V. cholerae manakala *tdh* dan *trh* tidak ditemui dalam koloni V. parahaemolyticus. Ujian ketahanan terhadap antibiotik menunjukkan bahawa semua koloni V. cholerae dan V. parahaemolyticus tahan terhadap sekurang-kurangnya satu antibiotik dengan purata indeks MAR 0.34 untuk V. cholerae dan 0.24 untuk V. parahaemolyticus. Indeks MAR 0.20 yang lebih tinggi menunjukkan bahawa antibiotik banyak mencemari air kolam udang. Koloni V. cholerae tidak menunjukkan sebarang ketahanan terhadap 10 µg imipenem, 30 µg tetracycline dan 10 µg norfloxacin. Bagi koloni V. parahaemolyticus pula, ianya tidak menunjukkan sebarang ketahanan terhadap 30 µg ceftazidime, 30 µg ceftriaxone, 30 µg amikacin, 30 µg neomycin, 30 µg nalidixic acid, 5 µg ciprofloxacin, 25 µg sulfamethoxazole-trimethoprim dan 30 µg chloramphenicol. Bagi rawatan Vibriosis di kolam udang, 5 µg ciprofloxacin didapati terbaik (dengan pengecualian terhadap antibiotik yang terlarang dalam haiwan makanan). Kajian ini menyumbang kepada pemahaman yang lebih baik tentang tahap taburan spesis Vibrio pada kolam udang, dan kemungkinan ianya penyebab penyakit. Penggunaan antibiotik yang betul dan rawatan air dalam kolam udang disyorkan bagi mengurangkan risiko jangkitan ketahanan terhadap antibiotik yang

disebabkan oleh V. cholerae dan V. parahaemolyticus di samping dapat mengurangi kesan negatif terhadap alam sekitar.

Kata kunci: Antibiotik, cyanobacteria, ladang udang, Vibrio cholerae, Vibrio parahaemolyticus

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LIST OF ABBREVIATIONS

°C	Degree Celcius
µL	Microlitres
AGE	Agarose Gel Electrophoresis
AHPND	Acute Hepatopancreatic Necrosis
APW	Alkaline Peptone Water
CCA	Canonical Correspondence Analysis
CTX	Cholera Toxin
DIC	Differential Interference Contrast
EMS	Early Mortality Syndrome
mL	Millilitre
mm	Millimetre
PAST	PAleontological STatistics
PCR	Canonical Correspondence Analysis
PPT	Parts per Thousand
TCBS	Thiosulfate-Citrate-Bile-Sucrose Agar
TDH	Thermostable Direct Hemolysin
TLH	Thermolabile Hemolysin
TRH	Thermostable Direct Hemolysin-Related Hemolysin
rpm	Rotation per Minute
RTX	Repeat in Toxin
UNIMAS	Universiti Malaysia Sarawak
UV	Ultra Violet

CHAPTER 1

INTRODUCTION

1.1 Study Background

Shrimp is one of the most popular seafood in the world's animal protein production alongside salmon and tilapia in aquaculture (Nunes et al., 2022). Due to the high demand for shrimp, governments in developing countries such as China, Thailand, Indonesia, India, Vietnam, Ecuador and Bangladesh have widely encouraged shrimp aquaculture to minimize poverty (World Wildlife Fund, 2022). In Malaysia, the data from the Fisheries Development of Authority Malaysia (LKIM), (2020) reported that shrimp export volumes in 2017 and 2018 totalled 27,674.10 and 33,667.54 metric tonnes, respectively, contributing RM 833,283,764 in profit in 2017 and RM 1,001,325,595 in 2018. Despite the high demand for shrimp and the significant profit generated from shrimp farming, the susceptibility of shrimp towards diseases, specifically vibriosis, remains a major concern in the industry. Often reared in a packed environment with water parameters fluctuations, relying of innate immune system and lack in species diversity in reared shrimps can make shrimps highly susceptible towards diseases.

Vibriosis is one of the common diseases that were caused by *Vibrio* species and belongs to the *Vibrionaceae* family. They are frequently found in warm seawater bodies. *Vibrio* species can infect shrimp at any stage of development and cause almost 100% death when an outbreak happens (Aguirre-Guzman et al., 2004; Harris & Owens, 1999; Prayitno & Latchford, 1995). Shrimp diseases that can be caused by *Vibrio* species in shrimp farms are White Spot Syndrome Virus (WSSV), Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis (AHPND), White Faeces Disease (WFD), loose shell syndrome,

white gut disease, red disease and luminous disease (de la Peña et al., 2015; Jayasree et al., 2006; Lavilla-Pitogo et al., 2000). Common *Vibrio* species that can be found in diseased shrimps are *Vibrio harveyi*, *V. parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio vulnificus* and *Vibrio splendidus* (Chatterjee & Haldar, 2012).

Vibrio species often coexist with cyanobacteria due to their similarity in their living habitat and nutritional needs. According to research conducted by Islam et al., (2004), cyanobacteria can act as a long-term reservoir for *V. cholerae* O139 for up to 15 months due to the cyanobacteria's ability to encase *V. cholerae* cells within mucilaginous sheaths, causing *V. cholerae* to enter a viable but non-culturable state (VBNC). Another research by Ward (2014) that studied the association between cyanobacteria and *V. parahaemolyticus* found out that the strain G445 was able to last long with the presence of cyanobacteria because the cells seemed to cluster randomly within the cyanobacterial mats. Eiler et al. (2007) also stated that cyanobacterial organic matters play an essential role in the growth and abundance of *V. cholerae* and *V. vulnificus*, strengthening the possibility of the interaction between these two bacterial species.

While many studies have found an association between *Vibrio* and cyanobacteria, there are also research that contradicts this, pointing to limited or no correlation between the two. For instance, the association is thought to differ according to regional factors, seasonal temperature and species involved (Greenfield et al., 2017; Paranjpye et al., 2015; Turner et al., 2009). Greenfield et al., (2017) reported that the presence of *Vibrio* species (*V. vulnificus* and *V. parahaemolyticus*) is dependent on seasonal temperature when they observed very low to no correlation between *Vibrio* species numbers with phytoplankton during winter. Chlorophyll a, essential for photosynthesis in cyanobacteria, also lacks correlation with *Vibrio* populations (Greenfield et al., 2017). Aside from seasonality, Turner et al., (2009)

also found out that species-specific taxa play a role in the determination of *Vibrio* species' population density and their associations with cyanobacteria. The study observed the antagonistic relationship between cyanobacteria, copepods and diatoms (Turner et al., 2009). Paranjpye et al., (2015) gathered no evidence of the association between the two bacteria groups with low density of cyanobacteria in Pacific Northwest in the USA in 2015 as opposed to the majorly stagnant *Vibrio* population density. Overall, the evidence on the association between cyanobacteria and *Vibrio* species is limited and inconclusive and involves complex multidimensional landscape to understand the relationship between these two groups.

The ability of a *Vibrio* species to cause diseases is determined by the presence of virulent genes in their genome. Several virulent genes that were known to be associated with *Vibrio* species are *tdh*, *trh*, *tlh*, *ctxB*, *rtxA* and *rtxC*. Thermostable direct haemolysin (*tdh*) and thermostable related haemolysin (*trh*) are majorly related to the toxicity of *V. parahaemolyticus* (Raghunath, 2015). The expression of *tdh* causes the formation of pore in red blood cell (Matsuda et al., 2010). *trh* genes are 70% similar to that of *tdh* and the expression results in abnormal secretion of chloride ion in human colon (Takahashi et al., 2000). *rtxA* and *rtxC* genes expression induces cell death in human intestine (Lee et al., 2008) while cholera toxin subunit B (*ctxB*) is the gene responsible for the watery diarrhoea symptom causing dehydration and severe electrolyte imbalance in human (Satitsri et al., 2016). *ctxB* was also detected in shrimp and is hazardous towards public health when consumed (Madhusudana & Surendran, 2013). Due to the high mortality rate of these illnesses, it is crucial to monitor them by looking for any possible virulence genes that the *Vibrio* species may have.

In shrimp farms, illnesses brought on by *Vibrio* species are typically treated with antibiotics. They were added in shrimp feed, or in the rearing pond water. However, due to the improper administration of antibiotics, the cases of antibiotic resistant bacteria have emerged (Fletcher, 2015; Holmström et al., 2003). Over the past few decades, the resistance towards antibiotics has developed and spread throughout many bacterial species as a result of the overuse of antibiotics in agricultural and aquaculture systems (Cabello, 2006). Antibiotic resistance can cause longer treatment duration, increase healthcare costs that can become a burden for families, society and the economy (Ventola, 2015). It is estimated that 10 million people will die from antibiotic resistance globally with the loss of £60 trillion by 2050 at the current rate of antibiotic resistance spread, making it more lethal than cancer (O'Neill, 2016). When infection can no longer be treated by first-line antibiotic, more expensive and toxic medication will come in play (Ventola, 2015).

This research is vital to understand the biotic factor that supports the growth population of *Vibrio* and to facilitate better understanding on the species of *Vibrio* that exist in local shrimp farm. It is hypothesized that the association between *Vibrio* species and cyanobacteria in shrimp farms, is highly dependent with the environmental factors such as water temperature, salinity and pH. Additionally, specific virulent genes associated with *Vibrio* species, such as *tdh*, *trh*, *ctxB*, *rtxA*, and *rtxC*, are expected to be present in the predominant *Vibrio* isolates in shrimp farms. Furthermore, the study hypothesizes that the widespread administration of antibiotics in shrimp farms contributes to the emergence of antibiotic-resistant *Vibrio* species. Methods such as Most Probable Number (MPN), Sedgwick-Rafter cell counting, Canonical Correspondence Analysis (CCA), Duplex-Polymerase Chain Reaction (D-PCR) were implemented in this study to achieve the objectives of quantifying and enumerating *Vibrio* species and cyanobacteria, determining

the interaction between the two bacteria genus and identifying the presence of *V. cholerae* and *V. parahaemolyticus*. This study also aimed to determine the virulence genes *tdh*, *trh*, *ctxB*, *rtxA* and *rtxC* from both predominant *Vibrio* species isolates in shrimp farm. The emergent of Multiple Antibiotic Resistance (MAR) index has been investigated and the antibiotics resistant profile has been established to mitigate the potentially infection in near future.

1.2 Problem Statement

The shrimp industry is a significant contributor to the global animal protein production and generates billions of dollars in profit annually. However, the susceptibility of shrimp towards vibriosis, a disease caused by *Vibrio* species, remains a major concern in the industry. The persistence of *Vibrio* species in shrimp farms is thought to be fuelled by the presence of cyanobacteria, however, there is limited research on the association between these two groups and the existing evidence is mixed. Understanding the possible interspecies correlation between *Vibrio* and cyanobacteria can contribute to better understanding of the microbial community in the shrimp farm, which led to the structuring of this study.

In shrimp farms, the use of antibiotics is high, and the presence of virulent genes is anticipated. When *Vibrio* species that are armed with virulence genes and the ability to resist antibiotics are present, there is an increase in their potential to cause severe infections, while also being resistant to treatment. This evolution of the bacterium is called the “superbug”. The evolution of superbugs is feared because of its difficulty to control and treat infections when they arise. This research aims to determine the association between cyanobacteria and *Vibrio* species in a local shrimp farm and the impact of virulent genes and antibiotic resistance on the potential for disease outbreak in a local shrimp farm in Sarawak. The

information that is gathered in this study is hoped to be able to minimize the risks of antibiotic-resistant infections in the shrimp industry. This research is crucial to ensure the sustainability and safety of the shrimp industry and to protect public health.

1.3 Objectives

The objectives of this study are as follows:

- i. To quantify and identify the presence of *Vibrio* and cyanobacteria species in two selected shrimp ponds, effluent, and influent water from the same shrimp farm.
- ii. To tabulate the correlation between the two bacterial genera (*Vibrio* species and cyanobacteria) in two selected shrimp ponds, effluent, and influent water from the same shrimp farm.
- iii. To detect the virulence genes *ctxB*, *rtxA* and *rtxC* from *V. cholerae* and *tdh*, *trh*, from *V. parahaemolyticus* isolates by using Duplex-Polymerase Chain Reaction (D-PCR).
- iv. To determine the Multiple Antibiotic Resistance (MAR) index of *V. cholerae* and *V. parahaemolyticus* isolates.

1.4 Chapter Summary

This thesis is divided into 5 chapters. Chapter 1 serves as an introduction for readers to understand the context of this research. It is meant to set the tone and enlighten the readers with the existing gaps in the study. Chapter 2 extensively discuss the established concept and niche of this study while incorporating existing theories and researches within the territory of this paper.

Chapter 3 occupies addresses i, ii and iii with the main title of “*Vibrio* species and Cyanobacteria: Understanding their Association in Local Shrimp Farm using Canonical Correspondence Analysis (CCA)” while Chapter 4 entitled “The Prevalence of *V. cholerae* and *V. parahaemolyticus* Virulence Genes and Multiple Antibiotics Resistant (MAR) Assessment from Local Shrimp Farm in Sarawak” is the extension of the study which addresses objective iv and v. Chapter 3 and Chapter 4 are comprised with their own workflow which are Introduction, Methodology, Result, Discussion and Conclusion.

Chapter 5 is meant to answer the gaps that were previously mentioned in Chapter 1 with the recommendations for future studies. The figures were placed in the main text while the reference list was labelled and presented at the last section of this thesis.

CHAPTER 2

LITERATURE REVIEW

2.1 Shrimp Farming in Malaysia: Economic Significance and Challenges

In Malaysia, shrimp farm is a significant industry. The warm tropical climate and long coastline of Malaysia provide ideal conditions for the farming of both freshwater and marine species of shrimp. According to the annual report for 2017 and 2018 from the Fisheries Development of Authority Malaysia (LKIM), (2020), the export quantities for shrimp in the same years reached up to 27,674.10 and 33,667.54 metric tonnes respectively, which contributed to RM 833,283,764 of profit in 2017 and RM 1,001,325,595 in 2018. Additionally, shrimp products were recorded to carry the highest export values compared to other fishery products (fishes, crustaceans, molluscs and others) from 2016 until 2018. For the year 2016, the data from the LKIM have shown the export quantity for the shrimp products was 21,719.33 metric tonnes which valued as much as RM 663,029,806. These indicate that shrimp farming can be one of the most critical leverages to boost the national economy with over a hundred million and billions of profits.

While shrimp farming is a significant industry, it is not without its challenges. Disease outbreaks, for example, is prominent in shrimp farms and can lead to significant shrimp morbidity and mortality, resulting in financial losses. Additionally, shrimp farming can have negative impacts on the environment, including the degradation of mangrove forests (de Lacerda et al., 2021), the pollution of water bodies (Nguyen et al., 2006), and the destruction of habitat for other species (Ozbay et al., 2014). There can also be social and

economic issues associated with shrimp farming, such as the exploitation of labour (Das, 2014) and the displacement of local communities (Ahmed, 2018).

To withstand the challenges and the negative impact brought by extensive shrimp farming, there is a need for continuous improvement and adaptation of sustainable practices. This includes ongoing research and development of innovative technologies and methods to address the environmental concerns associated with shrimp farming. To ensure the sustainability of shrimp farming, it is crucial to focus on disease prevention and management, sustainable feeding strategies, responsible water management practices, and adherence to environmental and social standards. By implementing these measures, Malaysia can mitigate the negative impacts of shrimp farming while still reaping the economic benefits that it provides.

2.2 Types of Diseases that can Occur in Shrimp Farm

Shrimp are vulnerable to a variety of diseases, which can be caused by a range of factors, including viruses, bacteria, fungi, and parasites. Their vulnerability can also be contributed to by the fact that they are invertebrates; therefore, they pose a non-specific immune system (Loker et al., 2004). Their bodies rely entirely on their first line of defence against infection. Their first line of protection includes haemocytes, plasma components, and multimeric systems (Aguirre-Guzman et al., 2009).

The types of diseases that commonly occur in shrimp farms include viral (Lee et al., 2022) and bacterial infections (Jayasree et al., 2006), as well as fungal (Karthikeyan et al., 2015) and parasitic diseases (Yu et al., 2022). Compared to bacterial and fungal-related infections, viral infections are the most prevalent and devastating diseases in shrimp farming,

accounting for more than 60% of losses (Flegel, 2012). These infections are primarily caused by viruses such as the white spot syndrome virus, the infectious hypodermal and haematopoietic necrosis virus, and the Taura syndrome virus (Lee et al., 2022; Lightner, 2011). These viruses can cause severe damage to shrimp, leading to high mortality rates and economic losses for farmers.

Bacterial infections pose another significant threat to shrimp farms. Vibriosis is a common bacterial disease that affects farmed shrimp. It is caused by several species of bacteria in the genus *Vibrio*, which are naturally present in the aquatic environment. Vibriosis can occur in both freshwater and marine shrimp farms (Cartwright and Griffin, 2012), and it has been reported in many countries around the globe. The disease can have profound consequences for shrimp farmers, as it can lead to significant morbidity and mortality in infected shrimp from eggs to brood stock, which can result in significant financial losses (Aguirre-Gusman et al., 2005; Harris & Owens, 1999; Prayitno & Latchford, 1995). Vibriosis can be difficult to control, as the bacteria that cause the disease are widespread in the aquatic environment and can be transmitted through contaminated water, feed, and equipment. The example of shrimp that is infected with vibriosis can be seen in Figure 2.1 while Table 2.1 shows some of the examples of shrimp diseases that are known to have been caused by *Vibrio* species:

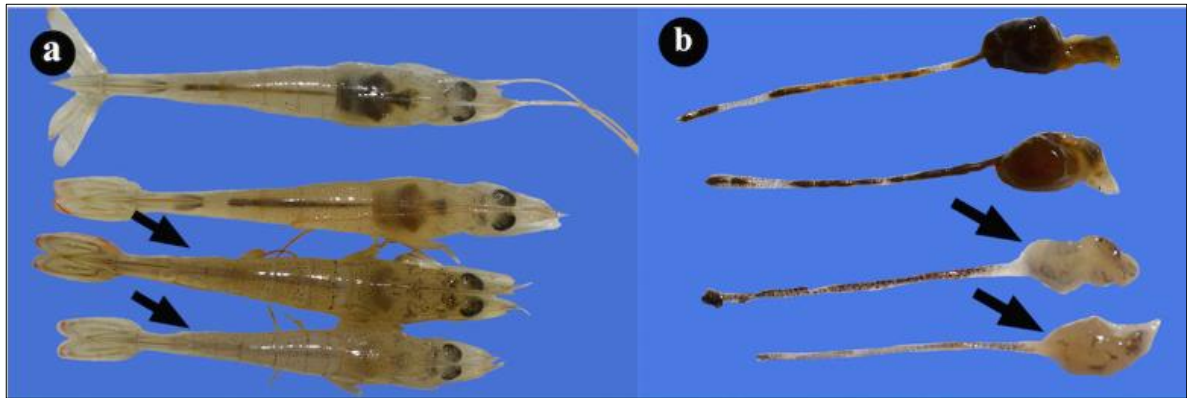


Figure 2.1: The photograph of a healthy shrimp hepatopancreas (no arrow) and the hepatopancreas of shrimp infected with Acute Hepatopancreatic Necrosis (AHPND) (arrows); (a) shows the hepatopancreas seen from the outer layer of shrimp while (b) shows the shrimp's hepatopancreas without the outer layer (Soto-Rodriguez et al., 2015).

Fungal and parasitic diseases are additional challenges that shrimp farmers face. While they account for fewer disease outbreaks compared to viral and bacterial infections, they still pose a threat to shrimp health and productivity. These infections can lead to significant damage to the shrimp's organs or tissues, impacting their growth and overall health (Ibarra-Gómez et al, 2023; Karthikeyan et al., 2015). Parasitic infections in farmed shrimp are also a concern. One example is hepatopancreatic microsporidiosis caused by the microsporidian parasite *Enterocytozoon hepatopenaei* (Ibarra-Gómez et al, 2023). This parasite specifically transmitted through fecal-oral pathway of shrimps, causing stunted growth and severe infections that can cause a poor production cycle and mortality (Ibarra-Gómez et al, 2023).

In conclusion, maintaining the health of shrimp farms is critical to the sustainability and profitability of aquaculture operations. Disease outbreaks caused by viruses, bacteria, fungi, and parasites can have devastating effects on shrimp populations and lead to significant economic losses for farmers. By understanding the various types of diseases that can occur in shrimp farming, farmers can take appropriate actions to minimize the impact

and spread of these infections. To mitigate these risks, shrimp farmers also must implement effective disease prevention and management strategies.

Table 2.1: The diseases that are caused by *Vibrio* species towards shrimps.

Disease name	<i>Vibrio</i> species	Symptoms and risk	Sources
White Spot Disease or White Spot Syndrome Virus (WSSV)	<i>V. parahaemolyticus</i>	Black Tiger shrimp affected by this disease have red discolouration and white spots ranging from 0.5 to 2.0 mm (in diameter) on the surface inside the carapaces. The white spots are deposits of calcium salts. Can wipe out shrimp stocks within 3-10 10 days of the onset of the signs especially juvenile shrimp of all ages and sizes.	(de la Peña et al., 2017). (Hashim & Kathamuthu, 2005).
Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis (AHPND)	<i>V. parahaemolyticus</i>	Pale to white discoloured hepatopancreas with the loss of more than 50% of the organ mass. Can cause mass mortality within 20 to 30 days of post-larvae stocking.	(de la Peña et al., 2015).
White Faeces Disease (WFD)	<i>V. vulnificus</i> , <i>Vibrio fluvialis</i> , <i>V. parahaemolyticus</i> , <i>V. alginolyticus</i> , <i>V. mimicus</i> and <i>V. cholerae</i> (nonO1)	Digestive system of shrimp malfunctions and normal shrimp faeces turns from brownish to pale white colour. Causes shrimp growth retardation, size disparities, reduced feeding and chronic mortalities.	(Somboon et al., 2012). (Shishehchian, 2020). (Aranguren et al., 2019).
Loose shell syndrome	<i>V. harveyi</i> , <i>V. parahaemolyticus</i> , <i>V. alginolyticus</i> , <i>V. vulnificus</i> , <i>V. anguillarum</i> , <i>V. splendidus</i> , <i>V. harveyi</i> and <i>V. campbelli</i> .	Infected shrimps shrunk in size and became frail. Causes mass mortality of shrimp.	(Jayasree et al., 2006). (Raja, 2015).
White Gut Disease		Causes mass mortality of shrimp.	(Jayasree et al., 2006).

Table 2.1 Continued

			(Anas et al., 2021).
Red disease		The body of shrimp turns red, and shrimp faecal matter turns brownish red.	(Jayasree et al., 2006).
		Causes mass mortality of shrimp.	(Lavilla-Pitogo et al., 2000).
Luminous disease	<i>V. harveyi</i> , <i>V. splendidus</i> .	Larvae become fragile and opaque white and can be seen exhibiting greenish luminescence when viewed under total darkness. Juvenile shrimp that are heavily infected also turn luminescence with their heads poised near the water surface.	(Lavilla-Pitogo et al., 2000).
		Can cause mass mortalities, especially in the first 45 days of cultures.	

2.3 Reported Cases of Vibriosis in Asian Shrimp Farm

The outbreaks of vibriosis are widespread across the globe. Asia, being one of the major hubs for shrimp farming, has witnessed a high occurrence of vibriosis in its aquaculture facilities. This has caused severe morbidity in farmed aquatic products, leading to a decline in shrimp productivity. Economic losses amounted to millions of dollars (Lundin, 1995), affecting not only individual farmers but also the overall industry in the region. This motivates the formation of the Association of Southeast Asian Nations (ASEAN) Shrimp Alliance (ASA), which serves as a platform for stakeholders in the industry to engage in discussions pertaining to challenges faced by shrimp farmers (ASEAN, 2020). This association aims at harmonizing efforts and facilitating cooperation among members of the shrimp farming community across Asia. By providing a space for dialogue, ASA fosters an environment conducive to addressing issues that affect the productivity and sustainability of shrimp production.

To fully understand the impact of vibriosis on global shrimp farming, it is important to examine specific case studies from different countries (Table 2.2). By examining specific instances from different areas, researchers can identify common trends, assess the severity of the problem, and propose effective measures for mitigating its detrimental effects on shrimp aquaculture. It is believed that many cases go undocumented due to a lack of monitoring and reporting measures. The reports of vibriosis cases are important for authorities to receive assistance and available compensation. A detailed examination of such specific cases will provide valuable insights into not only the widespread nature but also its socio-economic implications within varying contexts around the globe.

Table 2.2: Reported cases of vibriosis in shrimp farms.

Year	Country	Disease caused by <i>Vibrio</i> species	<i>Vibrio</i> species involved and their risks	Sources
2021	Jinnan District, Tianjin City, China.	AHPND	<i>V. parahaemolyticus</i> . The mortality of the cultured shrimps was over 70%.	(Zhang et al., 2021).
2021	Setiu, Terengganu, Malaysia.	Luminous disease	<i>V. harveyi</i> . The bacterium was confirmed to being resistant to various antibiotics.	(Wan Nurhafizah et al., 2021).
2017	Bohol, Philippines	AHPND and White Spot syndrome virus (WSSV).	<i>V. parahaemolyticus</i> . Mortalities up to 60% in <i>Litopenaeus vannamei</i> pond.	(de la Peña et al., 2017).
2012	Eastern part of Thailand	EMS	<i>V. parahaemolyticus</i> . The outbreak contributed to 7% of the loss in the total shrimp production of Thailand that year.	(Lightner et al., 2013).
2010, 2011 and 2012	Vietnam	EMS	<i>V. parahaemolyticus</i> . Shrimp product dropped from 70,000 (2010) tonnes to 40,000 (2011) and to 30,000 tonnes (2012).	(Zorriehzahra & Banaederakhshan, 2015).
2006	Andhra Pradesh, India	Loose shell syndrome (LSS), White gut disease (WGD), Red disease	Caused mass mortality in shrimp ponds.	(Jayasree et al., 2006).

2.4 Existing Disease Prevention and Control Measure

There are several measures that shrimp farmers can take to prevent and control diseases in their farms. They include biosecurity, anti-microbial, probiotics, immunostimulants (Biomin, 2012), vaccination (Amatul-Samahah et al., 2020) and bacteriophage therapy (Chellapandian et al., 2021). By implementing a combination of these measures, shrimp farmers can help to reduce the risk of disease outbreaks in their farms and improve the health and productivity of their shrimp.

Biosecurity in shrimp farming refers to the measures taken to prevent the introduction and spread of diseases in shrimp farms. It is also a non-medical means taken by shrimp farmers to combat diseases in shrimp farms. It often involves the filtering of water, sanitation, and disinfection (Biomin, 2012). The flushing of contaminated water and switching diseased shrimps into other species was also done in the sampling site of this research as a biosecurity measure to control vibriosis outbreaks. By implementing effective biosecurity measures, shrimp farmers can help to prevent the introduction and spread of diseases in their farms, which can lead to healthier shrimp and lower production costs.

Antibiotics are often regarded as a risky treatment as pathogens can evolve to develop resistance over time. Therefore, scientists in the microbiology community have long been in a constant state of searching for other available alternatives; essential oil is one of them. Essential oils are concentrated plant extracts that contain volatile compounds that give plants their characteristic smells (Dhifi et al., 2016). Domínguez-Borbor et al. (2020) uses essential oil (oregano), as a competent anti-virulence therapy to reduce the effects of toxic *Vibrio* species in shrimp. This is possible due to the ability of essential oil to disrupt quorum sensing, which is a form of bacterial communication (Defoirdt, 2018).

Certain strains of bacteria, known as probiotics, can be used to help prevent the proliferation of pathogenic bacteria, including *Vibrio* species. Probiotics will infiltrate shrimps' gut to promote competition for nutrients and space (Biomin, 2012). A recent study by Nguyen Thi Truc et al. (2021) found that lactic acid bacteria, *Lactobacillus plantarum*, successfully reduced the intensity of AHPND in shrimp caused by *V. parahaemolyticus*. The same study also stated that shrimps treated with this probiotic were seen to have improved in growth and the stimulation of their non-specific immune system.

Immunostimulants serve as a booster for shrimps' non-specific immune system. For example, research by Kilawati and Islamy (2021) used seaweed extract (*Gracilaria* species) to improve the total number of haemocytes in *Litopenaeus vannamei* (whiteleg shrimp) and were successful in lowering the risk of infection by *V. harveyi*. They work by activating the immune system of the shrimp, which helps to improve the shrimp's ability to fight off infections (Kumar et al., 2022).

Despite being innate, the shrimp immune system has been proven to have a simplified form of immune memory (Amatul-Samahah et al., 2020). This opens the gate for vaccine use for the treatment of shrimp diseases. Vaccines can be effective in preventing disease in shrimp, but they are not always 100% effective and may not provide long-lasting immunity because of their lack of true immunoglobulin (Amatul-Samahah et al., 2020). As a result, it is important for shrimp farmers to continue implementing other disease prevention and control measures.

Lastly, bacteriophage therapy is a specific, non-residual issues-form of therapy that works by using viruses to kill only the bacteria that had caused the infection (Chellapandian et al., 2021). It is one of the most effective treatments up to date with a promising result

within the aquaculture industry (Wang et al., 2017). In research conducted by Karunasagar et al., (2007), bacteriophages from the family *Siphoviridae* were able to reduce the population of *V. harveyi*.

In conclusion, various approaches have been explored to improve the health and productivity of farmed shrimp. The implementation of disease-resistant shrimp, specific pathogen-free shrimp, vaccines, immunostimulants such as probiotics, and bacteriophage therapy has shown promise in enhancing the immune response and reducing the incidence of diseases in shrimp farms. Given the high mortality rates that can be caused by vibriosis in shrimp farms, it remains essential to continually explore and create efficient approaches for preventing and managing diseases, especially the methods not only effectively protect against disease, but also minimize their impact on the environment.

2.5 *Vibrio* Species

Vibrio species, belonging to the family *Vibrionaceae*, represent a fascinating group of aquatic bacteria that predominantly inhabit warm saltwater environments (West & Lee, 1982; Canellas et al., 2021). These Gram-negative organisms exhibit a distinctive comma shape and possess a flagellum (Zhu et al., 2013), enabling them to navigate through their aqueous habitats with remarkable agility. Morphologically, they are comma-shaped bacteria with a flagellum at one end and are 0.5-0.8 µm in width and 2-3 µm in length (Figure 2.2) (Venkateswaran, 1999). They are also facultative anaerobes, which means they can live without oxygen in certain circumstances (Youngren-Grimes et al., 1988). *Vibrio* species hold significant relevance in human clinical microbiology and food safety due to their notorious capacity to cause a range of diseases. Infections usually occur in people who have eaten raw or undercooked seafood or after exposure of an open wound to seawater (Ramamurthy et al.,

1993; Johnson et al., 1994). *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *Vibrio mimicus* are among the *Vibrio* species that are known to have caused harm to the human body (Izumiya et al., 2011). From gastrointestinal inflammation (Ritchie et al., 2012) to septic shock (Mai et al., 2022) and wound infections (Yamazaki et al., 2022), these bacteria have been the focus of extensive research and scrutiny.



Figure 2.2: Pure culture of *V. cholerae* after Gram-staining (Kamel & Fawzi Jarjes, 2015).

Among the 48 species of *Vibrio* that have been recorded (Palit & Nair, 2014), *V. cholerae* is one of the most well-known. This is because the species caused seven cholera pandemics starting in 1817, with the 7th pandemic going on up to these days (Pollitzer, 1959; Islam et al., 1993). Cholera is also still endemic in certain countries around the globe. *V. cholerae*, which produces serogroup O1 and O139 toxin-producing strains, is responsible for most cholerae epidemics (Cartwright & Griffin, 2012). The next known *Vibrio* species covered in this study is *V. parahaemolyticus*. According to a review article by Raghunath (2015), thermostable direct hemolysin (TDH) or TDH-related hemolysin (TRH) genes are

important virulence factors for *V. parahaemolyticus*. However, some strains of this particular species reportedly do not contain these virulence factors (10% of the clinical strains) but are still highly cytotoxic to human gastrointestinal cells, therefore providing evidence for another virulence factor, according to the same article. Compared to *V. cholerae*, *V. parahaemolyticus* infections are more commonly associated with seafood consumption rather than waterborne transmission.

Vibrio species play an important ecological role in marine ecosystems by influencing the delicate balance of microbial communities and nutrient cycling processes (Zhang et al., 2018). These bacteria participate in intricate symbiotic relationships with various marine organisms, such as regular microflora in the haemolymph of bivalves (Pruzzo et al., 2005), planktonic organisms (Hartwick et al., 2021), and larger marine animals (Meighen, 1991). They can form mutualistic associations with bioluminescent organisms such as certain squid species and fish (Meighen, 1991). In return, these symbiotic partners provide an undisturbed habitat in which the bacterial species can multiply in their bodies (Lee & Ruby, 1994). In addition, *Vibrio* species are actively involved in the degradation of organic material (Zhang et al., 2018), contributing to the recycling of nutrients in the marine environment. Through their enzymatic activities, they contribute to the breakdown of intricate organic compounds, releasing vital nutrients that foster the development of other microbial communities (Zhang et al., 2018). By understanding the importance of *Vibrio* species for marine ecological dynamics, we can better appreciate the complex role played by these bacteria in maintaining ecological stability.

In conclusion, despite their unfavourable reputation for spreading a variety of diseases, *Vibrio* species are crucial to marine ecology. They have a significant impact on the

seafood sector because they have an influence on the world economy and face unique challenges as a result of *Vibrio* contamination. As *Vibrio* species thrive in warm seawater, ensuring seafood safety becomes paramount to preventing outbreaks and protecting consumer health. Stringent regulations, improved handling practices, and heightened awareness are essential for maintaining consumer confidence and sustaining the seafood industry's viability. Understanding the dynamics between *Vibrio* species and their environment is crucial for environmental conservation efforts. In studying *Vibrio* species, it becomes evident that these bacteria extend far beyond the confines of their microscopic world.

2.6 Cyanobacteria

Cyanobacteria are Gram-negative phytoplankton that are abundant in nature. They are one of the oldest groups of photosynthetic organisms on Earth (Biello, 2009). The cyanobacterial fossil record is among the oldest for any group of organisms, possibly reaching back to 3500 million years ago (Golubić & Lee, 1999). Today, cyanobacteria are found in diverse environments, from freshwater to marine environments, and are considered to be important sources of energy and nitrogen fixation for other organisms. A substantial amount of oxygen can be found in the atmosphere as a by-product of cyanobacterial photosynthesis (Cassier-Chauvat & Chauvat, 2014).

Morphologically, cyanobacteria can be found in coccoid unicellular, coccoid multicellular, or filamentous forms (Figure 2.3) (Mur et al., 1999). They are also commonly enveloped with a jelly-like structure called the mucilaginous sheath that protects the bacteria from ultraviolet rays, predation, and dehydration (Nienaber & Steinitz-Kannan, 2018). Some species can fix nitrogen under adverse conditions (Britannica, 2020) by dividing their

vegetative cells to undergo specialization and carry out specific functions (Nienaber & Steinitz-Kannan, 2018): akinetes to store ‘food’, apical cells (a reproductive cell that appears at the end terminal of cyanobacteria) to promote the formation of new filaments, and heterocysts to carry out nitrogen fixation under nitrogen-deficient conditions.

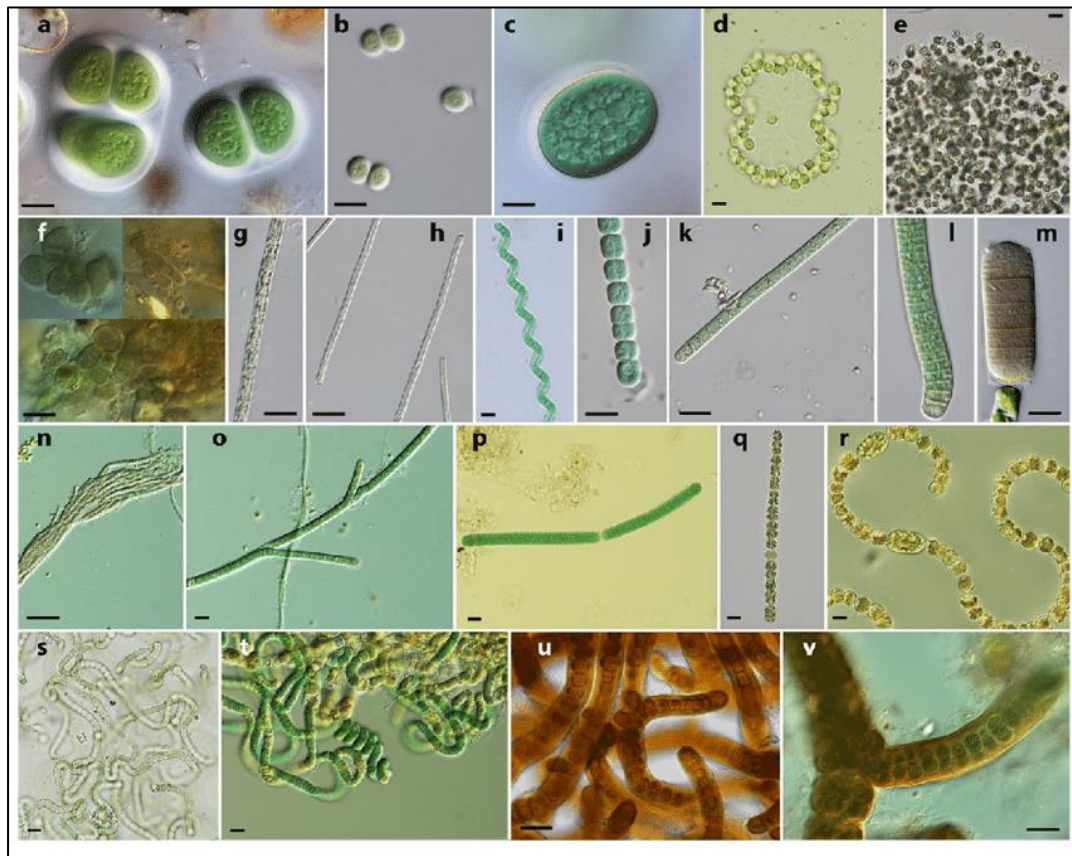


Figure 2.3: Morphological diversity of cyanobacteria (Dvorak et al., 2015). Scale bar 10 μm .

Cyanobacteria are important to the ecosystem in several ways. Whitton (2012) highlights the importance of cyanobacterial nitrogen fixation in the oceans and discusses several ecological subjects of current interest, including sensing the environment and other organisms and signaling between cyanobacterial cells and between cyanobacteria and other organisms. The ability of cyanobacteria to influence nutrient cycling and engage in

symbiotic relationships with other organisms earned them the title of primary producer (Allan & Castillo, 2007). The ability of cyanobacteria to cycle nutrients has also been beneficial for the formation of symbiotic relationships with other living organisms such as coral reefs and protozoa. Aside from supplying fixed nitrogen, coral reefs have been found to benefit from the association with cyanobacteria in their calcification and decalcification (Charpy et al., 2012). A mutualistic relationship also exists between an uncultivated unicellular cyanobacterium (UCYN-A) that fixes nitrogen, and a protozoon called a unicellular prymnesiophyte, which takes fixed nitrogen from the cyanobacteria in exchange for transferring fixed carbon (Thompson et al., 2016).

In addition to their ecological benefits, cyanobacteria are also important for various applications, including biotechnology, agriculture, and industry. They can be used as biofuels (Ducat et al., 2011), biofertilizers, wastewater treatment agents, and sources of value-added products such as pigments, enzymes, and exopolysaccharides (Garlapati, 2019). The diverse bioactive compounds found in cyanobacteria, which have antiviral, antibacterial, antifungal, and anticancer activities, can be used as food supplements (Ananya, 2014). The unique characteristics of cyanobacteria, including their ability to live in various environments, short generation time, and capability to fix atmospheric N₂, make them effective biofertilizer sources to improve soil physio-chemical characteristics such as water holding capacity and mineral nutrient status in degraded lands (Singh, 2016).

Despite the vast usefulness of cyanobacteria, they can also impact the ecosystem in a harmful way by creating algal blooms in certain conditions. Blooms of cyanobacteria are often attributed to two primary factors: natural processes such as circulation, upwelling relaxation, and river flow; and hazards caused by humans, leading to eutrophication (Sellner

et al., 2003). It is known that any type of harmful algae appears to be able to thrive when nutrient loads are not in proportion (Glibert & Burkholder, 2011). Harmful algal blooms (HABs) can release toxins called microcystins. Microcystins can contaminate all areas of aquatic ecosystems and enter the food chain in seafood, therefore posing health risks to humans (Pham & Utsumi, 2018). A study on Ukerewe Island in Tanzania found that concentrations of cyanobacteria cells in Lake Victoria were beyond acceptable limits, and water usage indicated that lake water users were more likely to experience gastrointestinal illness, vomiting, and throat irritation compared to those using other water sources, highlighting the negative impact of HABs on human health (Mchau et al., 2019). From a toxicological viewpoint, cyanotoxins span four major classes: neurotoxins, hepatotoxins, cytotoxins, and dermatotoxins (irritant toxins) (Pearson et al., 2010). Algal blooms can also have negative effects on the environment and society by promoting habitat destruction and causing higher water treatment costs (Burkholder, 2009). The challenges of monitoring microcystins in the environment are due to their susceptibility to abiotic and biotic processes that alter their toxicity and structure (Schmidt et al., 2014). Several genera of cyanobacteria can produce toxins that may be accessible for chemical warfare agents (Van der Merwe, 2015).

Several techniques have been developed to control HABs based on microorganism-based methods, including alga-bacterium and alga-fungus bioflocculation, algicidal bacteria, fungi, and actinomycete, and methods based on microbial aggregates such as periphytons and biofilms (Sun, 2018). The use of predatory bacteria as a biological control agent for HABs has also been studied (Gumbo, 2008). The use of a type of fungus, *Trichaptum abietinum* 1302BG, was able to destroy algal cells within 48 hours of co-incubation, possibly through encasing the algal cells with a mucous membrane secreted by the fungal mycelia

(Jia, 2010). Still, climate change can impact all mitigation strategies, despite these methods. Therefore, there may be a need to establish new targets and thresholds for reducing nutrient input and preventing nutrient blooms in impacted water bodies (Paerl et al., 2016).

2.7 *Vibrio*-Cyanobacteria Relationship

There are several researches about the association between *Vibrio* species and cyanobacteria, but the evidence is often mixed. Some studies have suggested that *Vibrio* species may be found close to cyanobacteria in marine environments, while others have found little or no association between the two groups. Many speculate the involvement of environmental reservoirs that facilitate the persistence of *Vibrio* species hinting at the connection with cyanobacteria in the scheme. Glazing the grounds as on how this might be possible, a taxonomical research were done by Jesser and Noble in 2018 by incorporating the method of amplicon sequencing of 16S rRNA and founded that there is a positive relationship between *Vibrio* species, predominantly *V. parahaemolyticus* with cyanobacteria with the relative abundance of $r=0.53$ for 16S rRNA data proving that there is indeed interspecies associations between the two.

Nguyen Thi Truc et al., (2021) noted that previously in Vietnam, shrimp farmers used the products that can promote the supply of carbon, nitrogen and phosphorus (CNP) as an effort to improve the growth of phytoplankton (a diverse group of aquatic microorganisms that include various types of algae, including cyanobacteria) which are known as a natural feed of shrimps. However, after 2 weeks, the shrimps were diagnosed with AHPND brought by *V. parahaemolyticus*. Earlier research by Ward (2014) studied the association between cyanobacteria and *V. parahaemolyticus* G445 and found out that the strain was able to last long with the presence of cyanobacteria and the cells can be seen clustering randomly within

the cyanobacterial mucilaginous sheath when viewed under a confocal microscope (Figure 2.4).

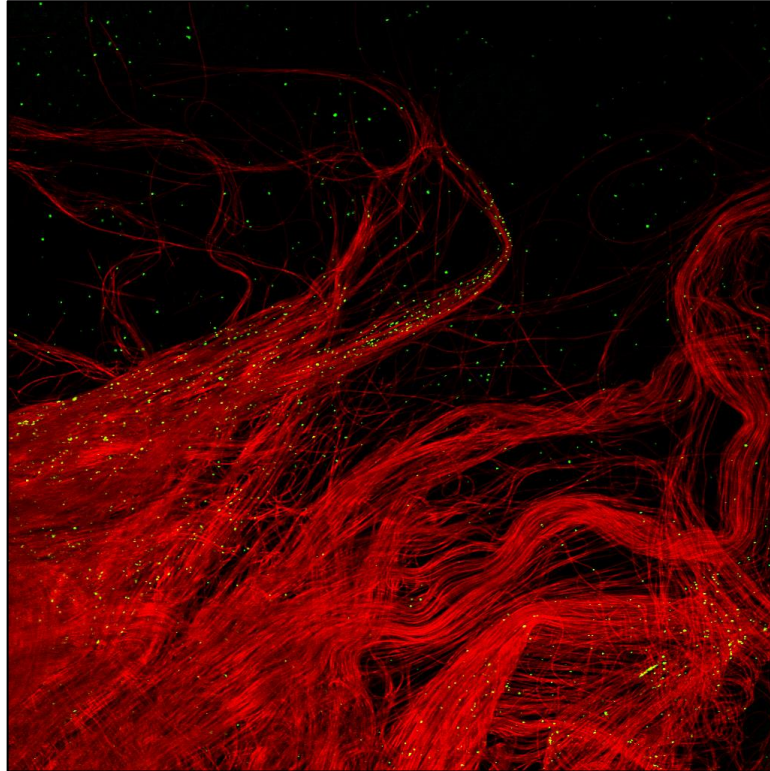


Figure 2.4: The interaction between cyanobacteria (red fluorescence) and *V. parahaemolyticus* G445 (green fluorescence) as viewed under a confocal microscope (Ward, 2014).

Islam et al., (2020) stated that cyanobacteria can be a long-term reservoir for *V. cholerae* O139 for up to 15 months due to the ability of the cyanobacteria (*Anabaena variabilis*) to encase the cells of *V. cholerae* within mucilaginous sheaths that causes *V. cholerae* to enter a Viable but non-Culturable state (VBNC) (Figure 5). Prior study by Islam et al., (2004) stated that *V. cholerae* O139 can be isolated for up to 12 days in a culturable form when cyanobacteria is present but were unable to be isolated after 2 days without the presence of cyanobacteria. Other study by Eiler et al. (2007) stated that cyanobacterial organic matters play an essential role in the growth and abundance of *V. cholerae* and *V.*

vulnificus, therefore, strengthening the possibility of the interaction that exists between these two bacterial species.

However, other studies have found no or limited associations between *Vibrio* species and cyanobacteria. The low density gathered of cyanobacteria in Pacific Northwest in the USA in 2015 suggests minor impact on the concentration of *Vibrio* (Paranjpye et al., 2015). The association is thought to differ according to regional factors, seasonal temperature and species involved. Another research reported the lack of evidence between the effect of planktonic bloom on *V. parahaemolyticus* existence (Julie et al., 2010). In the study, chlorophyll a were proven to be impactful to the *V. parahaemolyticus* present in mussels (Julie et al., 2010). However, since chlorophyll a can be found not only in cyanobacteria but also green algae and plants, this leaves open the possibility that cyanobacteria might not be the exact variable in affecting the population of *Vibrio* species.

Research by Greenfield et al., (2017) argued that the presence of *Vibrio* species (*Vibrio vulnificus* and *V. parahaemolyticus*) is dependent on seasonal temperature. The research concluded there was a very low to no correlation between *Vibrio* species population with phytoplankton during winter. *Vibrio* populations also lack the correlation with chlorophyll a (Greenfield et al., 2017). Turner et al., (2009) found out that seasonality and species-specific taxa plays a role in the determination of *Vibrio* species' population density and their associations with cyanobacteria. This result was consistent with their finding that the presence of copepods, cyanobacteria, and diatoms had an antagonistic relationship with *Vibrio* concentrations (Turner et al., 2009). Overall, the evidence on the association between cyanobacteria and *Vibrio* species is limited, inconclusive and involves complex multidimensional landscape to understand the relationship between these two groups.

2.8 Canonical Correspondence Analysis (CCA)

Canonical Correspondence Analysis (CCA) is a statistical method that is used to analyse the relationships between a set of predictor variables and a set of response variables (ter Braak, 1986). In microbiology, CCA is often used to study the relationships between a microbial community's composition and various environmental factors (Kuczynski et al., 2010). CCA also allows researchers to identify the environmental variables that are most strongly correlated with the composition of a microbial community. This is important because it helps researchers to understand how microbial communities survive in different environmental conditions and how these responses may be affected by external changes (Hou et al., 2017).

CCA has immensely contributed to our knowledge of the ecology and behaviour of microbial inhabitants (Zheng et al., 2019). In CCA, the predictor variables are typically the environmental variables thought to influence the microbial community, such as temperature, pH, and nutrient levels (Zheng et al., 2019). The response variables are the population of microbes that are present in the community, according to their taxa (ter Braak, 1986). For example, CCA has been used to study the impacts of land use change on soil microbial communities (Hou et al., 2017) and to investigate the relationships between aquatic microbial communities and water quality parameters (Breton-Deval et al., 2019).

2.9 Virulent Genes of *V. cholerae* and *V. parahaemolyticus*

Virulent genes play a role in determining the ability of a bacterium to cause disease. They encode to produce proteins or other molecules that allow the bacteria to survive and thrive in the host, often at the expense of the host's health. Some virulent genes encode for toxins production (Kobayashi et al., 2013). Some are responsible for creating enzymes that

help the bacteria to evade the host's immune system (Denzer et al., 2020), and help the bacteria to stick to and occupy host tissues (Deng et al., 2020; Pizarro-Cerdá & Cossart, 2006). In *Vibrio* species, the presence of virulent genes is known and studied extensively. Several virulent genes that were known to be associated with *Vibrio* species are *tdh*, *trh*, *tlh*, *ctxB*, *rtxA* and *rtxC*.

tdh and *trh* genes are genes that code to produce two related toxins called thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in certain *Vibrio* species, primarily *V. parahaemolyticus*. TDH and TRH are considered major virulence factors in *V. parahaemolyticus* (Raghunath, 2015). The presence of *tdh* and *trh* genes are commonly used as a marker to identify the virulent strain of *V. parahaemolyticus* (Sun et al., 2022). TDH is a toxin that can harm cells in many ways. It can break open red blood cells, harm other types of cells, and even damage the heart (Raghunath, 2015). TDH works by forming small openings, or pores on the surface of cells (Matsuda et al., 2010). These pores are about 2 nanometers in size, causing the unintentional flush of water and ions from the host cells (Matsuda et al., 2010; Honda et al., 1992). Both genes, *trh* and *tdh* share approximately 70% similarity in their structure (Kishishita et al., 1992). Similar to TDH, TRH activates chloride ion channels resulting in altered ion flux (Raghunath, 2015; Takahashi et al., 2000). *tdh* and *trh* are mostly found in clinical strains isolated from patients suffering from *V. parahaemolyticus* gastroenteritis (Klein et al., 2014) however, the detection rate of *trh* gene is relatively more in environmental strains (Raghunath, 2015). Both genes are considered as virulence factor, and play crucial role in causing infections like food poisoning in humans. The mechanism for *tdh* and *trh* toxin gene from *V. parahaemolyticus* can be observed in Figure 2.5.

Thermolabile hemolysin (TLH) is a toxin produced by certain strains of the bacterium *Vibrio*. The gene that encodes for the production of this toxin is called the *tlh* gene. The TLH toxin produced by *Vibrio* species can cause hemolysis, which is the rupture of red blood cells (Broberg et al., 2011). The gene encoding the toxin, *tlh*, encodes phospholipase A2, while its contribution to *V. parahaemolyticus* pathogenicity is unknown, expression of this gene is pertinent in the human intestine (Klein et al., 2014). It is important to note that the presence of *tlh* gene does not necessarily mean that the strain is pathogenic, it could be only a marker for certain *Vibrio* species (Hasrimi et al., 2018). The mechanism for *tlh* toxin gene from *V. parahaemolyticus* can be observed in Figure 2.5.

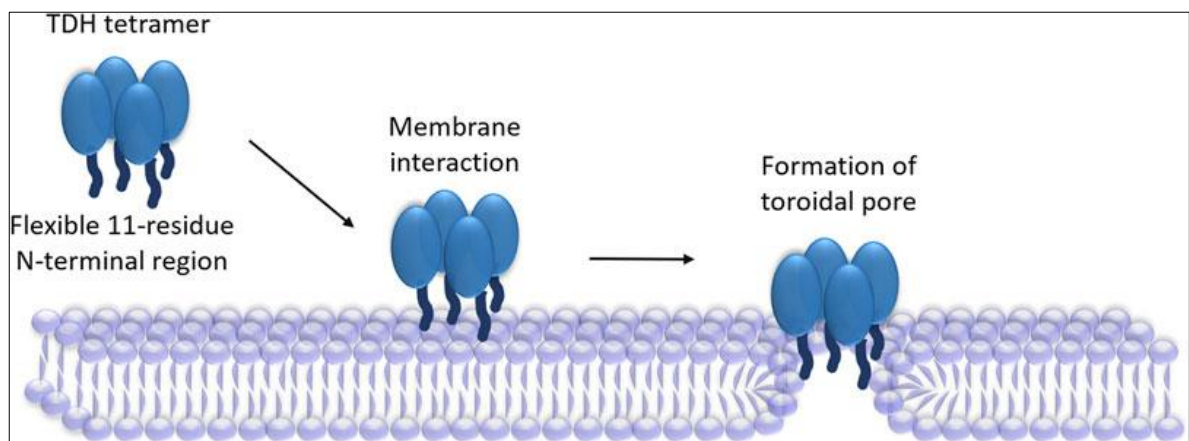


Figure 2.5: TDH works by forming pore on cell membrane, causing the burst of red blood cell. TRH and TLH can work in a similar way (Verma & Chattopadhyay, 2021).

ctx gene, (short for Cholera Toxin) encodes the production of cholera toxin in certain *Vibrio* species, most notably *V. cholerae*. CTX is thought to originate from a filamentous bacteriophage called the CTX ϕ (Mahboobi et al., 2020). The gene is found on a mobile phage, which can be transferred between different strains of *V. cholerae* through a process known as lysogeny (Davis et al., 1999). Cholera toxin is a protein that causes the symptoms of cholera, a severe diarrheal disease that can be fatal if left untreated (Holmgren, 1981).

Once inside the host (a human or other animal cell), the toxin *ctxB* works together with *ctxA* to bind to a holotoxin (specific receptor) on the cells lining the small intestine, which triggers an increase in the amount of water and electrolytes that are secreted into the intestinal lumen (Madhusudana & Surendran, 2013). This leads to severe diarrhoea, which can cause dehydration and electrolyte imbalance that can be fatal if not treated promptly (Guichard et al., 2013). The molecular mechanism for *ctxB* toxin gene from *V. cholerae* can be observed in Figure 2.6.

The *rtxA* and *rtxC* genes code to produce a type of toxin called RTX toxins in certain *Vibrio* species. RTX toxins are mostly known to pose the hemolytic activity (lysing blood) and cytotoxic ability that causes damage to cell membrane (Wiles & Mulvey, 2013). The unique feature of RTX is the way it exports proteins, and it is characterized by repeating sequences of nonapeptide, glycine, aspartate-rich repeats that bind with calcium ions (Linhartová et al., 2010). The RTX protein family contains secreted proteases and lipases that work together to cause damage to body tissues and cause inflammation when the bacteria infect the host (Linhartová et al., 2010). *rtxC* controls the expression of RTX toxin that is encoded by *rtxA* (Osickova et al., 2018). RTX exoproteins are shared between Gram-negative bacteria and are steadily growing and constructing various biological functions (Linhartová et al., 2010). The Gram-negative bacteria that were known to possess RTX toxins are of the genera *Escherichia*, *Bordetella*, *Proteus*, *Morganella*, *Moraxella*, *Vibrio* and *Kingella*, and members of the Pasteurellaceae family (*Mannheimia*, *Pasteurella* and *Aggregatibacter*) (Linhartová et al., 2010). The mechanism for *rtx* toxin gene from *V. cholerae* can be observed in Figure 2.6.

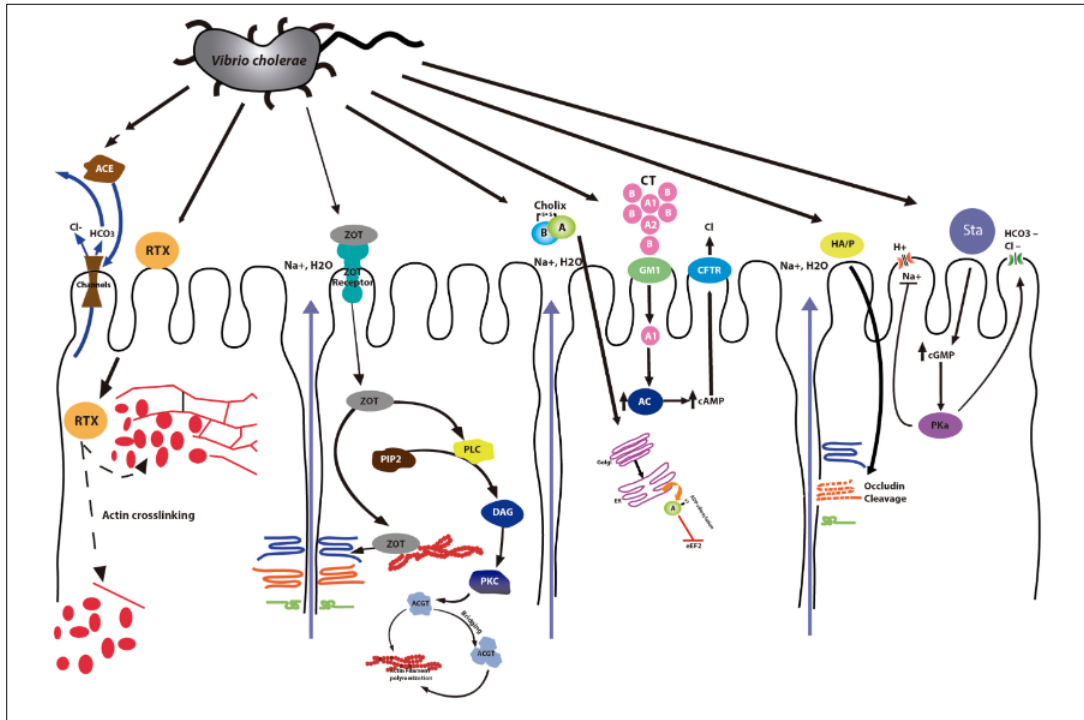


Figure 2.6: CTX (labelled as CT (Cholera Toxin)) binds to the specific receptor that cascades into event of the release of chloride ions into the lumen and causes diarrhea and dehydration. RTX proteins causes the disruption of actin crosslinking, leading to cytotoxicity and cell death (Pérez-Reytor et al., 2018).

Other factors, such as the overall genetic makeup of a bacterium and the susceptibility of the host, can also play a role in the ability of a bacteria to cause disease (Doron & Gorbach, 2008). It is worth noting that there is a great diversity among *Vibrio* species, and their virulence factors can vary depending on the strain or even the isolation. The molecular mechanisms of pathogenesis and host-microbe interactions in *Vibrio* species are still being studied, and many aspects are not fully understood.

2.10 Antibiotic Resistance of *Vibrio* Species in Shrimp Farm

The vulnerability of shrimps towards diseases are attributed to a range of factors, such as overfeeding and high stocking density in stagnant water which provide a breeding ground for the proliferation of *Vibrio* (Alfiansah et al., 2018) therefore compelled the use of antibiotics due to the lack of other alternative treatments (Thornber et al., 2020). Antibiotics such as tetracycline, norfloxacin, oxytetracycline, enrofloxacin, and sulphonamides (Holmström et al., 2003; Luu et al., 2021) are often used to fight off infections caused by pathogenic bacteria in shrimp farms. They are used often without a veterinary diagnosis, and are prescribed by feed dealers, drug sellers, and farmers themselves (Chowdhury et al., 2022). Research conducted by Holmström et al., (2003) confirmed that 74% of 76 farmers interviewed in the study used at least 13 different antibiotics to treat shrimps' diseases but rather loosely equipped with the proper knowledge of the handling practice of the antibiotics. An environment with high stress like a shrimp farm, the proliferation of antibiotic resistant bacterial strain is often anticipated. Antibiotic resistance is the term used to describe a bacteria's increased ability to withstand the effects of antibiotics, to which they were previously vulnerable (Osunla & Okoh, 2017). Despite the efficiency of antibiotics in eradicating shrimp diseases, improper antibiotic use in the aquaculture environment poses a risk of the potential transfer of resistance from aquaculture bacteria to human and animal pathogens (Abdullahi et al., 2013).

Just like any other bacteria, *Vibrio* species can develop antibiotic resistance as a naturally occurring survival technique (Figure 2.7). The way in which *Vibrio* species develop resistance to antibiotics can be broadly divided into two categories: intrinsic and acquired (Dutta et al., 2021). Intrinsic resistance is determined when the bacteria themselves have

evolved over time to become resistant to certain antibiotics, while acquired resistance occurs when the bacteria acquire resistance genes through the transfer of genetic material via horizontal and vertical gene transfer. Intrinsic resistance mechanism involves limiting the uptake of drugs by the adjustment in cell permeability, drugs efflux pump, modification in the drug's usual target site, the destruction of the drug and metabolism alteration (increase in tolerance towards drugs) (Dutta et al., 2021). Acquired resistance occurs when the *Vibrio* species is armed with mobile genetic elements like plasmid, integrating conjugative elements, superintegron, transposable elements, and insertion sequences, that contains genetic materials for antibiotic resistance genes (Dutta et al., 2021). These mobile genetic elements can bypass bacteria cells by using HGT and propagate them into the next generations (Dutta et al., 2021).

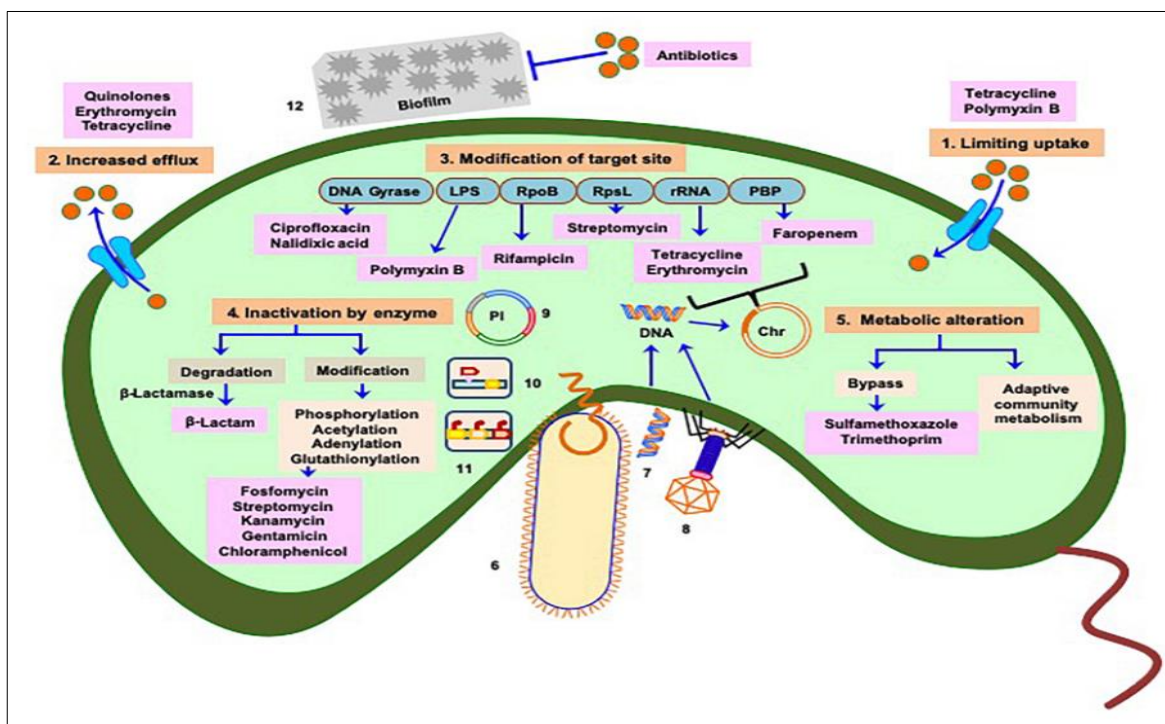


Figure 2.7: The molecular mechanism of antibiotic resistance genes in *Vibrio* species. 1: limiting the uptake of drugs by the adjustment in cell permeability, 2: drugs efflux pump, 3: modification in the drug's usual target site, 4: the destruction of the drug, 5: metabolism alteration, 6,7,8: acquirement of antibiotic resistant genes from conjugation, transformation, and transduction, respectively, 12: biofilm formation (Dutta et al., 2021).

As bacteria possess the ability to transfer their antibiotic-resistant genome across their cells (Thornber et al., 2020), they can become resistant to more than one antibiotic. This is known as Multiple Antibiotic Resistance (MAR). A MAR index greater than 0.2 indicates that isolates came from a highly contaminated source where antibiotics are frequently used (Davis & Brown, 2016). MAR cases have been reported in shrimp farms located in peninsular Malaysia, where 84% of the *Vibrio* species isolates harboured a MAR index value of more than 0.2 (Haifa-Haryani et al., 2022).

As MAR indices only indicate the number of antibiotics that the bacterial isolates are resistant to over the antibiotics used in the test, it is important for researchers to perform the profile and recognize the pattern that the isolates are most likely to show. Antibiotic patterns are also highly susceptible to changes (Ayobami, 2022); therefore, there is a need for a periodic update to their current profile. The pattern of antibiotic resistance varies between regions and countries according to the level of antibiotic consumption controlled and regulated by the antibiotic policy of a particular country (Zanichelli et al., 2018).

In shrimp farms, resistance towards antibiotics of *Vibrio* species issues raises several issues towards economy, human clinical health and the marine environment. The economic burden of treating, mitigating, and finding alternative treatments for infections caused by multiple antibiotic-resistant bacteria can be significant (Joy et al., 2017). There is also the possibility that the banned export can cause a significant drop in export product yield (Behera, 2018). In the human healthcare system, an infection brought on by antibiotic resistance can reduce the range of available treatments and raise the possibility of treatment failure. Patients may require longer hospital stays, more complex treatments, and more expensive medications, all of which contribute to higher healthcare costs (Dadgostar, 2019).

The presence of antibiotic-resistant strains in cooked shrimp was found in a study (Sharma, 2021), therefore raising concerns about food safety for human consumption. The ingestion of antibiotic residues in the environment causes alterations to the human microbiome, creating an enabling condition for bacteria resistance to emerge and propagate within the human body (Ben et al., 2019). The MAR index of aquaculture-related bacteria has been found to correlate with MAR indices from human clinical bacteria (Reverter et al., 2020). A systematic review and meta-analysis of point prevalence surveys in Asia revealed concerning levels of resistance to medically important antimicrobials in foodborne pathogens from aquatic food animals, including shrimp (Schar et al., 2021). The spread of antibiotic-resistant bacteria and genes in aquatic environments may be linked to the discharge of shrimp pond effluent into estuarine ecosystems or the exposure of harvested shrimps to antibiotic-resistant bacteria during processing (Hossain, 2012). Additionally, overuse can lead to selection pressure on environmental microbiomes, fostering reservoirs harbouring antibiotic-resistant strains that pose a potential threat to public health (Ben et al., 2019). The ecological consequences of MAR in shrimp farming present themselves as a form of disruption of microbial communities and the potential for the spread of resistance genes in the environment (Ben et al., 2019). Antibiotic residues, antibiotic-resistant bacteria, and resistance genes are considered environmental pollutants and are responsible for a tenacious public health crisis throughout the globe (Xi et al., 2009).

In conclusion, the extensive use of antibiotics in shrimp farming has contributed to the significant emergence and spread of multiple antibiotic-resistant bacteria strains. This has created a public health crisis that affects not only the aquaculture sector but also human populations, as bacteria resistant to medically important antibiotics can infect humans. Furthermore, the ecological consequences of this practice threaten environmental

microbiomes. The urgency of finding alternative treatments and sustainable farming practices in the aquaculture sector is now greater than ever. Finally, the efforts to mitigate antibiotic resistance in shrimp farming need a comprehensive approach that involves stakeholder engagement, regulatory efforts, and the search for alternative medicines.

CHAPTER 3

***Vibrio* SPECIES AND CYANOBACTERIA: UNDERSTANDING THEIR ASSOCIATION IN SELECTED LOCAL SHRIMP FARM USING CANONICAL CORRESPONDENCE ANALYSIS (CCA)**

3.1 Introduction

The Vibrionaceae genus, comprising diverse bacterial species, has captivated scientists due to its adaptability and wide-ranging habitats. They possess a curved morphology and exhibit flagellated motility on one end, measuring 0.5-0.8 μm in width and 2-3 μm in length (Venkateswaran, 1999). In clinical microbiology and food safety, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, and *V. mimicus* are among the *Vibrio* species that are potentially dangerous to humans (Izumiya et al., 2011). Their prevalence and pathogenicity in the environment raise concerns, as *Vibrio* is still endemic in certain countries (Cartwright & Griffin, 2012).

The common track record of *Vibrio* species in causing the infection is further enhanced by the fact that they can infect not only humans but also marine animals, such as shrimp, therefore leading to vibriosis. The shrimp farming industry regards vibriosis as a significant threat as it can affect the entire life cycle of shrimp, ranging from eggs to brood stock, which may lead to the complete eradication of an entire population in case there is an outbreak (Prayitno & Latchford, 1995; Harris & Owens, 1999). These infections can spread rapidly within shrimp ponds due to the close proximity and crowded conditions in aquaculture settings (Duc, 2015). Since *Vibrio* species are readily present in bodies of warm saltwater (Canellas et al., 2021), regular monitoring of their population is needed to prevent vibriosis infection in shrimp culture.

Shrimp farming is one of the most significant products for the Malaysian aquaculture industry. Among the Association of Southeast Asian Nations (ASEAN) countries, Malaysia has maintained its competitiveness in exporting fresh shrimp, alongside Thailand (Ismail & Abdullah, 2013). In Sarawak, the state's Modernization of Agriculture and Regional Development Minister aims to expand shrimp farming with the goal of producing RM 1 billion of exports by 2030 (Sulok Tawie, 2022). The shrimp farm selected for this study achieved the production of 165 metric tonnes of shrimp in 2021 (Lembaga Kemajuan Ikan Malaysia (LKIM), 2022), which marks great potential for the goal. As shrimp farming in Malaysia has the potential to be profitable and contribute to the country's economic expansion, effective disease prevention measures are essential to avoid profit loss from vibriosis-related infections.

It's been demonstrated through previous research that cyanobacteria play a crucial role in the ability of *Vibrio* to persist in our environment (Islam et al., 2004). These bacteria possess the unique ability to conduct photosynthesis, and they exist in either coccoid unicellular, coccoid multicellular, or filamentous forms (Mur et al., 1999). Some cyanobacterial species reside in the same habitat as *Vibrio*, supplying dissolved organic matter for the *Vibrio* community (Eiler et al., 2007). Both genera of bacteria can rapidly multiply under certain conditions, such as poor nutrition, oxygen depletion, and low water quality (Dawes, 1989), which can create a stressful environment for shrimp. As microorganisms are very much able to create symbiotic relationships, more research is required to better understand them.

Despite the evidence of their association, there have been a number of studies that have demonstrated contradictory results. Some isolated strains of cyanobacteria have been

found to exhibit antibacterial properties against pathogenic microorganisms, indicating that they can potentially eradicate *Vibrio* species (Rojas et al., 2020). Another study has found that the relationship between cyanobacteria and *Vibrio* species depends on the growth stage of the cyanobacteria (King et al., 2022). Therefore, there is a need to conduct research on the interaction between cyanobacteria and *Vibrio* species, particularly in the context of shrimp farms, where favourable conditions for the proliferation of both bacteria genera are naturally present.

Aside from the potential association with cyanobacteria, water parameters such as temperature, salinity, and pH can also influence the growth and abundance of *Vibrio* species (Larsen, 1984) in shrimp farms. For example, high water temperatures can provide optimal conditions for the growth of *Vibrio* species (Sheikh et al., 2022). Shrimps are equally sensitive to changes in their environment, and stressors such as suboptimal water quality can weaken their immune systems (Chen & He, 2019), making them more susceptible to infections from *Vibrio* species. On the contrary, the maintenance of optimum salinity can lead to better survival, weight gain, and protein retention rates in reared shrimp (Maicá, 2014).

To find out the correlation between both *Vibrio* species and cyanobacteria with water parameters such as pH, salinity, and temperature as additional variables, a statistical method called Canonical Correspondence Analysis (CCA) was employed. Previous studies have used CCA as a tool to analyze the microbial communities of aquatic environments, and it has been effective in elucidating the correlation between *Vibrio* populations, particulate organic matter, and environmental factors (Kopprio et al. 2020). By implementing CCA as

the statistical method for the study, the association between cyanobacteria and *Vibrio* species can be determined via their growth patterns.

This research is focused on shrimp farming and has several aims: Quantifying the number of *Vibrio* species and cyanobacteria in a nearby shrimp farm, detecting the presence of two major *Vibrio* species (*V. parahaemolyticus* and *V. cholerae*) that are known to be harmful to people and shrimp, figuring out which cyanobacterial genera are present there, and then calculating any possible relationships between the two genera and the water quality parameters. Methods such as Most Probable Number (MPN), Duplex-Polymerase Chain Reaction (D-PCR), Sedgwick-Rafter cell counting, Differential Interference Contrast (DIC) microscopy using an inverted microscope, and Canonical Correspondence Analysis (CCA) were implemented in this study to achieve the objectives of this study.

3.2 Methodology

3.2.1 Water Sampling

Water sampling was commenced biweekly (August – December 2021) in two White-leg shrimp (*Litopenaeus vannamei*) shrimp ponds (Pond A and Pond B), effluent and influent water (Figure 3.1) of Persatuan Nelayan Kawasan Satang Biru, Telaga Air, Kuching, Sarawak, Malaysia (1°40'34.7" N 110°12'10.8" E). The sampling campaign was executed within the shrimps' post-larvae stocking to harvesting time frame. A total of six ($n=6$) samples from Pond A, six ($n=6$) samples from Pond B, ten ($n=10$) samples from effluent and ten ($n=10$) samples from influent were successfully collected, making up to thirty-two ($n=32$) samples. A plankton net was used to sift water from shrimp ponds to collect a concentrated amount of cyanobacteria inside 50 mL Falcon tubes. Environmental parameters

such as water temperature, salinity and pH were also recorded during the sampling activity. The samples were then transported to the laboratory under aseptic conditions within 2 hours.



Figure 3.1: Map of the sampling sites at Persatuan Nelayan Kawasan Satang Biru, Telaga Air, Kuching, Sarawak, Malaysia: (A) Pond A; (B) Pond B; (C) Effluent; (D) Influent.

3.2.2 Enrichment of *Vibrio* Species

A total of one mL of the water samples were pipetted into 9 mL of Alkaline Peptone Water (APW) (HiMedia, India) to enrich *Vibrio* species in the samples. The cultures were then incubated overnight at 37 °C. Remaining water samples were treated with Lugol's solution for cyanobacteria counting step.

3.2.3 Dilution of *Vibrio* Species Culture

After enrichment, 1 mL of culture broths were diluted up to 10^{-5} . Each dilution was incubated overnight at 37 °C.

3.2.4 Most Probable Number (MPN)

MPN method from United States Food and Drug Administration's Bacterial Analytical Manual (BAM), (2020) was employed to enumerate the *Vibrio* species present in each water sample. This is done by making serial dilutions of the water samples and inoculating aliquots into broth media to detect bacterial growth. Firstly, 1 mL of the broths from the selected dilutions (10^{-2} , 10^{-3} and 10^{-4}) were inoculated into triplicates of 10 mL APW. The culture broths were then incubated for 24 hours at 37 °C. The tubes that went turbid from each triplicate of MPN tubes from every dilution indicate growth for *Vibrio* species. The number of tubes that went turbid from each triplicate was recorded and the result was referred from the MPN table (BAM, 2020). In this study, we used a different approach compared to the traditional MPN table for presenting our results. Instead of using dilutions of 10^{-1} , 10^{-2} and 10^{-3} like in a normal MPN table, we used dilutions of 10^{-2} , 10^{-3} and 10^{-4} . This modification allowed us to obtain accurate measurements and evaluate the microbial population more effectively. Therefore, each result for MPN/mL was multiplied by 10 to match the table configuration (Sutton, 2010). From each MPN tube that was incubated, a loopful of broth was streaked on the surface of Thiosulfate-citrate-bile salts sucrose (TCBS) agar (HiMedia, India) and subsequently incubated overnight at 37 °C for *Vibrio* species confirmation.

3.2.5 DNA Extraction of *Vibrio* Species

The method was modified from Peng et al., (2013) to fit the requirement of this study. 500 µL of the culture broth were pipetted into 1.5 mL microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded before adding 100 µL of deionized distilled water (ddH₂O). The mixture was then boiled for 10 minutes, and snap

cooled in ice for 5 minutes. Lastly, each tube was centrifuged for 10 minutes at 10,000 rpm. The clear solution is the product of the DNA extract.

3.2.6 Polymerase Chain Reaction (PCR)

PCR was done by adding exTEN PCR 2X Master Mix (Base Asia), 2 pairs of forward and reverse primers (Kim et al., 2015) as specified in Table 3.1, nuclease-free water and DNA extracts from each sampling. The volume of each reagent was shown in Table 3.2, while the amplification of DNA sequence was done by using thermal cycler (Eppendorf Mastercycler Gradient) with the conditions shown in Table 3.3.

Table 3.1: Primer pairs and their sources used for the identification of *V. cholerae* and *V. parahaemolyticus*.

Target genus or species	Primer name	Protein of target gene	Primer sequences	PCR base pair
<i>V. parahaemolyticus</i>	VP 1155272 F	Hypothetical protein VPA1095	5' - AGCTT ATTGG CGGTT TCTGT CGG – 3'	297
	VP 1155272 R		5' - CCAA GACCA AGAAA AGCCG TC – 3'	
<i>V. cholerae</i>	VC C634002 F	Hypothetical protein VCA0694	5' - CAAGC TCCGC ATGTC CAGAA GC – 3'	154
	VC C634002 R		5' - GGGGC GTGAC GCGAA TGATT – 3'	

Table 3.2: PCR reagent for the identification of *V. cholerae* and *V. parahaemolyticus*, forming 17 µL of aliquot.

PCR reagent	Volume per reaction (µL)
exTEN 2X PCR Master Mix (Base Asia)	12.5
10 pmol/µL of primer VP 1155272 F	0.6
10 pmol/µL primer VC C634002 F	0.6
10 pmol/µL primer VP 1155272 R	0.6
10 pmol/µL primer VC C634002 R	0.6
Nuclease-free water	1.1
DNA extract	1.0
Total volume	17

Table 3.3: PCR condition for the detection of *V. cholerae* and *V. parahaemolyticus*.

Step Cycle	Temperature/Time
Initial denaturation	94 °C (6 min)
Denaturation	94 °C (30 sec)
Annealing	62 °C (2 min) (35 cycles)
Extension	72 °C (1 min 50 sec)
Final extension	72 °C (6 min)

3.2.7 Agarose Gel Electrophoresis (AGE)

The PCR products were loaded into 1.5% agarose gel and were charged with 80V electric current for 1 hour prior to being stained for 45 minutes with 0.1% of Ethidium Bromide (EtBr). The bands formed were then observed by using UV transilluminator before being photographed.

3.2.8 Quantification and Morphological Identification of Cyanobacteria

A total of 1 mL of water samples that were previously preserved with Lugol's solution were pipetted by using Pasteur pipette into Sedgewick-Rafter counting chambers exactly filling it while avoiding the formation of air bubbles. The water sample were then given about 5-10 minutes to settle down before being counted grid by grid using compound microscope starting from left to right (Watzin et al., 2006). This process was repeated 3 times for each sample to gather the average count of the result. The number of cells per filament of the cyanobacteria were obtained by multiplying the total filaments counted with the average numbers of cells of the first 30 filaments (Chorus & Bartram, 1999). The morphological identification of cyanobacteria was done by using an Inverted Microscope: Olympus FluoView300 (Olympus Corporation, Japan). The microscope incorporates the Differential Interference Contrast (DIC) filter in the process that can produce high quality, pseudo-3D images of the cyanobacteria (Scientifica, n.d.). The camera used to capture the images were called Infinity 3 (Teledyne Lumenera, Ottawa, Canada) while the software used to analyse the image was Infinity Analyze (Teledyne Lumenera, Ottawa, Canada).

3.2.9 Canonical Corresponding Analysis (CCA)

Data regarding the environmental variables obtained during the sampling activity, *Vibrio* and cyanobacteria population was used to recognize any significant relationship between all the variables. The *p*-values are provided to indicate the statistical significance of *Vibrio* species and cyanobacteria. A *p*-value that is greater than 0.01 is considered not significant, while a *p*-value that is less than or equal to 0.01 indicates a significant relationship between the variables. The analysis was performed with PAST software version 4.03 which is available free on the internet at the time of writing.

3.3 Results

3.3.1 Environmental Parameters

Based on Table 3.4, the pond water pH ranges from 6.14 to 7.64, indicating that they were slightly acidic to neutral. While influent water was neutral, pond B was shown to develop the greatest amount of acidity in average. Minimal changes in water temperature for both ponds, effluent, and influent can be observed as they only slightly fluctuate. The salinity of ponds A and B both generally increased as the sampling process came to a close, contributing to the mean of 24. Evidently, the influent water had the lowest salinity compared to all sampling sites.

Table 3.4: A summary on the selected environmental parameters involved in this study.

Sampling sites	Duration of sampling	Total samples	Water pH (mean),	Water temperature (mean), (°C)	Water salinity (mean), (ppt)
Pond A	10 August -19 October 2020	± 6	± 6.59	± 31.5	± 24
Pond B	5 October – 14 December 2020	± 6	± 6.14	± 30.8	± 24
Effluent	10 August – 14 December 2020	± 10	± 6.57	± 30.9	± 23
Influent	10 August – 14 December 2020	± 10	± 7.64	± 30.9	± 17.4

3.3.2 Growth Pattern and Identification of *Vibrio* Species

3.3.2.1 Pond A

The growth of *Vibrio* showed a notable rise during the second sampling and remained consistent until the conclusion of all samplings. *Vibrio* species continued to be abundant until the end of the sampling cycle. In summary, PCR detection of *V. cholerae* and *V. parahaemolyticus* in Pond A shows 72% positive detection of *V. cholerae* and 78% of *V. parahaemolyticus* (Figure 3.2 and Table 3.5).

Table 3.5: The growth profile of *Vibrio* species in Pond A.

Number of samplings	Sampling site triplicates	MPN/10 mL
Sampling 1	A	>11000
	B	<30
	C	<30
Sampling 2	A	<30
	B	>11000
	C	<30
Sampling 3	A	>11000
	B	>11000
	C	>11000
Sampling 4	A	>11000
	B	>11000
	C	>11000
Sampling 5	A	>11000
	B	>11000
	C	>11000
Sampling 6	A	>11000
	B	>11000
	C	>11000

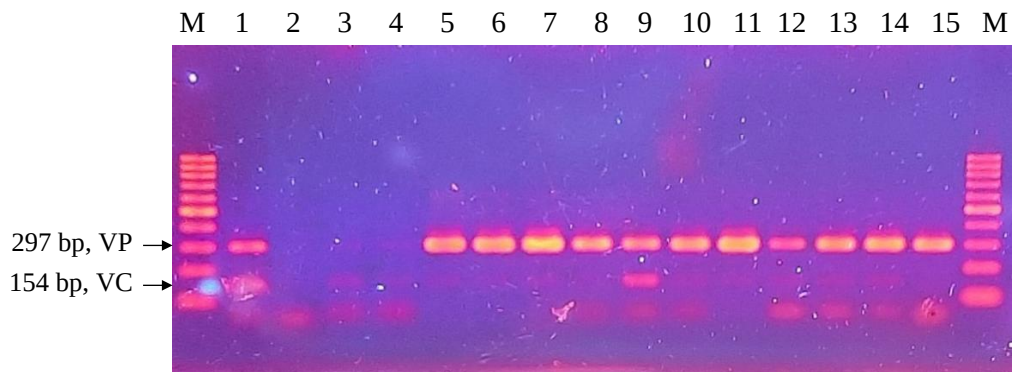


Figure 3.2: Duplex PCR result for the detection of *V. parahaemolyticus* and *V. cholerae* for Pond A viewed in 1.5% agarose gel. **M:** 100 bp DNA ladder, **1:** positive control of *V. cholerae* and *V. parahaemolyticus*, **2:** negative control, **3:** Sampling 1, **4:** Sampling 2, **5-7:** Sampling 3, **8-9:** Sampling 4, **10-12:** Sampling 5, **13-15:** Sampling 6. Positive detection for *V. parahaemolyticus* is seen in lane 3 to 15, while positive detection for *V. cholerae* is seen in lane 3 to 14. *V. cholerae* bands appears to be faint when compared to *V. parahaemolyticus*.

3.3.2.2 Pond B

The *Vibrio* species demonstrated rapid growth, reaching peak levels during each sampling period. According to the findings obtained through the Duplex PCR technique, *V. cholerae* and *V. parahaemolyticus* were detected with a prevalence of 61% and 100%, respectively (Figure 3.3 and Table 3.6).

Table 3.6: The growth profile of *Vibrio* species in Pond B.

Number of samplings	Sampling site triplicates	MPN/10 mL
Sampling 1	A	>11000
	B	>11000
	C	>11000
Sampling 2	A	>11000
	B	>11000
	C	>11000
Sampling 3	A	>11000
	B	>11000
	C	>11000
Sampling 4	A	>11000
	B	>11000
	C	>11000
Sampling 5	A	>11000
	B	>11000
	C	>11000
Sampling 6	A	>11000
	B	>11000
	C	>11000

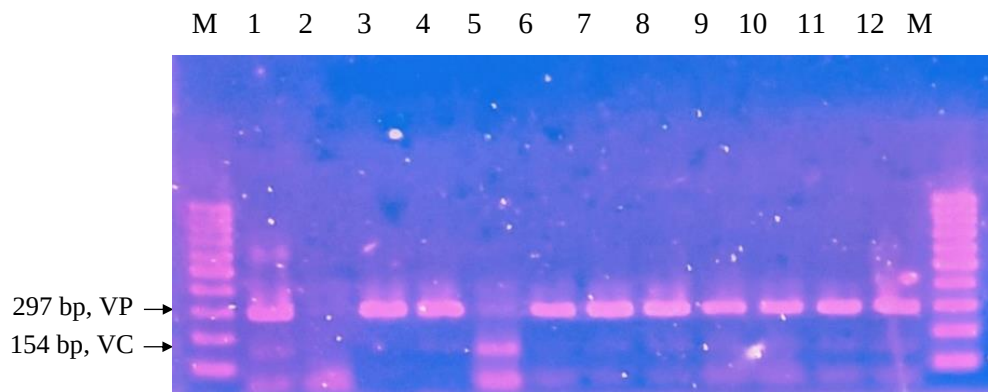


Figure 3.3: Duplex PCR result for the detection of *V. parahaemolyticus* and *V. cholerae* for Pond B viewed in 1.5% agarose gel. **M:** 100 bp DNA ladder, **1:** positive control of *V. cholerae* and *V. parahaemolyticus*, **2:** negative control, **3-4:** Sampling 1, **5:** Sampling 2, **6:** Sampling 3, **7-8:** Sampling 4, **9-10:** Sampling 5, **11-12:** Sampling 6. All samples show positive detection of *V. parahaemolyticus* while the positive detection of *V. cholerae* is shown in lane 4, 5, 7, 8, 9, 10, 11 and 12. *V. cholerae* bands appears to be faint when compared to *V. parahaemolyticus* with the exception of lane 5.

3.3.2.3 Effluent

The initial effluent water sampling demonstrated minimal *Vibrio* proliferation, while the following samples indicated a rapid escalation in bacterial growth that remained consistent until the completion of the sampling period. The detection of *V. cholerae* and *V. parahaemolyticus* is observed in Figure 3.4 while the summary from Table 3.7 gathered 90% of both species' prevalence.

Table 3.7: The growth profile of *Vibrio* species in effluent water.

Number of samplings	MPN/10 mL
Sampling 1	<30
Sampling 2	>11000
Sampling 3	>11000
Sampling 4	>11000
Sampling 5	>11000
Sampling 6	>11000
Sampling 7	>11000
Sampling 8	>11000
Sampling 9	>11000
Sampling 10	>11000

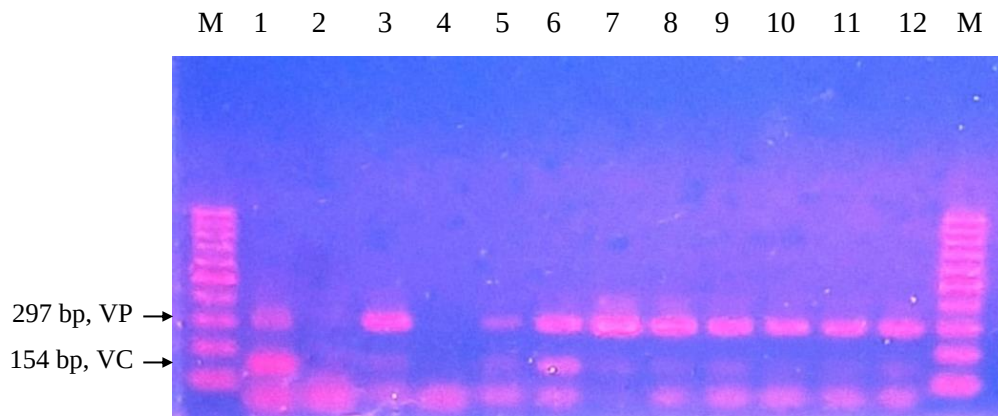


Figure 3.4: Duplex PCR result for the detection of *V. parahaemolyticus* and *V. cholerae* for effluent water viewed in 1.5% agarose gel. **M:** 100 bp DNA ladder, **1:** positive control of *V. cholerae* and *V. parahaemolyticus*, **2:** negative control, **3-4:** Sampling 2, **5:** Sampling 3, **6:** Sampling 4, **7:** Sampling 5, **8:** Sampling 6, **9:** Sampling 7, **10:** Sampling 8, **11:** Sampling 9, **12:** Sampling 10. Positive detection of *V. parahaemolyticus*. can be seen in lane 3, 5, 6, 7, 8, 9, 10, 11 and 12, same with *V. cholerae*. *V. cholerae* bands appears to be faint when compared to *V. parahaemolyticus* with the exception of lane 6.

3.3.2.4 Influent

Vibrio growth can be seen off to a gradual increase during earlier sampling before the number persistently soared from the third sampling until the end. As observed in Figure 3.5, the Duplex PCR result of *V. cholerae* and *V. parahaemolyticus* showed successful detection of both species with 70% of *V. cholerae* and 90% of *V. parahaemolyticus* prevalence (Table 3.8).

Table 3.8: The growth profile of *Vibrio* species in influent water.

Number of samplings	MPN/10 mL
Sampling 1	150
Sampling 2	350
Sampling 3	>11000
Sampling 4	>11000
Sampling 5	>11000
Sampling 6	>11000
Sampling 7	>11000
Sampling 8	>11000
Sampling 9	>11000
Sampling 10	>11000

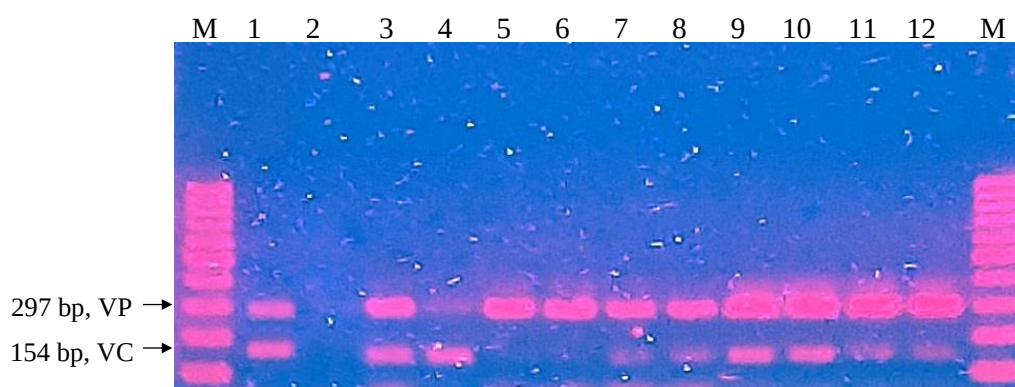


Figure 3.5: Duplex PCR result for the detection of *V. parahaemolyticus* and *V. cholerae* for influent water viewed in 1.5% agarose gel. **M:** 100 bp DNA ladder, **1:** positive control of *V. cholerae* and *V. parahaemolyticus*, **2:** negative control, **3:** Sampling 1, **4:** Sampling 2, **5:** Sampling 3, **6:** Sampling 4, **7:** Sampling 5, **8:** Sampling 6, **9:** Sampling 7, **10:** Sampling 8, **11:** Sampling 9, **12:** Sampling 10. Positive detection of *V. parahaemolyticus* can be seen on lane 3 to 12 while positive detection of *V. cholerae* can be seen on lane 3, 4, 7, 8, 9, 10, 11 and 12. *V. cholerae* bands are faint in comparison to *V. parahaemolyticus* with the exception of lane 4.

3.3.3 Growth Pattern and Morphological Identification of Cyanobacteria

Figure 3.6 shows the growth pattern of cyanobacteria in every sampling site, across their sampling period. Pond A's cyanobacterial growth curve gradually increased during the course of the sample weeks. The 4th and 6th week, however, show a stagnant growth development. As for Pond B, up until the 8th week, cyanobacterial growth indicated an overall increase. After that, there was a slight dip in the growth curve during week 10. Overall growth pattern for effluent displayed two peaks, one during the 4th week and the other at the 10th. However, it was clear that the peak of the 10th week was higher than the peak of the 4th week. The growth curve started to decline after the 10th week. The growth of cyanobacteria in the influent water can be observed to fluctuate, thus forming two peaks, which occurred during the 4th and 10th week. In comparison to the peak from the 4th week, the peak from the 10th week appeared to be higher. There was also a noticeable decline in

growth during the 6th week, which appeared to be at the lowest point throughout the entire sampling period of this site.

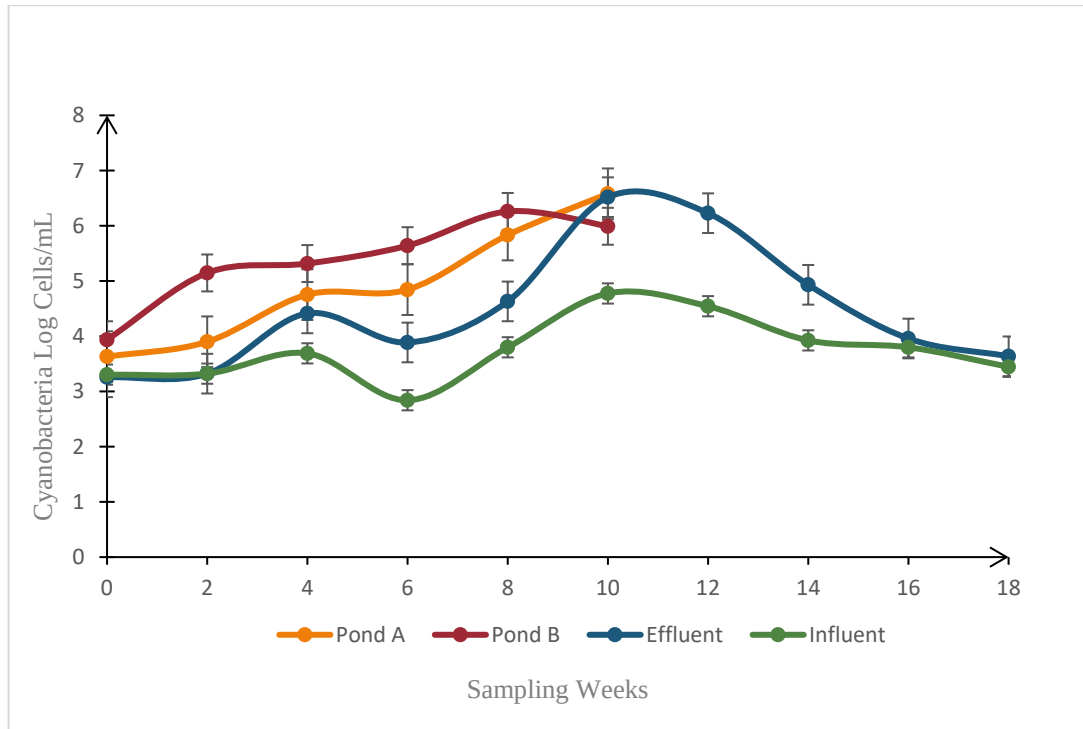


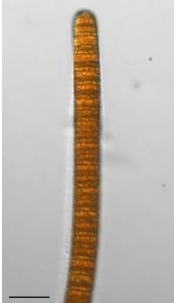
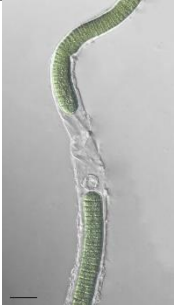
Figure 3.6: The cyanobacteria cell growth pattern in the shrimp farm.

A total of five members of the cyanobacteria genera were found residing in the shrimp farm water, namely *Chroococcus*, *Pseudanabaena*, *Phormidium*, *Oscillatoria* and *Lyngbya*. Based on cell counting with a Sedgwick-Rafter cell and morphological examination with an inverted microscope, it was evident that early samplings accumulate a greater diversity of cyanobacteria when compared to the conclusion of the sampling period, where *Pseudanabaena* genus can be seen dominating. The morphological assessment of the cyanobacterial species can be referred in Table 3.9.

Table 3.9: The morphological assessment of observed cyanobacteria in the shrimp farm water.

Labels, scale bar 100 µm	Cyanobacteria genus	Unicellular, colonial or filamentous	Cell shape	Mucilaginous sheath
	<i>Chroococcus</i>	Forms a colony of 2, 4 or 8 cells	Small, round cell with jelly- like consistency.	Colourless sheath sticks together to form a biofilm
	<i>Pseudanabaena</i> A	Filamentous and usually solitary.	Cylindrical, long cells.	Colourless.
	<i>Pseudanabaena</i> B	Filamentous and usually solitary.	Cylindrical, long cells.	Colourless.

Table 3.9 Continued

	<i>Phormidium</i>	Filamentous.	Almost uniform square stack of cells.	Colourless, thin but firm sheath.
	<i>Oscillatoria</i>	Filamentous.	Wide stack of cells that with no visible gaps.	No distinctive sheath.
	<i>Lyngbya</i>	Filamentous.	Disc-like stack of cells that appear short and wide.	Sheath can be seen extending beyond the row of cells (trichome).

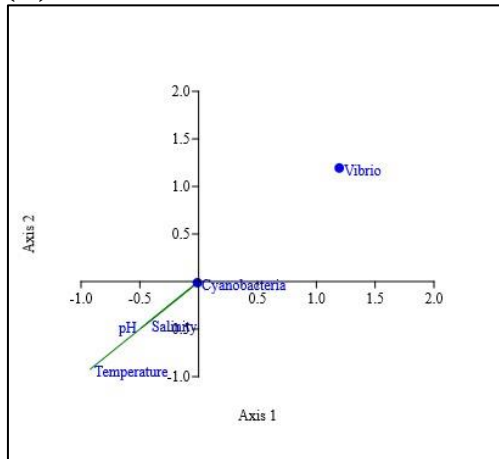
3.3.4 Canonical Correspondence Analysis (CCA)

The association between *Vibrio* species and cyanobacteria in Pond A, Pond B, effluent and influent water are shown in Figure 3.7. Canonical Correspondence Analysis (CCA) was used to calculate the p value for the correlation between both bacteria species. The water parameters were included to determine the distribution of *Vibrio* species and cyanobacteria according to abiotic factors. All p values are insignificant ($p > 0.01$).

Figure 3.10 (A) shows that the proliferation of cyanobacteria in pond A is predominantly associated with pH, salinity and temperature as opposed to *Vibrio* species. The CCA for pond B (Figure 3.10 (B)) shows that *Vibrio* growth gravitates towards pH while cyanobacteria growth gravitates towards salinity and temperature, although minimal correlation between both bacteria can be seen. In effluent water (Figure 3.10 (C)), a connection can be seen between *Vibrio* and cyanobacteria, yet, the growth of *Vibrio* is still primarily associated with pH levels rather than cyanobacteria, temperature, and salinity. Influent water shows no correlation between *Vibrio* species and cyanobacteria however, cyanobacteria correlates with all environmental parameters (Figure 3.10 (D)).

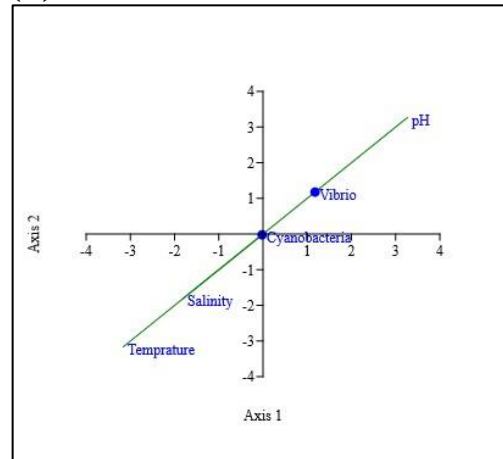
In summary, both genera have a stronger relationship with environmental factors than they do with one another. The link between both genera is proven to be inconsistent with every sampling site and are minor. Cyanobacteria can be seen to prefer salinity at all sample locations while *Vibrio* species were observed to prefer pH in pond B and effluent water. Compared to *Vibrio* species, cyanobacteria were seen to be more closely related to all environmental conditions.

(A) Pond A



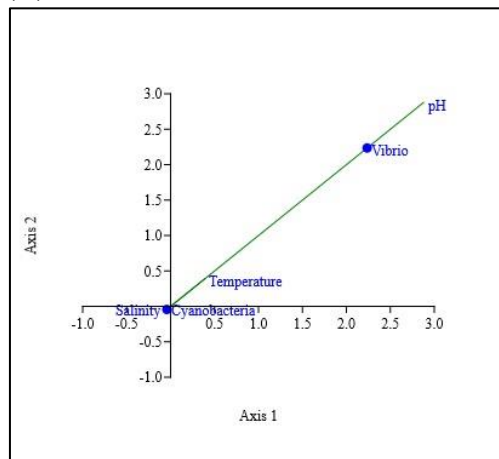
$$p = 0.847$$

(B) Pond B



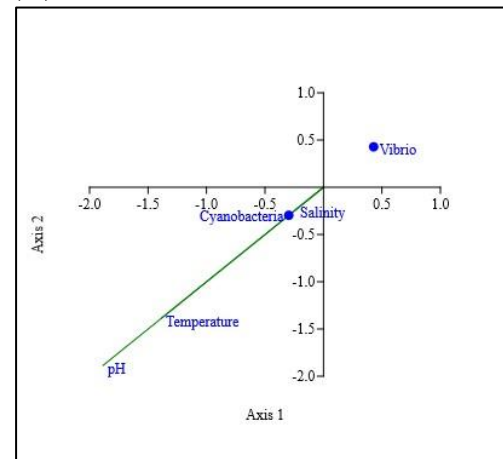
$$p = 0.255$$

(C) Effluent



$$p = 0.288$$

(D) Influent



$$p = 0.304$$

Figure 3.7: The products of canonical correspondence analysis as calculated using PAST software.

3.4 Discussion

Water quality parameters acts as a measure of health and wellbeing of shrimp culture (Carbajal-Hernández et al., 2013). Maintaining ideal environmental conditions can ensure shrimps' resilience to bacterial infections, to which they are particularly vulnerable with (Cheng et al., 2003). In the sampling location where this study was conducted, pH, salinity and temperature were monitored daily by the farmers. Mean pH for all the sampling sites were recorded to be along the line of 6.14 to 7.64. Moderate increase in acidity of the shrimp farm water was observed as the sampling progressed towards the end. This might indicate the accumulation of decaying organic matter in the shrimp farm waters (Zhang, 2017). This observation can be supported by the low pH displayed by pond A, pond B and effluent that are in contact with shrimps but not from influent where the water comes into the shrimp farm from outside source. Despite being slightly acidic, the level of pH remained in the normal range of 6.5 to 9.0 (Carbajal-Hernández et al., 2012).

In the past, shrimps have been known to be sensitive towards small changes in water temperature in a way that it can affect their sizes (Wyban et al., 1995). An increase in shrimp yield can be seen from shrimp water with the temperature between 23.5 °C and 25.5 °C or between 30 °C and 31.5 °C (Abdelrahman et al., 2019). Therefore, it has been reported that the ideal range for shrimp rearing is from 28 °C to 32 °C (Carbajal-Hernández et al., 2012). In the shrimp farm, water temperature ranges from 30.8 °C to 31.5 °C across all sampling sites. No significant fluctuations were recorded.

As for salinity, the optimum growing performance for shrimp was discovered between 20 and 30 ppt (Gao et al., 2016). Likewise, the recorded salinity of the ponds (17.4 ppt to 24 ppt) was well kept within the ideal range in all sampling sites. Shrimps can tolerate

changes in salinity level as long as proper acclimation was done before stocking the post larvae (Rahi et al., 2021). It is clear from the environmental parameters' data that daily monitoring in shrimp farms is essential in ensuring the best possible water quality for the shrimps' growth.

The growth of *Vibrio* and cyanobacteria were compared with various water parameters. Based on the canonical correspondence analysis, pH, salinity and temperature all influence cyanobacterial growth, but salinity had greater influence. This has also been proven from previous research (Silveira & Odebrecht, 2019). Salinity can affect cyanobacteria in many ways such as their cell growth and their rate of photosynthesis (Moisander et al., 2002). Cyanobacteria's tolerance towards salt might be due to the fact that they contain vast regulatory mechanism that allows them to acclimatize in various salt concentrations (Pade & Hagemann, 2014). Cyanobacteria's high tolerance towards salt has been proven beneficial in the past for treating saline soil to improve rice crop (Jan et al., 2018). In pond B and effluent water, *Vibrio* species were found to favour pH. However, other ponds (Pond A and influent) indicate no major effect of pH towards the growth of *Vibrio* species. The variation of the result might indicate the optimum pH where they grow best (Keenleyside, 2019).

In addition to water parameters, the monitoring of the population density of *Vibrio* species plays an important role in early prevention of shrimp diseases (de Souza Valente & Wan, 2021). Over the course of sampling period across multiple sampling sites, *Vibrio* species can be seen to either kick-start their growth slowly followed with a drastic increase in growth, or, maintaining a constant high growth pattern above 1,000 UFC/mL which is the maximum range of acceptable *Vibrio* population in healthy shrimp farm water (Carbajal-

Hernández et al., 2012). High concentration of *Vibrio* in water are commonly viewed as a rather undesirable attribute as they can cause outbreaks of shrimp diseases (de Souza Valente & Wan, 2021). In spite of that, it is important to consider that not all *Vibrio* species are pathogenic because they are naturally associated with shrimp gut microbiome (Zoqratt et al., 2018). Further testing for toxin genes was done to analyze the potential of the *Vibrio* species in causing infection due to the high abundance of their population.

Table 3.10: The summary of the PCR detection of *V. cholerae* and *V. parahaemolyticus* in Pond A, Pond B, effluent and influent.

<i>Vibrio</i> species	Number and percentage of positive detection			
	Pond A	Pond B	Effluent	Influent
<i>V. cholerae</i>	13/18 (72%)	11/18 (61%)	9/10 (90%)	7/10 (70%)
<i>V. parahaemolyticus</i>	14/18 (78%)	18/18 (100%)	9/10 (90%)	9/10 (90%)

According to the findings of this study, *V. cholerae* was detected at rates of 72%, 61%, 90%, and 70% in Pond A, Pond B, effluent, and influent (respectively), while *V. parahaemolyticus* was identified at levels of 78%, 100%, 90%, and 90% using Duplex-PCR methodology (Table 3.10). Since these *Vibrio* species are naturally found in water and the gut microbiome of shrimps, their detection in this study is expected (Garibay-Valdez et al., 2020). Recent studies have also proven the presence of both *Vibrio* species in aquaculture setting (Chen et al., 2022; Haifa-Haryani et al., 2022) and hypermarket setting ((Noorlis et al., 2011). The variation in the band's brightness can be explained by the halophilic profile of *V. parahaemolyticus* (Cartwright & Griffin, 2012) that thrives in saline water better than *V. cholerae*. *V. parahaemolyticus* can also sustain greater range of environmental differences such as pH and temperature in coastal water than *V.*

cholerae (Prasanthan et al., 2011). Since the sampling was done at a coastal region it is expected that *V. parahaemolyticus* population exceeds that of *V. cholerae*'s which explains the brighter bands displayed in duplex PCR.

When compared to overall *Vibrio* species, the growth curve of cyanobacteria displayed a clearer consistency. They have a structure resembling the lag phase, exponential or log phase, stationary phase, and death phase like a normal bacterial growth. This phenomenon can be attributed to the naturally slow growth rate of cyanobacteria due to their ability to carry on complex mechanism (such as the synthetization of amino acid and utilization of carbon dioxide for photosynthesis) causing the cells to prioritize cell function over reproduction (Burnap, 2015). In contrast to cyanobacteria, heterotrophic bacteria like *Vibrio* species use less energy to proliferate because they consume rather than produce their own food, leading to their quicker and more rapid development profile (Burnap, 2015). This repetitive nature of the data accounts for the persistently high *Vibrio* concentration as opposed to the assumed cyanobacterial growth trend.

Cyanobacteria may be very well distinguished from other prokaryotes based on the characteristics of their cells, tendency to form colonies and the structure of their filaments (Dvorak et al., 2017). It is apparent that *Pseudanabaena* genus dominated the shrimp farm water as we approach the end of the sampling, significantly reducing the diversity of cyanobacteria. Aside from the reason that *Pseudanabaena* genus can commonly be found inhabiting planktonic water, they also are highly tolerant towards disturbance and low light when compared to other cyanobacteria genus (Nienaber & Steinitz-Kannan, 2018). *Chroococcus*, *Phormidium*, *Oscillatoria* and *Lyngbya* were among the other species that were discovered during prior samplings.

Based on the canonical correspondence analysis, pH, salinity and temperature all influence cyanobacterial growth, but salinity had a greater influence. This has also been proven by previous research where high cell densities were preferred by a cyanobacteria *Nodularia spumigena* (Silveira & Odebrecht, 2019). The high degree of salt tolerance in cyanobacteria can be attributed to their extensive regulatory mechanism, which enables them to adapt and thrive under diverse salinity levels (Pade & Hagemann, 2014). In the past, cyanobacteria's remarkable ability to withstand high salinity levels has been found advantageous for enhancing rice cultivation by remediating saline soil (Jan et al., 2018). In pond B and effluent water, *Vibrio* species were found to favour pH. However, other ponds (Pond A and influent) indicate no major effect of pH on the growth of *Vibrio* species. The variation in the result might indicate the optimum pH where they grow best (Keenleyside, 2019).

The interspecies relationship between the two genera has been proven to be an extremely complex discussion. Our data demonstrated minimal interspecies correlation between *Vibrio* species and cyanobacteria, which is consistent with previous studies (Julie et al., 2010; Paranjpye et al., 2015; Greenfield et al., 2017) although several (Islam et al., 2004; Ward, 2014; Jesser & Noble, 2018) provide alternative evidence. This is because of the high population of *Vibrio* species in most sampling as opposed to the structured and more consistent growth pattern of cyanobacteria. It is also evident from the aforementioned studies that the interspecies relationship between the two genera is dependent on the species involved (Julie et al., 2010), seasonal temperature (Greenfield et al., 2017), and regional factors (Turner et al., 2009). However, this finding should not completely rule out the compatibility of *Vibrio* proliferation with cyanobacterial blooms as several studies have provided alternative evidence based on different regions and species involved.

It can be concluded that environmental considerations such as pH, temperature, and salinity are more important factors in determining the growth of *Vibrio* species and cyanobacteria than their relationship with each other. It is suggested that reducing exposure to environmental stressors may be a more effective way to limit *Vibrio* and cyanobacterial growth than focusing on controlling one bacterium over another. Further research is required to determine the precise mechanisms by which these bacteria interact with their environment and to develop more targeted interventions for managing them in aquaculture systems. A conclusive study involving a multidimensional landscape can link fragmentary knowledge regarding the relationship of both genera.

3.5 Conclusion

In this study, high *Vibrio* concentrations were detected in the shrimp farm; and molecular detection of *Vibrio* species showed a high percentage of *V. cholerae* and *V. parahaemolyticus* albeit the water parameters being within the acceptable range for shrimp farming. This calls for a regular *Vibrio* monitoring in the shrimp farming facility. Water treatments such as filtration, sanitation and disinfection can also be implemented to reduce the population of *Vibrio* species. Alternative treatments such as probiotics and phage therapy can be explored, as they have shown potential in controlling cell density of pathogenic *Vibrio* species. As for the *Vibrio*-cyanobacteria dynamic, the connection between *Vibrio* species and cyanobacteria appears to be minimal as suggested by their growth patterns. Since other factors affecting the persistence of *Vibrio* species in shrimp farms may be present still, further studies should also explore other factors such as species-specific relationships, regional dynamics, and other multidimensional landscapes that may be affecting the persistence of *Vibrio* species in shrimp farms.

CHAPTER 4

THE PREVALENCE OF *V. cholerae* AND *V. parahaemolyticus* VIRULENCE GENES AND MULTIPLE ANTIBIOTICS RESISTANT (MAR) ASSESSMENT FROM SELECTED LOCAL SHRIMP FARM IN SARAWAK

4.1 Introduction

A significant and valuable aquaculture product, shrimps are widely traded on a global scale. Governments in developing nations like China, Thailand, Indonesia, India, Vietnam, Ecuador, and Bangladesh are compelled to encourage shrimp farming as a way to fight poverty due to the high demand for shrimps (World Wildlife Fund, 2022). In Malaysia, shrimp export during 2017 and 2018 contributed RM 0.8 billion of profit in 2017 and RM 1 billion in 2018 (Fisheries Development Authority of Malaysia, 2020). According to the same source, shrimp goods also had the greatest export values when compared to other fishery products in those years, respectively. With high value of consumption of shrimp, there also comes the risk of the infection from shrimp sources. *Vibrio* species, including *V. parahaemolyticus* and *V. cholerae*, have been found to be associated with shrimp and pose a risk to human health.

Recent studies showed that *V. parahaemolyticus* have been prevalent in shrimp farms in Malaysia, Indonesia, India and Thailand (Yano et al., 2014; Narayanan et al., 2020; Sarjito & Sabdono, 2021; Haifa-Haryani et al., 2022). Ingesting seafood contaminated with pathogenic *V. parahaemolyticus* can cause gastroenteritis, an inflammation of the stomach and intestines that leads to symptoms such as abdominal pain, diarrhoea, vomiting, and fever (Broberg et al., 2011). While most cases of *V. parahaemolyticus* infection are mild, in rare cases it can cause a more severe form of the disease that requires hospitalization (Broberg et

al., 2011). The looming effects brought by *V. parahaemolyticus* serves as a motivation for it to be included in this study.

In addition to *V. parahaemolyticus*, the presence of *V. cholerae* in shrimp is also prominent (Joseph et al., 2015; Kriem et al., 2015; Shishehchian, 2020). The presence of *V. cholerae* in shrimp is a particularly concerning issue, as it is responsible for causing cholera infection towards human (Fu et al., 2019). Cholera infection causes severe dehydration with the onset of rice water diarrhoea and can cause a healthy person to die within 24 hours if not treated (Dick et al., 2012). Given the long track record of *V. cholerae* as a food-borne pathogen, (Reda et al., 2022), it is not surprising that this bacterial species was also included in the current study.

Vibrio is a natural occurring microbiota of shrimp guts; therefore, they are not necessarily pathogenic (Zoqratt et al., 2018). However, an unhealthy shrimp harboured 30% more *Vibrio* in their guts of overall shrimp gut microbiome, compared to a healthy shrimp (Kuthoose et al., 2021). Their ability to cause infections can be determined by the presence of virulence genes in their genome. Thermostable direct haemolysin (*tdh*) and thermostable-related haemolysin (*trh*) are majorly related to the toxicity of *V. parahaemolyticus* (Raghunath, 2015). The expression of *tdh* causes the formation of pore in red blood cell membrane (Matsuda et al., 2010). *trh* genes are 70% similar to that of *tdh* and the expression results in abnormal secretion of chloride ion in human colon (Takahashi et al., 2000). Repeat in toxin genes (*rtxA* and *rtxC*) are recently found in abundance in the genome of *V. cholerae* (Fu et al., 2020). *rtxA* and *rtxC* are present in many Gram-negative bacteria and can be transferred across their envelope (Linhartová et al., 2010). *rtxA* and *rtxC* genes expression induces cell death in human intestine (Lee et al., 2008). Cholera toxin subunit B (*ctxB*) is the gene responsible for the watery diarrhoea symptom causing dehydration and severe

electrolyte imbalance in human (Satitsri et al., 2016). *ctxB* was also detected in shrimp and is hazardous towards public health when consumed (Madhusudana & Surendran, 2013).

In shrimp farms, illnesses brought on by *Vibrio* species are typically treated with antibiotics such as tetracycline, norfloxacin, oxytetracycline, enrofloxacin and sulphonamides (Holmström et al., 2003; Luu et al., 2021). They were added in shrimp feed, or in the rearing pond water. However, due to the improper administration of antibiotics, the cases of antibiotic resistant bacteria have emerged (Fletcher, 2015; Holmström et al., 2003). Over the past few decades, the resistance towards antibiotics has developed and spread throughout many bacterial species as a result of the overuse of antibiotics in agricultural and aquaculture systems (Cabello, 2006). Antibiotic resistance is the term used to describe a bacteria increased in ability to withstand the effects of antibiotics, to which they were previously vulnerable to (Osunla & Okoh, 2017). Even non-pathogenic bacteria can harbour antibiotic resistance genes that then can transfer to pathogenic bacteria by a mechanism called horizontal gene transfer (Pérez-Rodríguez & Mercanoglu Taban, 2019). When infection can no longer be treated by first-line antibiotic, more expensive and toxic medication will come in play (Ventola, 2015). Antibiotic resistance can cause longer treatment duration, increase healthcare costs that can become a burden for families, society and the economy (Ventola, 2015).

This research project was conducted with great rigor, involving an in-depth analysis of a single, intensively sampled population of *Vibrio* isolates present in the local shrimp farm water. The study spanned a period of eight months and aimed to shed light on the virulence and antibiotic resistant profile of these bacterial strains. Specifically, this research aimed to identify several key virulence genes, including *tdh*, *trh*, *ctxB*, *rtxA*, and *rtxC*, which are associated with the pathogenicity of *Vibrio* species.

In addition to the investigation of virulence genes, the present study also focused on the emergence of Multiple Antibiotic Resistance (MAR) index. This index was used to identify the number of antibiotics that each isolate is resistant to, and their patterns of antibiotic resistance, with the aim of developing strategies to mitigate the potential for infection and ensure effective treatment in the near future. Overall, this study provides valuable new insights into the virulence and antibiotic resistance of *Vibrio* isolates present in shrimp farm water, and underscores the importance of ongoing research efforts to identify and mitigate potential health risks associated with these bacterial strains.

4.2 Methodology

4.2.1 Water Sampling

Water sampling was commenced biweekly in two shrimp ponds (Pond A and Pond B), effluent and influent water of Persatuan Nelayan Kawasan Satang Biru, Telaga Air, starting from August to December 2021. The sampling campaign was executed within the shrimps' post-larvae stocking to harvesting time frame. A total of six ($n=6$) samples from pond A, six ($n=6$) samples from pond B, ten ($n=10$) samples from effluent and ten ($n=10$) samples from influent were successfully collected, making up to ($n=32$) number of samples excluding triplicates samples. The samples were then transported to the laboratory under aseptic condition within 2 hours after sampling.

4.2.2 Enrichment of *Vibrio* Species

A total of one mL of the water samples was pipetted into 9 mL of Alkaline Peptone Water (APW) (HiMedia, India) to enrich *Vibrio* species in the samples. The cultures were then incubated overnight at 37 °C.

4.2.3 Isolation of *Vibrio* Species

A loopful of broth were streaked on the surface of TCBS agar (HiMedia, India) and subsequently incubated overnight at 37 °C. Ten yellow ($n=10$) and 10 green ($n=10$) colonies that were formed on the agar was reintroduced to 5 mL of APW and incubated overnight at 37 °C for the detection using PCR.

4.2.4 DNA Extraction of *Vibrio* Species

The method was adjusted from Peng et al., (2013) to fit the requirement of this study. 500 µL of the culture broth were pipetted into 1.5 mL microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded before adding 100 µL of deionized distilled water (ddH₂O). The mixture was then boiled for 10 minutes, and snap cooled in ice for 5 minutes. Lastly, each tube was centrifuged for 10 minutes at 10,000 rpm. The clear solution is the product of the DNA extract.

4.2.5 Polymerase Chain Reaction (PCR)

PCR was done by adding exTEN PCR 2X Master Mix (Base Asia), 2 pairs of forward and reverse primers as specified in Table 4.1, nuclease-free water and DNA extracts from each sampling. The volume of each reagent was shown in Table 4.2, while the amplification of DNA sequence was done by using thermal cycler (Eppendorf Mastercycler Gradient) with the conditions shown in Table 4.3. *trh* and *tdh* (Bio basic, Canada) PCR condition was derived from (Hossain et al., 2020) with slight modification. The PCR condition for *ctxB* (IDT, USA) was derived from (Said et al., 1995) while *rtxA*, *rtxC* (IDT, USA) was derived from (Chow et al., 2001), also with slight modification.

Table 4.1: Primer pairs for the detection of toxin genes *tdh*, *trh* *ctxB*, *rtxA* and *rtxC*.

Target genus or species	Target gene	Primer name	Primer sequences	PCR base pair
<i>V. parahaemolyticus</i>	<i>tdh</i>	<i>tdh</i> -F	5'- GTA AAG GTC TCT GAC TTT TGG AC -3'	623
		<i>tdh</i> -R	5'- TGG AAT AGA ACC TTC ATC TTC ACC -3'	
	<i>trh</i>	<i>trh</i> -F	5'- TTG GCT TCG ATA TTT TCA GTA TCT-3'	460
		<i>trh</i> -R	5' – CAT AAC AAA CAT ATG CCC ATT TCG G-3'	
<i>V. cholerae</i>	<i>ctxB</i>	<i>ctx</i> B2	5'- GGT TGC TTC TCA TCA TGG AAC CAC – 3'	460
		<i>ctx</i> B3	5' – GAT ACA CAT AAT AGA ATT AAG GAT G-3'	
	<i>rtxA</i>	<i>rtx</i> A-F	5'- CTG AAT ATG AGT GGG TGA CTT ACG -3'	417
		<i>rtx</i> A-R	5'- GTG TAT TGT TCG ATA TCC GCT ACG -3'	
	<i>rtxC</i>	<i>rtx</i> C-F	5'- CGA CGA AGA TCA TTG ACG AC -3'	263
		<i>rtx</i> C-R	5'- CAT CGT CGT TAT GTG GTT GC -3'	

Table 4.2: The volume of PCR reagent for the detection of *tdh*, *trh* *ctxB*, *rtxA* and *rtxC*.

PCR Reagent	Volume per reaction (μL)		
	<i>tdh</i> and <i>trh</i>	<i>ctxB</i>	<i>rtxA</i> and <i>rtxC</i>
exTEN 2X PCR Master Mix (Base Asia)		12.5	
10 pmol/μL of primer F- <i>tdh</i>	1.25		
10 pmol/μL primer R- <i>tdh</i>	1.25		
10 pmol/μL primer F- <i>trh</i>	1.25		
10 pmol/μL primer R- <i>trh</i>	1.25		
10 pmol/μL of primer <i>ctx</i> B2		1.5	
10 pmol/μL primer <i>ctx</i> B3		1.5	
10 pmol/μL of primer <i>rtx</i> A-F			1.5
10 pmol/μL primer <i>rtx</i> A-R			1.5
10 pmol/μL primer <i>rtx</i> C-F			1.5
10 pmol/μL primer <i>rtx</i> C-R			1.5
Nuclease-free water	3.0	1.5	3.5
DNA extract	2.0	3.0	3.0
Total volume	22.5	20	25

Table 4.3: PCR condition for *tdh*, *trh* *ctxB*, *rtxA* and *rtxC*.

Step Cycle	Temperature/Time	
	<i>tdh</i> and <i>trh</i>	<i>ctxB</i> , <i>rtxA</i> and <i>rtxC</i>
Initial denaturation	94 °C (5 min)	95 °C (5 min)
Denaturation	94 °C (1 min)	95 °C (1 min)
Annealing	58 °C (1 min) (30 cycles)	55 °C (1 min) (30 cycles)
Extension	72 °C (1 min)	72 °C (1 min)
Final extension	72 °C (10 min)	72 °C (10 min)

4.2.6 Agarose Gel Electrophoresis (AGE)

The PCR products were loaded into 1.5% agarose gel and were charged with 80V electric current for 1 hour prior to being stained for 45 minutes with 0.1% of Ethidium Bromide (EtBr). The bands formed were then observed by using UV transilluminator before being photographed.

4.2.7 Antibiotic Susceptibility Test (AST)

AST were done to determine bacterial sensitivity or resistance towards various antibiotics. The method used were disc diffusion method (Bauer et al., 1966). Firstly, healthy pure sample cultures of *V. cholerae* and *V. parahaemolyticus* were isolated and suspended in inoculums where their turbidity was compared with 0.5 Mcfarland standard of suspension. The pure bacterial cultures were then lawned by using sterile cotton swabs on the Mueller Hinton Agar (MHA) (HiMedia, India) plates that were supplied with 2% NaCl each. These plates were then left to settle and dry for 5 minutes (Uddin et al., 2018) Discs containing antibiotics were then secured on top of the agar by using sterile tweezer leaving a decent amount of spaces between each disc for accurate measurement of result. The antibiotics (Oxoid, UK) used in this experiment are listed in Table 4.4. The plates were then incubated overnight at 37 °C. The results were recorded by measuring the diameter of the inhibition zone in millimetre and interpreted as Resistant (R), Intermediate (I) or Sensitive (S) according to Clinical and Laboratory Standards Institute (CLSI), M45 guidelines (CLSI, 2015).

By quantifying the number of resistant isolates, information can be inferred, including the most effective and/or ineffective antibiotics for treating these isolates, the

Multiple Antibiotic Resistant (MAR) indices, and patterns indicating frequent antibiotic resistance among specific groups of antibiotics. This data allows for a comprehensive understanding of bacterial susceptibility to different drugs.

Table 4.4: Antibiotics used in this study for the Antibiotic Susceptibility Test (AST) for *V. cholerae* and *V. parahaemolyticus*.

Antimicrobial Class	Antibiotic Names	Abbreviation	Content (µg)
Penicillins and β- lactam/β- Lactamase Inhibitor Combinations	Ampicillin	AMP10	10
	Amoxycillin/Clavulanate	AMC30	30
Cephalosporins/Cephems	Ceftazidime	CAZ30	30
	Ceftriaxone	CRO30	30
	Cephalothin	KF30	30
Carbapenems	Imipenem	IPM10	10
	Meropenem	MEM10	10
Aminoglycosides	Amikacin	AK30	30
	Kanamycin	K30	30
	Neomycin	N30	30
	Streptomycin	S10	10
Tetracyclines	Tetracycline	TE30	30
Quinolones/Fluoroquinolones	Nalidixic Acid	NA30	30
	Norfloxacin	NOR10	10
	Ciprofloxacin	CIP5	5
Folate Pathway Inhibitors	Compound Sulphonamides	S3	300
	Sulfamethoxazole	RL25	25
	Sulfamethoxazole-Trimethoprim	SXT25	25
Phenicol	Chloramphenicol	C30	30
Rifamycin	Rifampicin	RD5	5

4.3 Results

4.3.1 Toxin Genes

a. *V. cholerae* (*ctxB*, *rtxA* and *rtxC*)

The genomes of all *V. cholerae* isolates lacked formation of any DNA bands for the detection of *ctxB*, therefore indicating a complete absence of the said gene in the isolates. As for *rtxA* and *rtxC*, 7 out of the 10 isolates showed a positive result for the detection of *rtxA*, indicating the presence of this virulence gene in those isolates. Similarly, 9 out of the 10 isolates tested positive for the detection of *rtxC*, suggesting the presence of this virulence gene in those isolates. These also mean that 70% of the *V. cholerae* isolates harbour *rtxA* genes and 90% of the *V. cholerae* isolates harbour *rtxC* genes. These findings are presented in Figure 4.1 and 4.2, which clearly shows the distribution of the positive results by the formation of stained DNA bands for each virulence gene across the lanes.



Figure 4.1: PCR result for the detection of *ctxB* toxin gene in *V. cholerae* isolates as viewed in 1.5% agarose gel. M: 100 bp DNA ladder, 1: positive control for *ctxB*, 2: negative control, 3: Isolate 1, 4: Isolate 2, 5: Isolate 3, 6: Isolate 4, 7: Isolate 5, 8: Isolate 6, 9: Isolate 7, 10: Isolate 8, 11: Isolate 9, 12: Isolate 10. No *ctxB* gene were detected in the *V. cholerae* isolates.

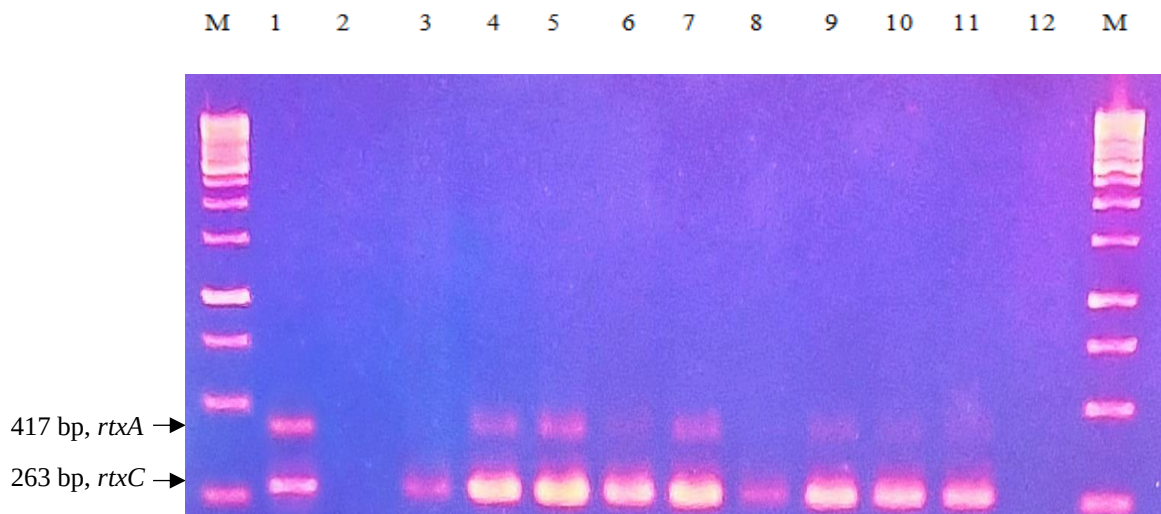


Figure 4.2: Duplex-PCR result for the detection of *rtxA* and *rtxC* toxin genes in *V. cholerae* isolates as viewed in 1.5% agarose gel. M: 100 bp DNA ladder, 1: positive control for *rtxA* and *rtxC*, 2: negative control, 3: Isolate 1, 4: Isolate 2, 5: Isolate 3, 6: Isolate 4, 7: Isolate 5, 8: Isolate 6, 9: Isolate 7, 10: Isolate 8, 11: Isolate 9, 12: Isolate 10. Lane 4, 5, 6, 7, 9, 10, and 11 appeared to have positive detection of *rtxA* while lane 3 to 11 showed positive detection of *rtxC* toxin gene.

b. *V. parahaemolyticus* (*tdh* and *trh*)

All 10 *V. parahaemolyticus* isolates showed no evidence of the *tdh* and *trh* gene in their genomes, as demonstrated in Figure 4.3. This is also determined by the lack of stained DNA bands formation indicating a complete absence of both genes in the isolates.

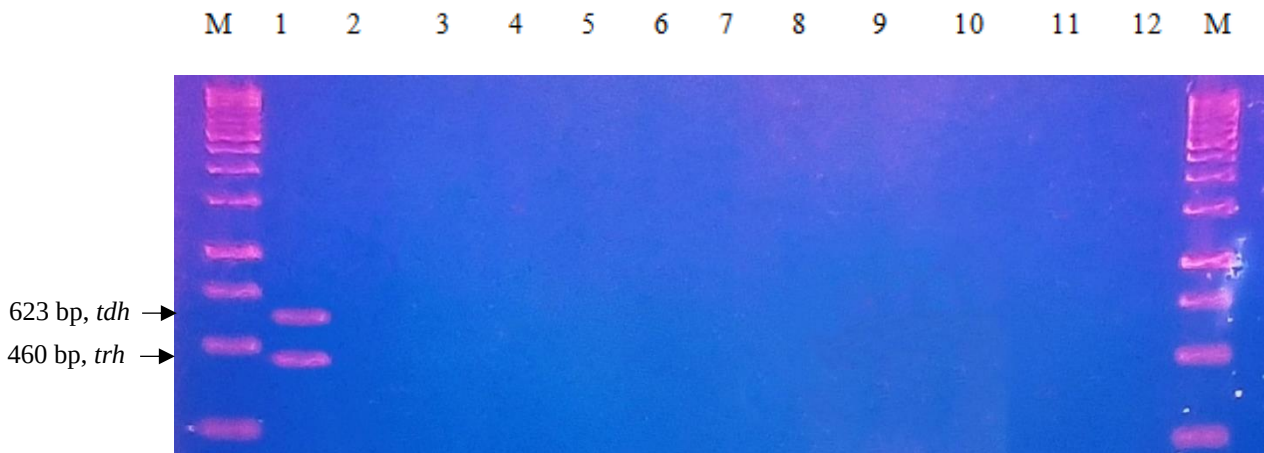


Figure 4.3: Multiplex-PCR result for the detection of *tdh*, *trh* and *tlh* toxin genes in *V. parahaemolyticus* isolates as viewed in 1.5% agarose gel. M: 100 bp DNA ladder, 1: positive control for *tdh* and *trh*, 2: negative control, 3: Isolate 1, 4: Isolate 2, 5: Isolate 3, 6: Isolate 4, 7: Isolate 5, 8: Isolate 6, 9: Isolate 7, 10: Isolate 8, 11: Isolate 9, 12: Isolate 10. Negative detection can be observed for *tdh* and *trh*.

4.3.2 Antibiotic Profile

a. *V. cholerae*

Table 4.5 presents the results of the antibiotic profiling of *V. cholerae* isolates against selected antibiotics. There appears to be no evidence of resistance from *V. cholerae* isolates, imipenem, tetracycline, norfloxacin thus awarding them as the most potent antibiotics. Amoxycillin/clavulanate, sulfamethoxazole-trimethoprim, and chloramphenicol are next in line and were recorded with 80% susceptibility of eradicating *V. cholerae* population. The least effective antibiotics at preventing the growth of *V. cholerae* is sulfamethoxazole (100% resistance), followed by cephalothin (80% resistance), neomycin (70% resistance), streptomycin (70% resistance) and ceftriaxone (60% resistance).

Table 4.5: Antibiotic profiling of *V. cholerae* isolates against selected antibiotics.

Antimicrobial Class	Antibiotic Names	Abbreviation	No. and Percentage (%) of <i>V. cholerae</i> according to their Susceptible, Intermediate and Resistance Distribution		
			Susceptible, S	Intermediate, I	Resistant, R
Penicillins and β -lactam/ β -Lactamase Inhibitor Combinations	Ampicillin	AMP10	4 (40%)	1 (10%)	5 (50%)
	Amoxycillin/Clavulanate	AMC20	8 (80%)	0 (0%)	2 (20%)
Cephalosporins/Cephems	Ceftazidime	CAZ30	7 (70%)	0 (0%)	3 (30%)
	Ceftriaxone	CRO30	4 (40%)	0 (0%)	6 (60%)
	Cephalothin	KF30	1 (10%)	1 (10%)	8 (80%)
Carbapenems	Imipenem	IPM10	10 100%	0 (0%)	0 (0%)
	Meropenem	MEM10	7 (70%)	2 (20%)	1 (10%)
Aminoglycosides	Amikacin	AK30	4 (40%)	3 (30%)	3 (30%)
	Kanamycin	K30	3	5	2

Table 4.5 Continued

			(30%)	(50%)	(20%)
	Neomycin	N30	3 (30%)	0 (0%)	7 (70%)
	Streptomycin	S10	1 (10%)	2 (20%)	7 (70%)
Tetracyclines	Tetracycline	TE30	10 (100%)	0 (0%)	0 (0%)
	Nalidixic Acid	NA30	5 (50%)	0 (0%)	5 (50%)
Quinolones/Fluoroquinolones	Norfloxacin	NOR10	10 (100%)	0 (0%)	0 (0%)
	Ciprofloxacin	CIP5	7 (70%)	3 (30%)	0 (0%)
	Compound Sulphonamides	S3	4 (40%)	4 (40%)	2 (20%)
Folate Pathway Inhibitors	Sulfamethoxazole	RL25	0 (0%)	0 (0%)	10 (100%)
	Sulfamethoxazole-Trimethoprim	SXT25	8 (80%)	0 (0%)	2 (20%)
Phenicols	Chloramphenicol	C30	8 (80%)	2 (20%)	0 (0%)

Table 4.5 Continued

Rifamycin	Rifampicin	RD5	6 (60%)	0 (0%)	4 (40%)
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b. *V. parahaemolyticus*

Table 4.6 presents the results of the antibiotic profiling of *V. parahaemolyticus* isolates against selected antibiotics. A total of 100% of *V. parahaemolyticus* isolates was susceptible with ceftazidime, ceftriaxone, amikacin, neomycin, nalidixic acid, ciprofloxacin, sulfamethoxazole-trimethoprim and chloramphenicol (Table 4.9). 90% of susceptibility was displayed by the antibiotic kanamycin, tetracycline and norfloxacin in eradicating *V. parahaemolyticus*. Others such as amoxycillin/clavulanate, meropenem and rifampicin were recorded to have 80% susceptibility towards *V. parahaemolyticus* isolates. The least effective antibiotics at preventing the growth of *V. parahaemolyticus* is cephalothin, streptomycin and sulfamethoxazole with 100% resistance recorded. The next antibiotic that the species were highly resistant to was compound sulphonamides which can be attributed to the high rates of resistance found in *V. parahaemolyticus* (80%).

Table 4.6: Antibiotic profiling of *V. parahaemolyticus* isolates against selected antibiotics.

Antimicrobial Class	Antibiotic Names	Abbreviation	No. and Percentage (%) of <i>V. parahaemolyticus</i> according to their Susceptible, Intermediate and Resistance Distribution		
			Susceptible, S	Intermediate, I	Resistance, R
Penicillins and β -lactam/ β -Lactamase Inhibitor Combinations	Ampicillin	AMP10	6 (60%)	0 (0%)	4 (40%)
	Amoxycillin/Clavulanate	AMC20	8 (80%)	0 (0%)	2 (20%)
Cephalosporins/Cephems	Ceftazidime	CAZ30	10 (100%)	0 (0%)	0 (0%)
	Ceftriaxone	CRO30	10 (100%)	0 (0%)	0 (0%)
	Cephalothin	KF30	0 (0%)	0 (0%)	10 (100%)
Carbapenems	Imipenem	IPM10	5 (50%)	4 (40%)	1 (10%)
	Meropenem	MEM10	8 (80%)	1 (10%)	1 (10%)
Aminoglycosides	Amikacin	AK30	10 (100%)	0 (0%)	0 (0%)

Table 4.6 Continued

	Kanamycin	K30	9 (90%)	1 (10%)	0 (0%)
	Neomycin	N30	10 (100%)	0 (0%)	0 (0%)
	Streptomycin	S10	0 (0%)	0 (0%)	10 (100%)
Tetracyclines	Tetracycline	TE30	9 (90%)	0 (0%)	1 (10%)
Quinolones/Fluoroquinolones	Nalidixic Acid	NA30	10 (100%)	0 (0%)	0 (0%)
	Norfloxacin	NOR10	9 (90%)	0 (0%)	1 (10%)
	Ciprofloxacin	CIP5	10 (100%)	0 (0%)	0 (0%)
Folate Pathway Inhibitors	Compound Sulphonamides	S3	1 (10%)	1 (10%)	8 (80%)
	Sulfamethoxazole	RL25	0 (0%)	0 (0%)	10 (100%)
	Sulfamethoxazole- Trimethoprim	SXT25	10 (100%)	0 (0%)	0 (0%)

Table 4.6 Continued

Phenicol	Chloramphenicol	C30	10 (100%)	0 (0%)	0 (0%)
Rifamycin	Rifampicin	RD5	8 (80%)	0 (0%)	2 (20%)

4.3.2 MAR Indices and Antibiotic Resistant Pattern

a. *V. cholerae*

The identification of the antibiotic resistant pattern from each *V. cholerae* isolates and their MAR indexes provides an insight on the diversity of the antibiotics that they are resistant with. Table 4.7 shows 9 profile patterns of antibiotic resistance with the most frequent pattern being C with the resistance profile of KF30N30RD5RL25S10. The frequency happened to be of 20% occurrence of all the 10 isolates. MAR indexes of *V. cholerae* isolates ranges from 0.15 to 0.55 while their mean is 0.34. The antibiotic pattern I has the highest MAR index at a staggering 0.55 while the pattern A showed the lowest MAR index at 0.15. Both occurrences were recorded to be at 10% frequency. From the 10 isolates, a total of 9 isolates showed MAR indexes greater than 0.2.

Table 4.7: Patterns of antibiotic resistance profile and MAR index of *V. cholerae* isolates.

Label	Isolate Number	^a Antibiotic Resistance Pattern	^b MAR Index	Percentage of Occurrence (No. of Isolates/Total of isolates) (%)
A	VC10	NRIS	0.15	10%
B	VC03	AmpCroKfNaRl	0.25	30%
C	VC07	KfNRdRIS	0.25	
	VC08		0.25	
D	VC02	AmcAmpCroKfNaRl	0.30	20%
E	VC09	KfNRdRlSSxt	0.30	
F	VC01	AmcAmpCroKfMemRlSxt	0.35	10%
G	VC05	AkCazCroKNNaRlSS3	0.45	10%
H	VC06	AkAmpCazCroKfNNaRdRIS	0.50	10%
I	VC04	AkAmpCazCroKKfNNaRlSS3	0.55	10%

^a Antibiotics used: Amp, Ampicillin; Amc, Amoxycillin/Clavulanate; Caz, Ceftazidime; Cro, Ceftriaxone; Kf, Cephalothin; Ipm, Imipenem; Mem, Meropenem; Ak, Amikacin; K, Kanamycin; N, Neomycin; S, Streptomycin; Te, Tetracycline; Na, Nalidixic Acid; Nor, Norfloxacin; Cip, Ciprofloxacin; S3, Compound Sulphonamides; Rl, Sulfamethoxazole; Sxt, Sulfamethoxazole-Trimethoprim; C, Chloramphenicol; Rd, Rifampicin

^b MAR Index = The number of antibiotics that the isolate is resistant to / Total number of antibiotics used

b. *V. parahaemolyticus*

The identification of the antibiotic resistant pattern and their MAR indexes provides an insight on the diversity of the antibiotics that each isolate is resistant with. From *V. parahaemolyticus* isolates, 8 profile patterns of antibiotic resistance were recorded (Table 4.8). The most frequent pattern was B with the resistance profile of KF30RL25S10S3. 100% of *V. parahaemolyticus* isolates were detected with the MAR indexes of 0.2 and higher. MAR indexes of *V. parahaemolyticus* isolates ranges from 0.20 to 0.35, making them all above 0.20 threshold. Their calculated mean is 0.24. The greatest MAR index is 0.35 from pattern H while the lowest is pattern A with 0.20 MAR index.

Table 4.8: Patterns of antibiotic resistance profile and MAR index of *V. parahaemolyticus* isolates.

Label	Isolate Number	^a Antibiotic Resistance Pattern	^b MAR Index	Percentage of Occurrence (No. of Isolates/Total of isolates) (%)
A	VP03	KfNorRIS	0.20	50%
	VP04		0.20	
B	VP05	KfRISS3	0.20	
	VP08		0.20	
C	VP10	AmpKfRIS	0.20	30%
D	VP02	KfMemRISS3	0.25	
E	VP06	KfRdRISS3	0.25	
F	VP09	AmcAmpKfRIS	0.25	
G	VP07	AmpKfRdRISS3	0.30	10%
H	VP01	AmcAmpIpmKfRISTe	0.35	10%

^a Antibiotics used: Amp, Ampicillin; Amc, Amoxycillin/Clavulanate; Caz, Ceftazidime; Cro, Ceftriaxone; Kf, Cephalothin; Ipm, Imipenem; Mem, Meropenem; Ak, Amikacin; K, Kanamycin; N, Neomycin; S, Streptomycin; Te, Tetracycline; Na, Nalidixic Acid; Nor, Norfloxacin; Cip, Ciprofloxacin; S3, Compound Sulphonamides; RI, Sulfamethoxazole; Sxt, Sulfamethoxazole-Trimethoprim; C, Chloramphenicol; Rd, Rifampicin

^b MAR Index = The number of antibiotics that the isolate is resistant to / Total number of antibiotics used

4.4 Discussion

In the present study, the impact of vibriosis on the global shrimp industry was examined, with a focus on the role of *V. parahaemolyticus* and *V. cholerae* in causing various diseases in shrimps. The presence of virulence genes, such as *tdh* and *trh* in *V. parahaemolyticus* and *rtxA* and *rtxC* in *V. cholerae*, can increase the toxicity and pathogenicity of these bacteria, leading to serious infections in shrimps. Table 4.9 shows the summary of PCR toxin genes detection in both *V. cholerae* and *V. parahaemolyticus* isolates that were done in this study.

Table 4.9: The summary of the PCR detection of toxin genes in the isolates of *V. cholerae* and *V. parahaemolyticus*.

<i>Vibrio</i> species	Toxin gene	Number of positive detections	Percentage of positive detection
<i>V. cholerae</i>	<i>ctxB</i>	0/10	0%
	<i>rtxA</i>	7/10	70%
	<i>rtxC</i>	9/10	90%
<i>V. parahaemolyticus</i>	<i>tdh</i>	0/10	0%
	<i>trh</i>	0/10	0%

trh and *tdh* genes were absent in *V. parahaemolyticus* isolates. Low detection of *tdh* and *trh* genes from aquaculture sample was also reported from previous study (Vieira et al., 2011). Since these virulence factors are major and can infect both people and aquatic organisms, it is crucial that their density remained low (Robert-Pillot et al., 2004). Despite the absence of *tdh* and *trh* genes, there is no certainty that *V. parahaemolyticus* does not possess other virulence components that are not covered in this study.

As for *V. cholerae* isolates, 70% *rtxA* and 90% *rtxC* genes were detected. RTX toxin are well known to be associated with gram-negative bacteria (Coote, 1992). Gram-negative

bacteria-related toxins have a number of effects, including haemolysin, pore formation, and cytotoxicity (Prithvisagar et al., 2021). The detection of *rtxA* and *rtxC* genes were also mentioned in recent studies (Igere et al., 2022; Fu et al., 2020; Xu et al., 2019). Since *rtx* genes can be present in not only *Vibrio* species but also in gram-negative bacteria, it is possible that horizontal gene transfer is widespread causing the high pathogenicity (Dell'Annunziata et al., 2021; Deng et al., 2019). In order to prevent a widespread infection from endangering the health of shrimp and people, a control measure should be carried out in response to the high proportion of *rtxA* and *rtxC* genes detected in the sampling location.

The classes of antibiotics that are the most effective for the treatment from *V. cholerae* are tetracycline (tetracycline), carbapenem (imipenem) and older quinolone (norfloxacin). As for *V. parahaemolyticus*, cephalosporins/cephems (ceftazidime and ceftriaxone), aminoglycosides (amikacin and neomycin), older quinolones (nalidixic acid and ciprofloxacin), folate pathway inhibitor (sulfamethoxazole-trimethoprim) and phenicol (chloramphenicol) are the most effective. Norfloxacin was the most successful antibiotic in eradicating 100% of *V. cholerae* and 90% of *V. parahaemolyticus* for the same reason it was banned for the use of food animals in Malaysia (Hassali et al., 2018). Avoparcin, chloramphenicol, nitrofurantoin, nitrofurazone, furazolidone, furaltadone, teicoplanin and vancomycin are also banned in food animals due to the level of harmful residues that can cause adverse impact on human health (Hassali et al., 2018). Antibiotic residues are proven to be difficult to extinguish entirely since they can remain in food even after cooking (Fathy Mahmoud, 2015).

Mean MAR indices of 0.34 for *V. cholerae* and 0.24 for *V. parahaemolyticus* were calculated in this study. 90% of *V. cholerae* isolated have recorded MAR index of higher

than 0.2 while 100% of *V. parahaemolyticus* have recorded MAR index of 0.2 and/or higher. MAR index greater than 0.2 indicate that isolates came from a highly contaminated source where antibiotics are frequently used (Davis & Brown, 2016). Based on the result, *V. parahaemolyticus* displayed significant resistance towards sulfamethoxazole, compound sulphonamides, cephalothin and streptomycin. It matches the antibiotics mentioned in CLSI-M45, (2015). As for *V. cholerae*, they are the most resistant towards sulfamethoxazole, cephalothin, neomycin, streptomycin, and ceftriaxone. This result is in favour with previous research that found the same antibiotics resistance from *V. cholerae* towards listed antibiotics except for neomycin (Adesiyan et al., 2021; Fu et al., 2020; Mandal et al., 2012; Noorlis et al., 2011). Low resistance (Verma et al., 2019) and high susceptibility (Mrityunjy et al., 2013) of neomycin for the treatment of *V. cholerae* have been recorded in previous research. Decrease in neomycin resistant from the year 2000 to 2022 were also documented (Wu et al., 2022). Regardless, the use of neomycin for treating vibriosis in aquaculture and cholera infection is indeed described by other studies (Adesiyan et al., 2022; Farooq & Unno, 2018; Manjusha & Sarita, 2011).

The distribution of antibiotic resistance genes is linked to various factors such as potential host genera and biomes, sample collection year, environmental factors influenced by human activities and collection countries (Lin et al., 2022). As the shrimp farmers in this study denied any use of treatment for the shrimps, it is possible that the bacteria acquired antibiotics resistance from the environment or from the post-larvae that were imported from other hatcheries. This observation correlates with a study which stated that higher frequency of MAR occurs in hatcheries compared to in shrimp rearing pond (Zhang et al., 2011). Nevertheless, the high susceptibility of *Vibrio* species towards banned antibiotics in this

study indicate proper obligation and understanding of shrimp farmers and aquaculture organisations towards food safety.

Several control measures can be taken before bigger conflict involving shrimp diseases develop. To avoid prolong effect of MAR, the proper usage and constant supervision of antibiotics use in shrimp farm should be implemented. The use of alternative treatments such as detergents (Elexson et al., 2014), probiotics (Ngo & Fotedar, 2010), and phage therapy (Chen et al., 2019) have all been shown to be equally effective, if not superior, in treating vibriosis in the shrimp farming industry. Water treatment should be implemented before being released back to the environment to lessen the negative impact brought by the rearing of shrimp from a highly contaminated source. These precautions should be properly considered as the shrimp farming industry develops to help safeguard the welfare of our natural environment. Finally, it is important for everyone participating in the shrimp farming sector, as well as the general public, to have a sufficient awareness of food safety.

4.5 Conclusion

Toxin genes *rtxA* and *rtxC* were prominent in *V. cholerae* isolates, proving the pathogenicity of the bacteria. High MAR indices from both bacterial species isolates indicate the consequences of improper use of antibiotics from highly contaminated sources, whether from imported shrimp larvae from external nurseries, or other bacteria in the environment via water current. Despite the high MAR indices, the banned antibiotics are still highly susceptible towards the isolates, therefore proving the obligation of shrimp handlers towards banned antibiotics. Alternative shrimp disease treatments with lower risk towards the environment and human consumption can slowly be implemented for treating diseases caused by *Vibrio* species. Of all the antibiotics tested in this study, ciprofloxacin is the most

suitable antibiotic for the treatment of vibriosis. Overall, the research presented in this study provides important insights into the pathogenicity and antibiotic resistance of *Vibrio* species isolates in aquaculture, underscoring the need for ongoing surveillance and monitoring of these bacterial strains to prevent the spread of antibiotic-resistant infections and protect the health of shrimp handlers and consumers. With the implementation of preventative measures and more sustainable practices, the impact of *Vibrio*-related illnesses on public health can be properly mitigated.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

From this study, it is apparent that most sampling showed that the population of *Vibrio* species were above the considered normal density of 1000 UFC/mL. From the total samples of thirty-two ($n=32$), *V. parahaemolyticus* showed 78%, 100%, 90%, and 90% prevalence while *V. cholerae* showed 72%, 61%, 90% and 70% prevalence from Pond A, Pond B, effluent, and influent, respectively. The quantification of cyanobacteria using Sedgwick-Rafter counting chamber and inverted microscope with DIC filter revealed the presence of *Pseudanabaena*, *Chroococcus*, *Phormidium*, *Oscillatoria* and *Lyngbya* genus in our local shrimp farm with *Pseudanabaena* dominating the shrimp farm towards the end of sampling.

This study also provided an overview of the relationship between *Vibrio* species and cyanobacteria in a local shrimp farm. Results from CCA showed an insignificant correlation between *Vibrio* species and cyanobacteria with p -values of 0.847, 0.255, 0.288, and 0.304 for Pond A, Pond B, effluent, and influent water, respectively. The insignificant correlation of *Vibrio* species and cyanobacteria in a local shrimp farm in Sarawak proves that other factors such as regional factors and species-specific bacteria plays the role in determining their correlation. Further research should be conducted in order to better understand the relationship between *Vibrio* species and cyanobacteria.

Aside from that, the emergence of virulent genes and antibiotic resistance in *Vibrio* species detected in this study can pose as potential health risks in the shrimp industry. It is apparent that 70% of *rtxA* and 90% of *rtxC* virulence genes were present in *V. cholerae*

isolates while *tdh* and *trh* were absent in *V. parahaemolyticus* isolates. Antibiotic susceptibility testing showed that all *V. cholerae* and *V. parahaemolyticus* isolates were resistant to at least one antibiotic with the mean MAR indices of 0.34 for *V. cholerae* and 0.24 for *V. parahaemolyticus*. The MAR index of 0.20 and greater indicates that antibiotics are heavily contaminating the shrimp farm water.

Based on the results of this study, it is recommended that *Vibrio* monitoring is performed periodically in shrimp farms so that disease prevention measure can be done. Due to the high *Vibrio* population in the shrimp farm water, the treatments such as water filtration, sanitation, and disinfection are recommended. Aside from that, the import of shrimp larvae from other hatcheries leaves open the possibility of the hatcheries to be contaminated with antibiotics as well. As such, it is also important for farmers to limit the use of antibiotics in their shrimp farms to mitigate the emergence of superbugs. It is also advised that alternative treatment of diseases in the aquaculture industry to be implemented before antibiotic resistance becomes the new normal. This research has provided valuable insight into the risks of antibiotic-resistant infections, which can be applied by or suggested for the shrimp industry in Sarawak and beyond.

REFERENCES

- A guide to Differential Interference Contrast (DIC). (n.d.). *Scientifica*.
<https://www.scientifica.uk.com/learning-zone/differential-interference-contrast>
- Abdelrahman, H., Abebe, A., & Boyd, C. (2019). Influence of variation in water temperature on survival, growth and yield of Pacific white shrimp *Litopenaeus vannamei* in inland ponds for low-salinity culture. *Aquaculture Research*, 658–672.
<https://doi.org/10.1111/are.13943>
- Abdullahi, R., Lihan, S., Carlos, B., Bilung, M., Mikal, M., & Collick, F. (2013). Detection of *oprL* gene and antibiotic resistance of *Pseudomonas aeruginosa* from aquaculture environment. *European Journal of Experimental Biology*, 3(6), 148-152. Retrieved from https://www.researchgate.net/publication/259189297_Detection_of_oprL_gene_and_antibiotic_resistance_of_Pseudomonas_aeruginosa_from_aquaculture_environment
- Adams, D. H., & Duggan, P. S. (2007). Cyanobacteria-bryophyte symbioses. *Journal of Experimental Botany*, 59(5), 1047–1058. <https://doi.org/10.1093/jxb/ern005>
- Adesiyun, I. M., Bisi-Johnson, M. A., & Okoh, A. I. (2022). Incidence of antibiotic resistance genotypes of *Vibrio* species recovered from selected freshwaters in Southwest Nigeria. *Scientific Reports*, 12(1), Article 1.
<https://doi.org/10.1038/s41598-022-23479-0>
- Adesiyun, I. M., Bisi-Johnson, M. A., Ogunfowokan, A. O., & Okoh, A. I. (2021). Occurrence and antibiogram signatures of some *Vibrio* species recovered from selected rivers in South West Nigeria. *Environmental Science and Pollution Research*, 28(31), 42458–42476. <https://doi.org/10.1007/s11356-021-13603-4>
- Aguirre-Guzmán, G., Ascencio, F., & Saulnier, D. (2005). Pathogenicity of *Vibrio penaeicida* for white shrimp *Litopenaeus vannamei*: A cysteine protease-like exotoxin as a virulence factor. *Diseases of Aquatic Organisms*, 67, 201–207.
<https://doi.org/10.3354/dao067201>

- Aguirre-Guzman, G., Mejia Ruiz, H., & Ascencio, F. (2004). A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. *Aquaculture Research*, 35(15), 1395–1404. <https://doi.org/10.1111/j.1365-2109.2004.01165.x>
- Aguirre-Guzman, G., Sánchez-Martínez, J. G., & Campa-Córdova, Á. L., & Luna, A., & Ascencio, F. (2009). Penaeid shrimp immune system. *The Thai veterinary medicine*, 39(3), 205-215. https://www.researchgate.net/publication/261323302_Penaeid_Shrimp_Immune_System
- Ahmed, S. (2018). Shrimp farming at the interface of land use change and marginalization of local farmers: Critical insights from southwest coastal Bangladesh. *Journal of Land Use Science*, 13(3), 251–258. <https://doi.org/10.1080/1747423X.2018.1529833>
- Albrecht, M., Pröschold, T., & Schumann, R. (2017). Identification of cyanobacteria in a eutrophic coastal lagoon on the Southern Baltic Coast. *Frontiers in Microbiology*, 8. <https://doi.org/10.3389/fmicb.2017.00923>
- Alfiansah, Y. R., Hassenrück, C., Kunzmann, A., Arief Taslihan, Harder, J., & Gärdes, A. (2018). Bacterial abundance and community composition in pond water from shrimp aquaculture systems with different stocking densities. *Frontiers in Microbiology*, 9. <https://doi.org/10.3389/fmicb.2018.02457>
- Allan, J. D., & Castillo, M. M. (2007). Primary producers. In: *Stream Ecology*. Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-5583-6_6
- Amatul-Samahah, Md. A., Wan Omar, W. H. H., Mohd Ikhsan, N. F., Amal Azmai, M. N., Zamri-Saad, M., & Ina-Salwany, Md. Y. (2020). Vaccination trials against vibriosis in shrimp: A review. *Aquaculture Reports*, 18, 100471. <https://doi.org/10.1016/j.aqrep.2020.100471>
- Ananya, Kamal, A., & Ahmad, I.Z. (2014). Cyanobacteria "the blue green algae" and its novel applications: A brief review. *International Journal of Innovation and Applied*

Studies, 7, 251-261. <https://www.issr-journals.org/xplore/ijias/0007/001/IJIAS-14-174-05.pdf>

Anas, A., Sukumaran, V., Nampullipurackal Devarajan, D., Maniyath, S., Chekidhenkuzhiyil, J., Mary, A., Parakkaparambil Kuttan, S., & Tharakan, B. (2021). Probiotics inspired from natural ecosystem to inhibit the growth of *Vibrio* spp. causing white gut syndrome in *Litopenaeus vannamei*. *3 Biotech*, 11(2), 66. <https://doi.org/10.1007/s13205-020-02618-2>

Aranguren, L. F., Mai, H., Pichardo, O., Hanggono, B., Dhar, A. K. (2019). White feces syndrome in shrimp: Predictor of EHP? *Global Seafood Alliance*. Retrieved from <https://www.globalseafood.org/advocate/white-feces-syndrome-shrimp-predictor-ehp/>

Association of Southeast Asian Nations (ASEAN). (2020). *Fisheries cooperation*. Retrieved from <https://asean.org/our-communities/economic-community/enhanced-connectivity-and-sectoral-development/asean-food-agriculture-and-forestry/fisheries-cooperation/>

Ayobami, O., Brinkwirth, S., Eckmanns, T., & Markwart, R. (2022). Antibiotic resistance in hospital-acquired ESKAPE-E infections in low- and lower-middle-income countries: A systematic review and meta-analysis. *Emerging Microbes and Infections*, 11(1), 443–451. <https://doi.org/10.1080/22221751.2022.2030196>

Babu, B., Sathiyaraj, G., Mandal, A., Kandan, S., Biju, N., Palanisamy, S., You, S., Nisha, R. G., & Prabhu, N. M. (2021). Surveillance of disease incidence in shrimp farms located in the east coastal region of India and *in-vitro* antibacterial efficacy of probiotics against *Vibrio parahaemolyticus*. *Journal of Invertebrate Pathology*, 179, 107536. <https://doi.org/10.1016/j.jip.2021.107536>

BAM Appendix 2: Most probable number from serial dilutions. (2020). Retrieved from <https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions>

- Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, 45(4_ts), 493–496. https://doi.org/10.1093/ajcp/45.4_ts.493
- Behera, N. (2018). EU ambassador rules out possibility of a ban on Indian shrimp imports. *Business Standard*. Retrieved from https://www.business-standard.com/article/economy-policy/eu-ambassador-rules-out-possibility-of-a-ban-on-indian-shrimp-imports-118092500916_1.html
- Bell, B., Schellevis, F. G., Stobberingh, E. E., Goossens, H., & Pringle, M. (2014). A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. *BMC Infectious Disease*, 14(1). <https://doi.org/10.1186/1471-2334-14-13>
- Ben, Y., Fu, C., Hu, M., Liu, L., Ming Hung Wong, & Zheng, C. (2019). Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: A review. *Environmental Research*, 169, 483–493. <https://doi.org/10.1016/j.envres.2018.11.040>
- Biello, D. (2009). The Origin of Oxygen in Earth's Atmosphere. *Scientific American*. Retrieved from <https://www.scientificamerican.com/article/origin-of-oxygen-in-atmosphere/>
- Biomin. (2012). *Vibriosis in shrimp aquaculture: Causes, symptoms and prevention measures*. Retrieved from <https://www.biomin.net/species/aquaculture/vibriosis/>
- Breton-Deval, L., Sanchez-Flores, A., Juárez, K., & Vera-Estrella, R. (2019). Integrative study of microbial community dynamics and water quality along the Apatlaco river. *Environmental Pollution*, 255, 113158. <https://doi.org/10.1016/j.envpol.2019.113158>
- Britannica. (2020). Nitrogen-fixing bacteria. *Encyclopedia Britannica*. Retrieved from <https://www.britannica.com/science/nitrogen-fixing-bacteria>

- Broberg, C. A., Calder, T. J., & Orth, K. (2011). *Vibrio parahaemolyticus* cell biology and pathogenicity determinants. *Microbes and Infection*, 13(12–13), 992–1001. <https://doi.org/10.1016/j.micinf.2011.06.013>
- Burkholder, J. M. (2009). Harmful Algal Blooms. *Encyclopedia of Inland Waters*, 264–285. <https://doi.org/10.1016/b978-012370626-3.00239-8>
- Burnap, R. L. (2015). Systems and photosystems: Cellular limits of autotrophic productivity in cyanobacteria. *Frontiers in Bioengineering and Biotechnology*, 3, 1. <https://doi.org/10.3389/fbioe.2015.00001>
- Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. *Environmental Microbiology*, 8(7), 1137–1144. <https://doi.org/10.1111/j.1462-2920.2006.01054.x>
- Canellas, A. L. B., Lopes, I. R., Mello, M. P., Paranhos, R., de Oliveira, B. F. R., & Laport, M. S. (2021). *Vibrio* species in an urban tropical estuary: Antimicrobial susceptibility, interaction with environmental parameters, and possible public health outcomes. *Microorganisms*, 9(5), 1007. <https://doi.org/10.3390/microorganisms9051007>
- Carbajal-Hernández, J. J., Sánchez-Fernández, L. P., Carrasco-Ochoa, J. A., & Martínez-Trinidad, J. F. (2012). Immediate water quality assessment in shrimp culture using fuzzy inference systems. *Expert Systems with Applications*, 39(12), 10571–10582. <https://doi.org/10.1016/j.eswa.2012.02.141>
- Carbajal-Hernández, J. J., Sánchez-Fernández, L. P., Villa-Vargas, L. A., Carrasco-Ochoa, J. A., & Martínez-Trinidad, J. Fco. (2013). Water quality assessment in shrimp culture using an analytical hierarchical process. *Ecological Indicators*, 29, 148–158. <https://doi.org/10.1016/j.ecolind.2012.12.017>
- Cartwright, E. J., & Griffin, P. M. (2012). 159—Other *Vibrio* species. In S. S. Long (Ed.), *Principles and Practice of Pediatric Infectious Diseases (Fourth Edition)* (pp. 854–856.e1). Elsevier. <https://doi.org/10.1016/B978-1-4377-2702-9.00161-6>

- Cassier-Chauvat, C., & Chauvat, F. (2014). Function and regulation of ferredoxins in the cyanobacterium, *Synechocystis* PCC6803: Recent advances. *Life*, 4(4), 666–680. <http://dx.doi.org/10.3390/life4040666>
- Charpy, L., Casareto, B. E., Langlade, M. J., & Suzuki, Y. (2012). Cyanobacteria in coral reef ecosystems: A Review. *Journal of Marine Biology*, 2012, 1–9. <https://doi.org/10.1155/2012/259571>
- Chatterjee, S., & Haldar, S. (2012). *Vibrio* related diseases in aquaculture and development of rapid and accurate identification methods. *J Marine Sci Res Dev*, 1(2). <https://doi.org/10.4172/2155-9910.S1-002>
- Chellapandian, H., Sivakamavalli, J., Vijay, A. A., & Balasubramanian, B. (2021). Challenges in controlling vibriosis in shrimp farms. *IntechOpen*. <https://doi.org/10.5772/intechopen.97018>
- Chen, L., Fan, J., Yan, T., Liu, Q., Yuan, S., Zhang, H., Yang, J., Deng, D., Huang, S., & Ma, Y. (2019). Isolation and characterization of specific phages to prepare a cocktail preventing *Vibrio* sp. Va-F3 infections in shrimp (*Litopenaeus vannamei*). *Frontiers in Microbiology*, 10, 2337. <https://doi.org/10.3389/fmicb.2019.02337>
- Chen, Y., Ai, X., & Yang, Y. (2022). *Vibrio cholerae*: A pathogen shared by human and aquatic animals. *The Lancet Microbe*, 3(6), e402. [https://doi.org/10.1016/S2666-5247\(22\)00125-2](https://doi.org/10.1016/S2666-5247(22)00125-2)
- Chen, Y.-H., & He, J. (2019). Effects of environmental stress on shrimp innate immunity and white spot syndrome virus infection. *Fish & Shellfish Immunology*, 84, 744–755. <https://doi.org/10.1016/j.fsi.2018.10.069>
- Cheng, W., Chen, S.-M., Wang, F.-I., Hsu, P.-I., Liu, C.-H., & Chen, J.-C. (2003). Effects of temperature, pH, salinity and ammonia on the phagocytic activity and clearance efficiency of giant freshwater prawn *Macrobrachium rosenbergii* to *Lactococcus garvieae*. *Aquaculture*, 219(1–4), 111–121. [https://doi.org/10.1016/S0044-8486\(03\)00017-6](https://doi.org/10.1016/S0044-8486(03)00017-6)

- Chorus, I., & Bartram, J. (Eds.). (1999). *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring, and management*. E & FN Spon. Retrieved from <https://www.who.int/publications/m/item/toxic-cyanobacteria-in-water---second-edition>
- Chow, K. H., Ng, T. K., Yuen, K. Y., & Yam, W. C. (2001). Detection of RTX Toxin Gene in *Vibrio cholerae* by PCR. *Journal of Clinical Microbiology*, 39(7), 2594–2597. <https://doi.org/10.1128/JCM.39.7.2594-2597.2001>
- Chowdhury, S. R., Shafiq Rheman, Debnath, N. C., Delamare-Deboutteville, J., Akhtar, Z., Ghosh, S., Parveen, S., Islam, K., Md. Ariful Islam, Rashid, M., Zobaidul Haque Khan, Rahman, M., Vishnumurthy Mohan Chadag, & Chowdhury, F. (2022). Antibiotics usage practices in aquaculture in Bangladesh and their associated factors. *One Health*, 15, 100445–100445. <https://doi.org/10.1016/j.onehlt.2022.100445>
- CLSI. (2015). Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. 3rd ed. CLSI guideline M45. Wayne, PA. Retrieved from [https://goums.ac.ir/files/deputy_treat/md_labs_ef39a/files/CLSI-M45ed3e-2018\(1\).pdf](https://goums.ac.ir/files/deputy_treat/md_labs_ef39a/files/CLSI-M45ed3e-2018(1).pdf)
- Coote, J. G. (1992). Structural and functional relationships among the RTX toxin determinants of Gram-negative bacteria. *FEMS Microbiology Reviews*, 8(2), 137–161. <https://doi.org/10.1111/j.1574-6968.1992.tb04961.x>
- Dadgostar P. (2019). Antimicrobial resistance: Implications and costs. *Infection and drug resistance*, 12, 3903–3910. <https://doi.org/10.2147/IDR.S234610>
- Das, R. (2014). Low-wage capitalism, social difference, and nature-dependent production: A study of the conditions of workers in shrimp aquaculture. *Human Geography*, 7, 17–34. <https://doi.org/10.1177/194277861400700109>
- Davis, B. M., Kimsey, H. H., Chang, W., & Waldor, M. K. (1999). The *Vibrio cholerae* O139 calcutta bacteriophage CTX ϕ is infectious and encodes a novel repressor. *Journal of Bacteriology*, 181(21), 6779–6787. <https://doi.org/10.1128/jb.181.21.6779-6787.1999>

- Davis, R., & Brown, P. D. Y. (2016). Multiple antibiotic resistance index, fitness and virulence potential in respiratory *Pseudomonas aeruginosa* from Jamaica. *Journal of Medical Microbiology*, 65(4), 261–271. <https://doi.org/10.1099/jmm.0.000229>
- Dawes, E.A. (1989). Growth and survival of bacteria. In: Poindexter, J.S., Leadbetter, E.R. (eds) *bacteria in nature. Bacteria in Nature*, vol 3. Springer, Boston, MA. https://doi.org/10.1007/978-1-4613-0803-4_2
- de la Peña, L. D., Cabillon, N. A. R., Catedral, D. D., Amar, E. C., Usero, R. C., Monotilla, W. D., Calpe, A. T., Fernandez, D. D., & Saloma, C. P. (2015). Acute hepatopancreatic necrosis disease (AHPND) outbreaks in *Penaeus vannamei* and *P. monodon* cultured in the Philippines. *Diseases of Aquatic Organisms*, 116(3), 251–254. <https://doi.org/10.3354/dao02919>
- de la Peña, L., Cabillon, N. A., Amar, E., Catedral, D., Usero, R., Faisan, J., Arboleda, J., Monotilla, W., Calpe, A., Fernandez, D., & Saloma, C. (2017). Mortality of pond-cultured *Litopenaeus vannamei* associated with acute hepatopancreatic necrosis disease (AHPND) and white spot syndrome virus (WSSV) infection in the Philippines. *Fish Pathology*, 52, 38–41. <https://doi.org/10.3147/jsfp.52.38>
- de Lacerda, L. D., Ward, R. D., Godoy, M. D. P., de Andrade Meireles, A. J., Borges, R., & Ferreira, A. C. (2021). 20-Years cumulative impact from shrimp farming on mangroves of northeast Brazil. *Frontiers in Forests and Global Change*, 4. <https://www.frontiersin.org/articles/10.3389/ffgc.2021.653096>
- de Souza Valente, C., & Wan, A. H. L. (2021). *Vibrio* and major commercially important vibriosis diseases in decapod crustaceans. *Journal of Invertebrate Pathology*, 181, 107527. <https://doi.org/10.1016/j.jip.2020.107527>
- Defoirdt, T. (2018). Quorum-sensing systems as targets for antivirulence therapy. *Trends in Microbiology*, 26(4), 313–328. <https://doi.org/10.1016/j.tim.2017.10.005>
- Dell’Annunziata, F., Folliero, V., Giugliano, R., De Filippis, A., Santarcangelo, C., Izzo, V., Daglia, M., Galdiero, M., Arciola, C. R., & Franci, G. (2021). Gene Transfer

- Potential of Outer Membrane Vesicles of Gram-Negative Bacteria. *International Journal of Molecular Sciences*, 22(11), 5985. <https://doi.org/10.3390/ijms22115985>
- Deng, Y., Xu, H., Su, Y., Liu, S., Xu, L., Guo, Z., Wu, J., Cheng, C., & Feng, J. (2019). Horizontal gene transfer contributes to virulence and antibiotic resistance of *Vibrio harveyi* 345 based on complete genome sequence analysis. *BMC Genomics*, 20, 761. <https://doi.org/10.1186/s12864-019-6137-8>
- Deng, Y., Xu, L., Chen, H., Liu, S., Guo, Z., Cheng, C., Ma, H., & Feng, J. (2020). Prevalence, virulence genes, and antimicrobial resistance of *Vibrio* species isolated from diseased marine fish in South China. *Scientific Reports*, 10(1), Article 1. <https://doi.org/10.1038/s41598-020-71288-0>
- Denzer, L., Schrotten, H., & Schwerk, C. (2020). From gene to protein—how bacterial virulence factors manipulate host gene expression during infection. *International Journal of Molecular Sciences*, 21(10), 3730. <https://doi.org/10.3390/ijms21103730>
- Dhifi, W., Bellili, S., Jazi, S., Bahloul, N., & Mnif, W. (2016). Essential oils' chemical characterization and investigation of some biological activities: A critical review. *Medicines*, 3(4), 25. <https://doi.org/10.3390/medicines3040025>
- Dick, M. H., Guillermin, M., Moussy, F., & Chaignat, C.-L. (2012). Review of two decades of cholera diagnostics – How far have we really come? *PLOS Neglected Tropical Diseases*, 6(10), e1845. <https://doi.org/10.1371/journal.pntd.0001845>
- Domínguez-Borbor, C., Sánchez-Rodríguez, A., Sonnenholzner, S., & Rodríguez, J. (2020). Essential oils mediated antivirulence therapy against vibriosis in *Penaeus vannamei*. *Aquaculture*, 529, 735639. <https://doi.org/10.1016/j.aquaculture.2020.735639>
- Doron, S., & Gorbach, S. L. (2008). Bacterial infections: Overview. *International Encyclopedia of Public Health*, 273–282. <https://doi.org/10.1016/B978-012373960-5.00596-7>
- Duc, P. M., Hoa, T. T., Phuong, N. T., Bosma, R. H., Huỳnh, V. H., & Tran, N. T. (2015). Virus diseases risk-factors associated with shrimp farming practices in rice-shrimp

- and intensive culture systems in Mekong Delta Viet Nam. *International journal of scientific and research publications*, 5, 1-6. Retrieved from <https://www.ijsrp.org/research-paper-0815/ijsrp-p4434.pdf>
- Ducat, D. C., Way, J. C., & Silver, P. A. (2011). Engineering cyanobacteria to generate high-value products. *Applied Microbiology*, 29(2), 95–103. <https://doi.org/10.1016/j.tibtech.2010.12.003>
- Dutta, D., Kaushik, A., Kumar, D., & Bag, S. (2021). Foodborne pathogenic vibrios: Antimicrobial resistance. *Frontiers in Microbiology*, 12. <https://www.frontiersin.org/articles/10.3389/fmicb.2021.638331>
- Dvorak, P., Casamatta, D., Hasler, P., Jahodářová, E., Norwich, A., & Pouličková, A. (2017). Diversity of the cyanobacteria. *Modern Topics in the Phototrophic Prokaryotes: Environmental and Applied Aspects*, 3–46. https://doi.org/10.1007/978-3-319-46261-5_1
- Dvorak, P., Pouličková, A., Hasler, P., Belli, M., Casamatta, D., & Papini, A. (2015). Species concepts and speciation factors in cyanobacteria, with connection to the problems of diversity and classification. *Biodiversity and Conservation*. <https://doi.org/10.1007/s10531-015-0888-6>
- Eiler, A., Gonzalez-Rey, C., Allen, S., & Bertilsson, S. (2007). Growth response of *Vibrio cholerae* and other *Vibrio* spp. to cyanobacterial dissolved organic matter and temperature in brackish water: Cyanobacterial DOM, temperature and *Vibrio* growth. *FEMS Microbiology Ecology*, 60(3), 411–418. <https://doi.org/10.1111/j.1574-6941.2007.00303.x>
- Elexson, N., Afsah-Hejri, L., Rukayadi, Y., Soopna, P., Lee, H. Y., Tuan Zainazor, T. C., Nor Ainy, M., Nakaguchi, Y., Mitsuaki, N., & Son, R. (2014). Effect of detergents as antibacterial agents on biofilm of antibiotics-resistant *Vibrio parahaemolyticus* isolates. *Food Control*, 35(1), 378–385. <https://doi.org/10.1016/j.foodcont.2013.07.020>

- Farooq, A., & Unno, T. (2018). Plasmid profiling of multi-drug resistant *Vibrio* sp. Isolated from influent and effluent water samples of fish farms in Jeju, South Korea. *Korean Journal of Microbiology*, 54(1), 53–59. <https://doi.org/10.7845/kjm.2018.7095>
- Fathy Mahmoud, F. (2015). Effect of Cooking Methods on Antibiotic Residues in Broiler Chicken Meat. *Conference: 2nd International conference of Food Safety, Suez Canal University At: Faculty of Veterinary Medicine- Suez Canal University*, 1(2015), 76–81. https://www.researchgate.net/publication/322631993_Effect_of_Cooking_Methods_on_Antibiotic_Residues_in_Broiler_Chicken_Meat
- Fisheries Development Authority of Malaysia. (2020). *Fisheries Development Authority of Malaysia*. Annual Report. <https://lkim.gov.my/en/annual-report/>
- Flegel, T. W. (2012). Historic emergence, impact and current status of shrimp pathogens in Asia. *Journal of Invertebrate Pathology*, 110(2), 166–173. <https://doi.org/10.1016/j.jip.2012.03.004>
- Fletcher, S. (2015). Understanding the contribution of environmental factors in the spread of antimicrobial resistance. *Environmental Health and Preventive Medicine*, 20(4), Article 4. <https://doi.org/10.1007/s12199-015-0468-0>
- Fu, H., Yu, P., Liang, W., Kan, B., Peng, X., & Chen, L. (2020). Virulence, resistance, and genomic fingerprint traits of *Vibrio cholerae* isolated from 12 species of aquatic products in Shanghai, China. *Microbial Drug Resistance*, 26(12), 1526–1539. <https://doi.org/10.1089/mdr.2020.0269>
- Fu, S., Hao, J., Jin, S., Wu, K., Wang, Y., Ye, S., Liu, Y., & Li, R. (2019). A human intestinal infection caused by a novel non-O1/O139 *Vibrio Cholerae* genotype and its dissemination along the river. *Frontiers in Public Health*, 7. <https://www.frontiersin.org/articles/10.3389/fpubh.2019.00100>
- Gao, W., Tian, L., Huang, T., Yao, M., Hu, W., & Xu, Q. (2016). Effect of salinity on the growth performance, osmolarity and metabolism-related gene expression in white shrimp *Litopenaeus vannamei*. *Aquaculture Reports*, 4, 125–129. <https://doi.org/10.1016/j.aqrep.2016.09.001>

- Garibay-Valdez, E., Martínez-Córdova, L. R., López-Torres, M. A., Almendariz-Tapia, F. J., Martínez-Porchas, M., & Calderón, K. (2020). The implication of metabolically active *Vibrio* spp. in the digestive tract of *Litopenaeus vannamei* for its post-larval development. *Scientific Reports*, 10(1), Article 1. <https://doi.org/10.1038/s41598-020-68222-9>
- Garlapati, D., Chandrasekaran, M., Devanesan, A., Mathimani, T., & Pugazhendhi, A. (2019). Role of cyanobacteria in agricultural and industrial sectors: an outlook on economically important byproducts. *Applied Microbiology and Biotechnology*, 103, 4709–4721. <https://doi.org/10.1007/s00253-019-09811-1>
- Gellert, M. R., Kim, B. J., Reffsin, S. E., Jusuf, S., Wagner, N., Winans, S. C., & Wu, M. (2018). Nanobiotechnology for the environment: Innovative solutions for the management of harmful algal blooms. *Journal of Agricultural and Food Chemistry*, 66(26), 6474–6479. <https://doi.org/10.1021/acs.jafc.7b04271>
- Glibert, P. M., & Burkholder, J. M. (2011). Harmful algal blooms and eutrophication: Strategies for nutrient uptake and growth outside the Redfield comfort zone. *Chinese Journal of Oceanology and Limnology*, 29(4), 724–738. <https://doi.org/10.1007/s00343-011-0502-z>
- Golubic, S., & Seong-Joo, L. (1999). Early cyanobacterial fossil record: preservation, palaeoenvironments and identification. *European Journal of Phycology*, 34(4), 339–348. <https://doi.org/10.1080/09670269910001736402>
- Greenfield, D. I., Gooch Moore, J., Stewart, J. R., Hilborn, E. D., George, B. J., Li, Q., Dickerson, J., Keppler, C. K., & Sandifer, P. A. (2017). Temporal and environmental factors driving *Vibrio vulnificus* and *V. parahaemolyticus* populations and their associations with harmful algal blooms in South Carolina detention ponds and receiving tidal creeks. *GeoHealth*, 1(9), 306–317. <https://doi.org/10.1002/2017GH000094>
- Guichard, A., Moreno, B. C., Aguilar, B., van Sorge, N. M., Kuang, J., Kurkciyan, A. A., Wang, Z., Hang, S., Pineton de Chambrun, G. P., McCole, D. F., Watnick, P., Nizet, V., & Bier, E. (2013). Cholera toxin disrupts barrier function by inhibiting exocyst-

- mediated trafficking of host proteins to intestinal cell junctions. *Cell Host & Microbe*, 14(3), 294–305. <https://doi.org/10.1016/j.chom.2013.08.001>
- Gumbo, R., Ross, G., & Cloete, E. (2008). Biological control of Microcystis dominated harmful algal blooms. *African Journal of Biotechnology*, 7(25), 4765–4773. [https://repository.up.ac.za/bitstream/handle/2263/10181/Gumbo_Biological\(2008\).pdf](https://repository.up.ac.za/bitstream/handle/2263/10181/Gumbo_Biological(2008).pdf)
- Haifa-Haryani, W. O., Amatul-Samahah, M. A., Azzam-Sayuti, M., Chin, Y. K., Zamri-Saad, M., Natrah, I., Amal, M. N. A., Satyantini, W. H., & Ina-Salwany, M. Y. (2022). Prevalence, antibiotics resistance and plasmid profiling of *Vibrio* spp. isolated from cultured shrimp in peninsular Malaysia. *Microorganisms*, 10(9), Article 9. <https://doi.org/10.3390/microorganisms10091851>
- Harris, L., & Owens, L. (1999). Production of exotoxins by two luminous *Vibrio harveyi* strains known to be primary pathogens of *Penaeus monodon* larvae. *Diseases of Aquatic Organisms*, 38, 11–22. <https://doi.org/10.3354/dao038011>
- Hartwick, M. A., Berenson, A., Whistler, C. A., Naumova, E. N., & Jones, S. E. (2021). The seasonal microbial ecology of plankton and plankton-associated *Vibrio parahaemolyticus* in the northeast United States. *Applied and Environmental Biology*, 87(15). <https://doi.org/10.1128/aem.02973-20>
- Hashim, M. & Kathamuthu, S. (2005). Shrimp farming in Malaysia. In: Regional Technical Consultation on the Aquaculture of *P. vannamei* and Other Exotic Shrimps in Southeast Asia, Manila, Philippines (pp. 50-56). Tigbauan, Iloilo, Philippines: *SEAFDEC Aquaculture Department*. <http://hdl.handle.net/10862/851>
- Hasrimi, A. N., Budiharjo, A., & Jannah, S. N. (2018). Detection of *tlh* and *tdh* genes in *Vibrio Parahaemolyticus* inhabiting farmed water ecosystem used for *L. Vannamei* aquaculture. *Journal of Physics: Conference Series*, 1025, 012058. <https://doi.org/10.1088/1742-6596/1025/1/012058>
- Hassali, M. A. A., Yann, H. R., Verma, A. K., Hussain, R., & Sivaraman, S. (2018). Antibiotic use in food animals: Malaysia overview. *School of Pharmaceutical*

Sciences Universiti Sains Malaysia, 33. Retrieved from https://www.reactgroup.org/wp-content/uploads/2018/11/Antibiotic_Use_in_Food_Animals_Malaysia_Overview_2018web.pdf

- Holmgren, J. (1981). Actions of cholera toxin and the prevention and treatment of cholera. *Nature*, 292(5822), Article 5822. <https://doi.org/10.1038/292413a0>
- Holmström, K., Gräslund, S., Wahlström, A., Pongshompoo, S., Bengtsson, B.-E., & Kautsky, N. (2003). Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International Journal of Food Science & Technology*, 38, 255–266. <https://doi.org/10.1046/j.1365-2621.2003.00671.x>
- Honda, T., Ni, Y., Miwatani, T., Adachi, T., & Kim, J. (1992). The thermostable direct hemolysin of *Vibrio parahaemolyticus* is a pore-forming toxin. *Canadian Journal of Microbiology*, 38(11), 1175–1180. <https://doi.org/10.1139/m92-192>
- Hong To, T. T., Yanagawa, H., Khanh Thuan, N., Hiep, D. M., Cuong, D. V., Khai, L. T. L., Taniguchi, T., Kubo, R., & Hayashidani, H. (2020). Prevalence of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease of shrimp in shrimp, molluscan shellfish and water samples in the Mekong delta, Vietnam. *Biology*, 9(10), 312. <https://doi.org/10.3390/biology9100312>
- Hossain, M. S., Aktaruzzaman, M., Fakhruddin, A. N. M., Uddin, M. J., Rahman, S. H., Chowdhury, M. A. Z., & Alam, M. K. (2012). Antimicrobial susceptibility of *Vibrio* species isolated from brackish water shrimp culture environment. *Journal of Bangladesh Academy of Sciences*, 36(2), 213–220. <https://doi.org/10.3329/jbas.v36i2.12964>
- Hossain, Md. M. M., Uddin, Md. I., Islam, H., Fardoush, J., Rupom, Md. A. H., Hossain, Md. M., Farjana, N., Afroz, R., Hasan-Uj-Jaman, Roy, H. S., Shehab, Md. A. S., & Rahman, Md. A. (2020). Diagnosis, genetic variations, virulence, and toxicity of AHPND-positive *Vibrio parahaemolyticus* in *Penaeus monodon*. *Aquaculture International*, 28(6), 2531–2546. <https://doi.org/10.1007/s10499-020-00607-z>

- Hou, D., Huang, Z., Zeng, S., Liu, J., Wei, D., Deng, X., Weng, S., He, Z., & He, J. (2017). Environmental factors shape water microbial community structure and function in shrimp cultural enclosure ecosystems. *Frontiers in Microbiology*, 8, 2359. <https://doi.org/10.3389/fmicb.2017.02359>
- Ibarra-Gómez, J., Rubio-García, M., Sánchez-Díaz, R., Casillas-Hernández, R., & Martínez-Ibarra, D. (2023). Identification techniques to prevent the current emerging disease hepatopancreatic microsporidiosis in white shrimp *Penaeus vannamei*: an overview. *Latin American Journal of Aquatic Research*, 51(1), 1-11. <http://dx.doi.org/10.3856/vol51-issue1-fulltext-2693>
- Igere, B. E., Okoh, A. I., & Nwodo, U. U. (2022). Atypical and dual biotypes variant of virulent SA-NAG-*Vibrio cholerae*: An evidence of emerging/evolving patho-significant strain in municipal domestic water sources. *Annals of Microbiology*, 72(1), 3. <https://doi.org/10.1186/s13213-021-01661-5>
- Islam, M. S., Drasar, B. S., & Sack, R. B. (1993). The aquatic environment as a reservoir of *Vibrio cholerae*: A review. *Journal of Diarrhoeal Diseases Research*, 11(4), 197–206. Retrieved from <https://www.jstor.org/stable/23498278>
- Islam, M. S., Mahmuda, S., Morshed, M. G., Bakht, H. B. M., Khan, M. N. H., Sack, R. B., & Sack, D. A. (2004). Role of cyanobacteria in the persistence of *Vibrio cholerae* O139 in saline microcosms. *Canadian Journal of Microbiology*, 50(2), 127–131. <https://doi.org/10.1139/w03-114>
- Islam, M., Zaman, M., Islam, M., Ahmed, N., & Clemens, J. (2020). Environmental reservoirs of *Vibrio cholerae*. *Vaccine*, 38, A52-A62. <https://doi.org/10.1016/j.vaccine.2019.06.033>
- Ismail, M. M., & Abdullah, A. M. (2013). Shrimp trade competitiveness of Malaysia and selected ASEAN countries. *Journal of International Food & Agribusiness Marketing*, 25(sup1), 98–115. <https://doi.org/10.1080/08974438.2013.800009>
- Izumiya, H., Matsumoto, K., Yahiro, S., Lee, J., Morita, M., Yamamoto, S., Arakawa, E., & Ohnishi, M. (2011). Multiplex PCR assay for identification of three major pathogenic

Vibrio spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. *Molecular and Cellular Probes*, 25(4), 174–176. <https://doi.org/10.1016/j.mcp.2011.04.004>

Jan, Z., Ali, S., Sultan, T., Khan, M. J., Shah, Z., & Khan, F. (2018). Impact of different strains of cyanobacteria on rice crop growth and nutrients uptake under saline soil condition. *Sarhad Journal of Agriculture*, 34(2). <https://doi.org/10.17582/journal.sja/2018/34.2.450.458>

Jayasree, L., Janakiram, P., & Madhavi, R. (2006). Characterization of *Vibrio* spp. associated with diseased shrimp from culture ponds of Andhra Pradesh (India). *Journal of the World Aquaculture Society*, 37(4), 523–532. <https://doi.org/10.1111/j.1749-7345.2006.00066.x>

Jesser, K. J., & Noble, R. T. (2018). *Vibrio* ecology in the neuse river estuary, North Carolina, characterized by next-generation amplicon sequencing of the gene encoding heat shock protein 60 (*hsp60*). *Applied and Environmental Microbiology*, 84(13), e00333-18. <https://doi.org/10.1128/AEM.00333-18>

Jesser, K. J., & Noble, R. T. (2018). *Vibrio* ecology in the Neuse river estuary, North Carolina, characterized by next-generation amplicon sequencing of the gene encoding heat shock protein 60 (*hsp60*). *Applied and Environmental Microbiology*, 84(13), e00333-18. <https://doi.org/10.1128/AEM.00333-18>

Jia, Y., Han, G., Wang, C., Guo, P., Jiang, W., Li, X.-N., & Tian, X. (2010). The efficacy and mechanisms of fungal suppression of freshwater harmful algal bloom species. *Journal of Hazardous Materials*, 183(1-3), 176–181. <https://doi.org/10.1016/j.jhazmat.2010.07.009>

Johnson, J. A., Salles, C. A., Panigrahi, P., Albert, M. J., Wright, A. C., Johnson, R. J., & Morris, J. G. (1994). *Vibrio cholerae* O139 synonym bengal is closely related to *Vibrio cholerae* El Tor but has important differences. *Infection and Immunity*, 62(5), 2108–2110. <https://doi.org/10.1128/iai.62.5.2108-2110.1994>

- Joseph, T., Vaiyapuri, M., Reghunathan, D., Peeralil, Shaheer. P., Akhilnath, P. G., & Lalitha, K. (2015). Isolation and characterization of *Vibrio cholerae* O139 associated with mass mortality in *Penaeus monodon* and experimental challenge in postlarvae of three species of shrimp. *Aquaculture*, 442. <https://doi.org/10.1016/j.aquaculture.2015.02.020>
- Joy, Schreier, H. J., Lauma Lanska, & Hale, M. S. (2017). The rising tide of antimicrobial resistance in aquaculture: Sources, sinks and solutions. *Marine Drugs*, 15(6), 158–158. <https://doi.org/10.3390/md15060158>
- Julie, D., Solen, L., Antoine, V., Jaufrey, C., Annick, D., & Dominique, H.-H. (2010). Ecology of pathogenic and non-pathogenic *Vibrio parahaemolyticus* on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll a: Ecology of *Vibrio parahaemolyticus* in France. *Environmental Microbiology*, 12(4), 929–937. <https://doi.org/10.1111/j.1462-2920.2009.02136.x>
- Kamel, F., & Fawzi Jarjes, S. (2015). *Essentials of Bacteriology and Immunology*. Retrieved from https://www.researchgate.net/publication/290911856_Essentials_of_Bacteriology_and_Immunology
- Karthikeyan, V., Periyasamy Selvakumar, & Gopalakrishnan, A. (2015). A novel report of fungal pathogen *Aspergillus awamori* causing black gill infection on *Litopenaeus vannamei* (pacific white shrimp). *Aquaculture*, 444, 36–40. <https://doi.org/10.1016/j.aquaculture.2015.03.021>
- Karunasagar, I., Shivu, M., Girisha, S., Krohne, G., & Karunasagar, I. (2007). Biocontrol of pathogens in shrimp hatcheries using bacteriophages. *Aquaculture*, 268(1-4), 288-292. <https://doi.org/10.1016/j.aquaculture.2007.04.049>
- Keenleyside, W. (2019). 9.5 *The Effects of pH on Microbial Growth*. <https://ecampusontario.pressbooks.pub/microbio/chapter/the-effects-of-ph-on-microbial-growth/>
- Kilawati, Y., & Islamy, R. A. (2021). Immunostimulant activity of *Gracilaria* sp. and *Padina* sp. on immune system of vannamei Shrimp (*Litopenaeus vannamei*) against *Vibrio*

- harveyi*. *Journal of Aquaculture and Fish Health*, 10(2), 252-257. <https://doi.org/10.20473/jafh.v10i2.23009>
- Kim, H.-J., Ryu, J.-O., Lee, S.-Y., Kim, E.-S., & Kim, H.-Y. (2015). Multiplex PCR for detection of the *Vibrio* genus and five pathogenic *Vibrio* species with primer sets designed using comparative genomics. *BMC Microbiology*, 15(1), 239. <https://doi.org/10.1186/s12866-015-0577-3>
- King, K., Bramucci, A. R., Labbate, M., Raina, J.-B., & Seymour, J. R. (2022). Heterogeneous growth enhancement of *Vibrio cholerae* in the presence of different phytoplankton species. *Applied and Environmental Microbiology*, 88(17), e01158-22. <https://doi.org/10.1128/aem.01158-22>
- Kishishita, M., Matsuoka, N., Kumagai, K., Yamasaki, S., Takeda, Y., & Nishibuchi, M. (1992). Sequence variation in the thermostable direct hemolysin-related hemolysin (trh) gene of *Vibrio parahaemolyticus*. *Applied and environmental microbiology*, 58(8), 2449–2457. <https://doi.org/10.1128/aem.58.8.2449-2457.1992>
- Klein, S. L., Gutierrez West, C. K., Mejia, D. M., & Lovell, C. R. (2014). Genes similar to the *Vibrio parahaemolyticus* virulence-related genes *tdh*, *tlh*, and *vscC2* occur in other *Vibrionaceae* species isolated from a pristine estuary. *Applied and Environmental Microbiology*, 80(2), 595–602. <https://doi.org/10.1128/AEM.02895-13>
- Kobayashi, N., Lee, K., Yamazaki, A., Saito, S., Furukawa, I., Kono, T., Maeda, E., Isobe, J., Sugita-Konishi, Y., & Hara-Kudo, Y. (2013). Virulence gene profiles and population genetic analysis for exploration of pathogenic serogroups of Shiga toxin-producing *Escherichia coli*. *Journal of Clinical Microbiology*, 51(12), 4022–4028. <https://doi.org/10.1128/JCM.01598-13>
- Kopprio, G. A., Neogi, S. B., Rashid, H., Alonso, C., Yamasaki, S., Koch, B. P., Gärdes, A., & Lara, R. J. (2020). *Vibrio* and bacterial communities across a pollution gradient in the Bay of Bengal: Unraveling their biogeochemical drivers. *Frontiers in Microbiology*, 11, 594. <https://doi.org/10.3389/fmicb.2020.00594>

- Kriem, M. R., Banni, B., El Bouchtaoui, H., Hamama, A., El Marrakchi, A., Chaouqy, N., Robert-Pillot, A., & Quilici, M. L. (2015). Prevalence of *Vibrio* spp. in raw shrimps (*Parapenaeus longirostris*) and performance of a chromogenic medium for the isolation of *Vibrio* strains. *Letters in Applied Microbiology*, 61(3), 224–230. <https://doi.org/10.1111/lam.12455>
- Kua, B. C., Iar, A., Siti Zahrah, A., Irene, J., Norazila, J., Nik Haiha, N. Y., Fadzilah, Y., Mohammed, M., Siti Rokhaiya, B., Omar, M., & Teoh, T. P. (2016). *Current status of acute hepatopancreatic necrosis disease (AHPND) of farmed shrimp in Malaysia*. 55–59. <https://repository.seafdec.org.ph/handle/10862/3090>
- Kuczynski, J., Liu, Z., Lozupone, C., McDonald, D., Fierer, N., & Knight, R. (2010). Microbial community resemblance methods differ in their ability to detect biologically relevant patterns. *Nature Methods*, 7(10), 813–819. <https://doi.org/10.1038/nmeth.1499>
- Kumar, S., Verma, A., Shivesh Pratap Singh, & Abhishek Kumar Awasthi. (2022). Immunostimulants for shrimp aquaculture: paving pathway towards shrimp sustainability. *Environmental Science and Pollution Research*, 30(10), 25325–25343. <https://doi.org/10.1007/s11356-021-18433-y>
- Kuthoose, M. F. A., Suhaimi, N. S. M., Ibrahim, D., & Kamarul Zaman Zarkasi. (2021). Microbial community diversity associated with healthy and unhealthy shrimp (early mortality syndrome) at Malaysian shrimp farm. *Songklanakarin Journal of Science and Technology (SJST)*, 43, 897902. <https://doi.org/10.14456/SJST-PSU.2021.118>
- Lampert, W. (1987). Laboratory studies on zooplankton-cyanobacteria interactions. *New Zealand Journal of Marine and Freshwater Research*, 21(3), 483–490, DOI: 10.1080/00288330.1987.9516244
- Larsen, J. P. (1984). *Vibrio anguillarum*: Influence of temperature, pH, NaCl concentration and incubation time on growth. *Journal of Applied Microbiology*, 57(2), 237–246. <https://doi.org/10.1111/j.1365-2672.1984.tb01388.x>

- Lavilla-Pitogo, C. R., Lio-Po, G. D., Cruz-Lacierda, E. R., Alapide-Tendencia, E. V., & De la Peña, L. D. (2000). Diseases of penaeid shrimps in the Philippines. (2nd ed.). Tigbauan, Iloilo, Philippines: *Aquaculture Department, Southeast Asian Fisheries Development Center*. Retrieved from <https://repository.seafdec.org.ph/bitstream/handle/10862/1510/aem16-2ndEd.pdf?sequence=1&isAllowed=y>
- Lee, B. C., Choi, S. H., & Kim, T. S. (2008). *Vibrio vulnificus* RTX toxin plays an important role in the apoptotic death of human intestinal epithelial cells exposed to *Vibrio vulnificus*. *Microbes and Infection*, 10(14), 1504–1513. <https://doi.org/10.1016/j.micinf.2008.09.006>
- Lee, D., Yu, Y. B., Choi, J. H., Jo, A. H., Hong, S. M., Kang, J. C., & Kim, J. H. (2022). Viral shrimp diseases listed by the OIE: A Review. *Viruses*, 14(3), 585. <https://doi.org/10.3390/v14030585>
- Lee, K., & Ruby, E. G. (1994). Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri*. *Nature*, 60(5), 1565–1571. <https://doi.org/10.1126/aem.60.5.1565-1571.1994>
- Lembaga Kemajuan Ikan Malaysia Negeri Sarawak (LKIM). (2022). Retrieved from https://www.facebook.com/profile/100063528072203/search/?q=telaga%20&locale=ms_MY
- Lightner, D. V. (2011). Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): A review. *Journal of Invertebrate Pathology*, 106(1), 110–130. <https://doi.org/10.1016/j.jip.2010.09.012>
- Lightner, D. V., Redman, R. M., Pantoja, C. R., Tang, K., Noble, B. L., Schofield, P. N., Mohny, L. L., Nunan, L. M., & Navarro, S. (2012). Historic emergence, impact and current status of shrimp pathogens in the Americas. *Journal of Invertebrate Pathology*, 110(2), 174–183. <https://doi.org/10.1016/j.jip.2012.03.006>
- Lin, Q., Xavier, B. B., Alako, B. T. F., Mitchell, A. L., Rajakani, S. G., Glupczynski, Y., Finn, R. D., Cochrane, G., & Malhotra-Kumar, S. (2022). Screening of global microbiomes implies ecological boundaries impacting the distribution and

- dissemination of clinically relevant antimicrobial resistance genes. *Communications Biology*, 5(1), Article 1. <https://doi.org/10.1038/s42003-022-04187-x>
- Linhartová, I., Bumba, L., Mašín, J., Basler, M., Osička, R., Kamanová, J., Procházková, K., Adkins, I., Hejnová-Holubová, J., Sadílková, L., Morová, J., & Šebo, P. (2010). RTX proteins: A highly diverse family secreted by a common mechanism. *Fems Microbiology Reviews*, 34(6), 1076–1112. <https://doi.org/10.1111/j.1574-6976.2010.00231.x>
- Loker, E. S., Adema, C. M., Zhang, S.-M., & Kepler, T. B. (2004). Invertebrate immune systems – not homogeneous, not simple, not well understood. *Immunological Reviews*, 198, 10–24. <https://doi.org/10.1111/j.0105-2896.2004.0117.x>
- Lundin, C. (1995). *Global Attempts to Address Shrimp Disease*. Retrieved from <http://library.enaca.org/Shrimp/Publications/ShrimpDisease.pdf>
- Luu, Q. H., Nguyen, T. B. T., Nguyen, T. L. A., Do, T. T. T., Dao, T. H. T., & Padungtod, P. (2021). Antibiotics use in fish and shrimp farms in Vietnam. *Aquaculture Reports*, 20, 100711. <https://doi.org/10.1016/j.aqrep.2021.100711>
- Madhusudana, R. B., & Surendran, P. K. (2013). Detection of *ctx* gene positive non-O1/non-O139 *V. cholerae* in shrimp aquaculture environments. *Journal of Food Science and Technology*, 50(3), 496–504. <https://doi.org/10.1007/s13197-011-0374-4>
- Mahboobi, M., Mirnejad, R., Sedighian, H., Piranfar, V., & Imani Fooladi, A. A. (2020). Genetic diversity of *ctxB* gene among classical O1 and El Tor strains of *Vibrio cholerae* using high-resolution melting curve analysis. *Iranian Journal of Pathology*, 15(4), 320–325. <https://doi.org/10.30699/ijp.2020.127793.2393>
- Mai, A. T., Chung, D., Ngo, L., Huynh, K. H., & Dinh, L. T. (2022). Multiorgan dysfunction with severe cardiac injury secondary to septic cellulitis due to *Vibrio parahaemolyticus*. *Cureus*, 14(11), e31673. <https://doi.org/10.7759/cureus.31673>
- Maicá, P. F., Borba, M. R. de, Martins, T. G., & Wasielesky Junior, W. (2014). Effect of salinity on performance and body composition of Pacific white shrimp juveniles

- reared in a super-intensive system. *Revista Brasileira de Zootecnia*, 43(7), 343–350. <https://doi.org/10.1590/s1516-35982014000700001>
- Mandal, J., Sangeetha, V., Ganesan, V., Parveen, M., Preethi, V., Harish, B. N., Srinivasan, S., & Parija, S. C. (2012). Third-generation cephalosporin-resistant *Vibrio cholerae*, India. *Emerging Infectious Diseases*, 18(8), 1326–1328. <https://doi.org/10.3201/eid1808.111686>
- Manjusha, S., & Sarita, G. B. (2011). Plasmid associated antibiotic resistance in *Vibrios* isolated from coastal waters of Kerala. *International Food Research Journal*, 18(3), 1171-1181. [http://www.ifrj.upm.edu.my/18%20\(03\)%202011/\(45\)IFRJ-2010-143.pdf](http://www.ifrj.upm.edu.my/18%20(03)%202011/(45)IFRJ-2010-143.pdf)
- Matsuda, S., Kodama, T., Okada, N., Okayama, K., Honda, T., & Iida, T. (2010). Association of *Vibrio parahaemolyticus* thermostable direct hemolysin with lipid rafts is essential for cytotoxicity but not hemolytic activity. *Infection and Immunity*, 78(2), 603–610. <https://doi.org/10.1128/IAI.00946-09>
- Mchau, G. J., Makule, E. E., Machunda, R. L., Gong, Y., & Kimanya, M. E. (2019). Harmful algal bloom and associated health risks among users of Lake Victoria freshwater: Ukerewe Island, Tanzania. *Journal of Water and Health*, 17(5), 826–836. <https://doi.org/10.2166/wh.2019.083>
- Mehrabadi, J. F., Morsali, P., Nejad, H. R., & Imani Fooladi, A. A. (2012). Detection of toxigenic *Vibrio cholerae* with new multiplex PCR. *Journal of Infection and Public Health*, 5(3), 263–267. <https://doi.org/10.1016/j.jiph.2012.02.004>
- Meighen, E. A. (1991). Molecular biology of bacterial bioluminescence. *Microbiology and Molecular Biology Review*, 55(1), 123–142. <https://doi.org/10.1128/mr.55.1.123-142.1991>
- Moisander, P. H., McClinton, E., & Paerl, H. W. (2002). Salinity effects on growth, photosynthetic parameters, and nitrogenase activity in estuarine planktonic cyanobacteria. *Microbial Ecology*, 43(4), 432–442. <https://doi.org/10.1007/s00248-001-1044-2>

- Morii, D., Tomono, K., & Imanaka, Y. (2020). Economic impact of antimicrobial-resistant bacteria outbreaks on Japanese hospitals. *American Journal of Infection Control*, 48(10), 1195–1199. <https://doi.org/10.1016/j.ajic.2019.12.006>
- Mrityunjey, A., Kaniz, F., Fahmida, J., Shanzida, J. S., Md. Aftab, U., & Rashed, N. (2013). Prevalence of *Vibrio cholerae* in different food samples in the city of Dhaka, Bangladesh. *International Food Research Journal*. 20(2), 1017-1022. https://www.researchgate.net/publication/230687315_Prevalence_of_Vibrio_cholerae_in_different_food_samples_in_the_city_of_Dhaka_Bangladesh
- Muang Chonburi, Chonburi, Thailand, & Shinn, A. P. (2018). Asian shrimp production and the economic costs of disease. *Asian Fisheries Science*, 31S. <https://doi.org/10.33997/j.afs.2018.31.S1.003>
- Mur, L.R., Skulberg, O.M., et al. (1999). Cyanobacteria in the environment. In: Chorus, I. and Bartram, J., Eds., *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management*, London, E. and FN Spon (on Behalf of WHO), 15-40. Retrieved from <https://www.who.int/publications/m/item/toxic-cyanobacteria-in-water---second-edition>
- Narayanan, S. V., Joseph, T. C., Peeralil, S., Koombankallil, R., Vaiyapuri, M., Mothadaka, M. P., & Lalitha, K. V. (2020). Tropical shrimp aquaculture farms harbour pathogenic *Vibrio parahaemolyticus* with high genetic diversity and carbapenam resistance. *Marine Pollution Bulletin*, 160, 111551. <https://doi.org/10.1016/j.marpolbul.2020.111551>
- Ngo, H., & Fotedar, R. (2010). A review of probiotics in shrimp aquaculture. *Journal of Applied Aquaculture*, 22, 251–266. <https://doi.org/10.1080/10454438.2010.500597>
- Nguyen Thi Truc, L., Nguyen Thanh, T., Tran Thi Hong, T., Pham Van, D., Vo Thi Tuyet, M., Nguyen Trong, N., Phan Cong, M., Cao Ngoc, D., & Truong Quoc, P. (2021). Effects of feed mixed with lactic acid bacteria and carbon, nitrogen, phosphorus supplied to the water on the growth and survival rate of white leg shrimp (*Penaeus vannamei*) infected with acute hepatopancreatic necrosis disease caused by *Vibrio parahaemolyticus*. *Biology*, 10(4), 280. <https://doi.org/10.3390/biology10040280>

- Nguyen, T., Momtaz, S., & Zimmerman, K. (2006). Water pollution concerns in shrimp farming in Vietnam: A case study of Can Gio, Ho Chi Minh City. *The International Journal Environmental, Cultural, Economic & Social Sustainability*, 3, 129–138. <http://dx.doi.org/10.18848/1832-2077/CGP/v03i02/54339>
- Nienaber, M. A., & Steinitz-Kannan, M. (2018). *A guide to cyanobacteria: Identification and impact*. University Press of Kentucky. Retrieved from <https://www.perlego.com/book/874533/a-guide-to-cyanobacteria-identification-and-impact-pdf>
- Noorlis, A., Ghazali, F., & Cheah, Y. K. (2011). Antibiotic resistance and biosafety of *Vibrio cholerae* and *Vibrio parahaemolyticus* from freshwater fish at retail level. *International Food Research Journal*, 18(4), 1523-1530. [http://www.ifrj.upm.edu.my/18%20\(04\)%202011/\(45\)IFRJ-2011-266.pdf](http://www.ifrj.upm.edu.my/18%20(04)%202011/(45)IFRJ-2011-266.pdf)
- Norasma, D. & Saleem, M. (2007). Health management of shrimp farming in Sabah. *Malaysian Fisheries Journal*, 6(2), 81-96. Retrieved from http://fri.dof.gov.my/fri/resources/MFJ/2007Vol2/Vol_6_2_1.pdf
- Nunes, A. J. P., Dalen, L. L., Leonardi, G., & Burri, L. (2022). Developing sustainable, cost-effective and high-performance shrimp feed formulations containing low fish meal levels. *Aquaculture Reports*, 27, 101422. <https://doi.org/10.1016/j.aqrep.2022.101422>
- Nwankwegu, A. S., Li, Y.-P., Huang, Y., Wei, J., Eyram Norgbey, Sarpong, L., Lai, Q., & Wang, K. (2019). Harmful algal blooms under changing climate and constantly increasing anthropogenic actions: the review of management implications. 3 *Biotech*, 9(12). <https://doi.org/10.1007/s13205-019-1976-1>
- O’neill, J. (2016). Tackling drug-resistant infections globally: Final report and recommendations. *Review on Antimicrobial Resistance*. Wellcome Trust and HM Government. Retrieved from https://amrreview.org/sites/default/files/160518_Final%20paper_with%20cover.pdf

- Osickova, A., Balashova, N., Masin, J., Sulc, M., Roderova, J., Wald, T., Brown, A. C., Koufos, E., Chang, E. H., Giannakakis, A., Lally, E. T., & Osicka, R. (2018). Cytotoxic activity of *Kingella kingae* rtxA toxin depends on post-translational acylation of lysine residues and cholesterol binding. *Emerging Microbes & Infections*, 7, 178. <https://doi.org/10.1038/s41426-018-0179-x>
- Osunla, C., & Okoh, A. (2017). *Vibrio* pathogens: A public health concern in rural water resources in sub-Saharan Africa. *International Journal of Environmental Research and Public Health*, 14(10), 1188. <https://doi.org/10.3390/ijerph14101188>
- Ozbay, G., Blank, G., & Thunjai, T. (2014). Impacts of aquaculture on habitats and best management practices (BMPs). In *Sustainable Aquaculture Techniques*. IntechOpen. <https://doi.org/10.5772/57471>
- Pade, N., & Hagemann, M. (2014). Salt acclimation of cyanobacteria and their application in biotechnology. *Life*, 5(1), 25–49. <https://doi.org/10.3390/life5010025>
- Paerl, H. W., Gardner, W. S., Havens, K. E., Joyner, A. R., McCarthy, M. I., Newell, S. E., Qin, B., & Scott, J. F. (2016). Mitigating cyanobacterial harmful algal blooms in aquatic ecosystems impacted by climate change and anthropogenic nutrients. *Harmful Algae*, 54, 213–222. <https://doi.org/10.1016/j.hal.2015.09.009>
- Palit, A., & Nair, G. B. (2014). Bacteria: Other *Vibrios*. Y. Motarjemi (Ed.), *Encyclopedia of Food Safety* (570–573). Retrieved from <https://doi.org/10.1016/B978-0-12-378612-8.00120-7>
- Pandey, N., & Shallu. (2020). Cyanobacteria act as nitrogen-fixing organisms: A review. *Asian Journal of Plant and Soil Sciences*, 5(1), 9–17. Retrieved from <https://ikprress.org/index.php/AJOPSS/article/view/5092>
- Paranjpye, R. N., Nilsson, W. B., Liermann, M., Hilborn, E. D., George, B. J., Li, Q., Bill, B. D., Trainer, V. L., Strom, M. S., & Sandifer, P. A. (2015). Environmental influences on the seasonal distribution of *Vibrio parahaemolyticus* in the Pacific Northwest of the USA. *FEMS Microbiology Ecology*, 91(12), fiv121. <https://doi.org/10.1093/femsec/fiv121>

- Pearson, L. A., Mihali, T. K., Moffitt, M. C., Kellmann, R., & Neilan, B. A. (2010). On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. *Marine Drugs*, 8(5), 1650-1680. <https://doi.org/10.3390/md8051650>
- Peng, X., Yu, K.-Q., Deng, G.-H., Jiang, Y.-X., Wang, Y., Zhang, G.-X., & Zhou, H.-W. (2013). Comparison of direct boiling method with commercial kits for extracting fecal microbiome DNA by Illumina sequencing of 16S rRNA tags. *Journal of Microbiological Methods*, 95(3), 455–462. <https://doi.org/10.1016/j.mimet.2013.07.015>
- Pérez-Reytor, D., Jaña, V., Pavez, L., Navarrete, P., & García, K. (2018). Accessory toxins of *Vibrio* pathogens and their role in epithelial disruption during infection. *Frontiers in Microbiology*, 9, 2248. <https://doi.org/10.3389/fmicb.2018.02248>
- Pérez-Rodríguez, F., & Mercanoglu Taban, B. (2019). A state-of-art review on multi-drug resistant pathogens in foods of animal origin: Risk factors and mitigation strategies. *Frontiers in Microbiology*, 10, 2091. <https://doi.org/10.3389/fmicb.2019.02091>
- Pham, T.-L., & Utsumi, M. (2018). An overview of the accumulation of microcystins in aquatic ecosystems. *Journal of Environmental Management*, 213, 520–529. <https://doi.org/10.1016/j.jenvman.2018.01.077>
- Pizarro-Cerdá, J., & Cossart, P. (2006). Bacterial adhesion and entry into host cells. *Cell*, 124(4), 715–727. <https://doi.org/10.1016/j.cell.2006.02.012>
- Pollitzer, R. (1959). *Cholera*. Monograph Series 43. Geneva, Switzerland, World Health Organization
- Prasanthan, V., Purayath, U., Sarathkumar, & Ouseph, P. P. (2011). Influence of abiotic environmental factors on the abundance and distribution of *Vibrio* species in coastal waters of Kerala, India. *Indian Journal of Marine Sciences*, 40, 587–592. Retrieved from https://www.researchgate.net/publication/286382075_Influence_of_abiotic_environmental_factors_on_the_abundance_and_distribution_of_Vibrio_species_in_coastal_waters_of_Kerala_India

- Prayitno, S. B., & Latchford, J. W. (1995). Experimental infections of crustaceans with luminous bacteria related to photobacterium and *Vibrio*: Effect of salinity and pH on infectiosity. *Aquaculture*, 132(1–2), 105–112. [https://doi.org/10.1016/0044-8486\(94\)00374-W](https://doi.org/10.1016/0044-8486(94)00374-W)
- Prithvisagar, K. S., Krishna Kumar, B., Kodama, T., Rai, P., Iida, T., Karunasagar, I., & Karunasagar, I. (2021). Whole genome analysis unveils genetic diversity and potential virulence determinants in *Vibrio parahaemolyticus* associated with disease outbreak among cultured *Litopenaeus vannamei* (Pacific white shrimp) in India. *Virulence*, 12(1), 1936–1949. <https://doi.org/10.1080/21505594.2021.1947448>
- Pruzzo, C., Gallo, G., & Canesi, L. (2005). Persistence of vibrios in marine bivalves: the role of interactions with haemolymph components. *Environmental Microbiology*, 7(6), 761–772. <https://doi.org/10.1111/j.1462-2920.2005.00792.x>
- Raghunath, P. (2015). Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. *Frontiers in Microbiology*, 5, 805. <https://doi.org/10.3389/fmicb.2014.00805>
- Rahi, Md. L., Azad, K. N., Tabassum, M., Irin, H. H., Hossain, K. S., Aziz, D., Moshtaghi, A., & Hurwood, D. A. (2021). Effects of salinity on physiological, biochemical and gene expression parameters of black tiger shrimp (*Penaeus monodon*): Potential for farming in low-salinity environments. *Biology*, 10(12), 1220. <https://doi.org/10.3390/biology10121220>
- Raja, K. (2015). Loose shell syndrome (LSS) in *Litopenaeus vannamei* grow-out ponds and its effect on growth and production. *Fisheries and Aquaculture Journal*, 4(4). <https://doi.org/10.4172/2150-3508.1000151>
- Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya, S. K., Nair, G. B., Shimada, T., Takeda, T., Karasawa, T., Kurazano, H., & Pal, A. (1993). Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. *Lancet (London, England)*, 341(8846), 703–704. [https://doi.org/10.1016/0140-6736\(93\)90480-5](https://doi.org/10.1016/0140-6736(93)90480-5)

- Reda, A., Sah, R., Abdelaal, A., Shrestha, S., & Rodriguez-Morales, A. J. (2022). The emergence of cholera in multiple countries amidst current COVID-19 pandemic: Situation and implications for public health and travel medicine. *Travel Medicine and Infectious Disease*, 49, 102423. <https://doi.org/10.1016/j.tmaid.2022.102423>
- Reverter, M., Sarter, S., Caruso, D., Jean-Christophe Avarre, Combe, M., Elodie Pepey, Laurent Pouyaud, Sarahi Vega-Heredía, Hugues de Verdal, & Gozlan, R. E. (2020). Aquaculture at the crossroads of global warming and antimicrobial resistance. *Nature Communications*, 11(1). <https://doi.org/10.1038/s41467-020-15735-6>
- Ritchie, J. M., Rui, H., Zhou, X., Iida, T., Toshio Kodoma, Ito, S., Davis, B. M., Bronson, R. T., & Waldor, M. K. (2012). *Inflammation and Disintegration of Intestinal Villi in an Experimental Model for Vibrio parahaemolyticus-Induced Diarrhea*. PLoS Pathogens 8(3), e1002593–e1002593. <https://doi.org/10.1371/journal.ppat.1002593>
- Robert-Pillot, A., Guénolé, A., Lesne, J., Delesmont, R., Fournier, J.-M., & Quilici, M.-L. (2004). Occurrence of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two France coastal areas and from seafood imported into France. *International Journal of Food Microbiology*, 91, 319–325. <https://doi.org/10.1016/j.ijfoodmicro.2003.07.006>
- Rojas, V., Rivas, L., Cárdenas, C., & Guzmán, F. (2020). Cyanobacteria and eukaryotic microalgae as emerging sources of antibacterial peptides. *Molecules*, 25, 24. <https://doi.org/10.3390/molecules25245804>
- Said, B., Smith, H. R., Scotland, S. M., & Rowe, B. (1995). Detection and differentiation of the gene for toxin co-regulated pili (*tcpA*) in *Vibrio cholerae* non-01 using the polymerase chain reaction. *FEMS Microbiology Letters*, 125, 5. [https://doi.org/10.1016/0378-1097\(94\)00499-H](https://doi.org/10.1016/0378-1097(94)00499-H)
- Saini, D. K., Pabbi, S., & Shukla, P. (2018). Cyanobacterial pigments: Perspectives and biotechnological approaches. *Food and Chemical Toxicology*, 120, 616–624. <https://doi.org/10.1016/j.fct.2018.08.002>

- Sarjito, S., & Sabdono, A. (2021). Associated *Vibrio* species in shrimp vibriosis from traditional brackish water pond in the North Coastal of Central Java, Indonesia. *Genetics of Aquatic Organisms*, 5(2), 45–54. https://doi.org/10.4194/2459-1831-v5_2_01
- Satitsri, S., Pongkorpsakol, P., Srimanote, P., Chatsudthipong, V., & Muanprasat, C. (2016). Pathophysiological mechanisms of diarrhea caused by the *Vibrio cholerae* O1 El Tor variant: An in vivo study in mice. *Virulence*, 7(7), 789–805. <https://doi.org/10.1080/21505594.2016.1192743>
- Schar, D., Zhao, C., Wang, Y., Larsson, D. G. J., Gilbert, M., & Van Boeckel, T. P. (2021). Twenty-year trends in antimicrobial resistance from aquaculture and fisheries in Asia. *Nature communications*, 12(1), 5384. <https://doi.org/10.1038/s41467-021-25655-8>
- Schmidt, J., Wilhelm, S. W., & Boyer, G. L. (2014). The fate of microcystins in the environment and challenges for monitoring. *Toxins*, 6(12), 3354–3387. <https://doi.org/10.3390/toxins6123354>
- Sellner, K. G., Doucette, G. J., & Kirkpatrick, G. J. (2003). Harmful algal blooms: Causes, impacts and detection. *Journal of Industrial Microbiology and Biotechnology*, 30(7), 383–406. <https://doi.org/10.1007/s10295-003-0074-9>
- Sharma, L., Nagpal, R., Jackson, C., Patel, D., & Singh, P. (2021). Antibiotic-resistant bacteria and gut microbiome communities associated with wild-caught shrimp from the United States versus imported farm-raised retail shrimp. *Scientific Reports* 11, 3356(2021). <https://doi.org/10.1038/s41598-021-82823-y>
- Sheikh, H. I., Najiah, M., Fadhline, A., Laith, A. A, Nor, M. M., Jalal, K. C. A., & Kasan, N. A. (2022). Temperature upshift mostly but not always enhances the growth of *Vibrio* species: A systematic review. *Frontiers in Marine Science*, 9(2022). <https://doi.org/10.3389/fmars.2022.959830>
- Shishehchian, F. (2020). *Prevention and Solution for White Feces Disease (WFD) in Litopenaeus vannamei Culture*. Retrieved from <https://medium.com/@nathalie>

_74709/prevention-and-solution-for-white-feces-disease-wfd-in-litopenaeus-vannamei-culture-31e4dc393410

- Silveira, S. B., & Odebrecht, C. (2019). Effects of salinity and temperature on the growth, toxin production, and akinete germination of the cyanobacterium *Nodularia spumigena*. *Frontiers in Marine Science*, 6. <https://www.frontiersin.org/articles/10.3389/fmars.2019.00339>
- Singh, J., Kumar, A., Rai, A. N., & Singh, D. (2016). Cyanobacteria: A precious bio-resource in agriculture, ecosystem, and environmental sustainability. *Frontiers in Microbiology*, 7. <https://doi.org/10.3389/fmicb.2016.00529>
- Somboon, M., Purivirojkul, W., Limsuwan, C., & Chuchird, N. (2012). Effect of *Vibrio* spp. in white feces infected shrimp in Chanthaburi, Thailand. *Journal of Fisheries and Environment*, 36(1), 7–15. Retrieved from <https://li01.tci-thaijo.org/index.php/JFE/article/view/80769>
- Somboonwiwat, K., Chaikerasak, V., Wang, H., Lo, C. F., & Tassanakajon, A. (2010). Proteomic analysis of differentially expressed proteins in *Penaeus monodon* hemocytes after *Vibrio harveyi* infection. *Proteome Science*, 8, 39. <https://doi.org/10.1186/1477-5956-8-39>
- Soto-Rodriguez, S. A., Gomez-Gil, B., Lozano-Olvera, R., Betancourt-Lozano, M., & Morales-Covarrubias, M. S. (2015). Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in north-western Mexico. *Applied and Environmental Microbiology*, 81(5), 1689–1699. <https://doi.org/10.1128/AEM.03610-14>
- Sulok Tawie. (2022). Sarawak aims to open up 7,000 hectares for shrimp farming, says state minister. *Malay Mail*. Retrieved from <https://www.malaymail.com/news/malaysia/2022/09/07/sarawak-aims-to-open-up-7000-hectares-for-shrimp-farming-says-state-minister/26969>

- Sun, J., Li, X., Hu, Z., Xue, X., Zhang, M., Wu, Q., Zhang, W., Zhang, Y., & Lu, R. (2022). Characterization of *Vibrio parahaemolyticus* isolated from stool specimens of diarrhea patients in Nantong, Jiangsu, China during 2018–2020. *PLOS ONE*, 17(8), e0273700. <https://doi.org/10.1371/journal.pone.0273700>
- Sun, R., Sun, P., Zhang, J., Esquivel-Elizondo, S., & Wu, Y. (2018). Microorganisms-based methods for harmful algal blooms control: A review. *Bioresource Technology*, 248, 12–20. <https://doi.org/10.1016/j.biortech.2017.07.175>
- Sutton, S. (2010). The most probable number method and its uses in enumeration, qualification, and validation. *Journal of Validation Technology*, 4. Retrieved from <https://www.semanticscholar.org/paper/The-Most-Probable-Number-Method-and-Its-Uses-in-and-Sutton/2185acb4e79908a9e68e4b80f5b484398042dfff>
- Takahashi, A., Kenjyo, N., Imura, K., Myonsun, Y., & Honda, T. (2000). Cl[−] secretion in colonic epithelial cells induced by the *Vibrio parahaemolyticus* hemolytic toxin related to thermostable direct hemolysin. *Infection and Immunity*, 68(9), 5435–5438. <https://doi.org/10.1128/iai.68.9.5435-5438.2000>
- ter Braak, C. J. F. (1986). Canonical correspondence analysis: A new eigenvector technique for multivariate direct gradient analysis. *Ecology*, 67(5), 1167–1179. <https://doi.org/10.2307/1938672>
- ter Braak, C. J. F., & Verdonschot, P. F. M. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. *Aquatic Sciences*, 57(3), 255–289. <https://doi.org/10.1007/BF00877430>
- Thompson, A.W., Foster, R.A., Krupke, A., Carter, B.J., Musat, N., Vaultot, D., Kuypers, M.M., & Zehr, J.P. (2012). Unicellular Cyanobacterium Symbiotic with a Single-Celled Eukaryotic Alga. *Science*, 337, 1546 - 1550. <https://doi.org/10.1126/science.1222700>
- Thornber, K., Verner-Jeffreys, D. W., Hinchliffe, S., Muhammad Sabbir Rahman, Bass, D., & Tyler, C. R. (2020). Evaluating antimicrobial resistance in the global shrimp

- industry. *Reviews in Aquaculture*, 12(2), 966–986. <https://doi.org/10.1111/raq.12367>
- Tran, P. H., Kumar, V., Tran, P. T. N., & Bossier, P. (2020). Do acute hepatopancreatic necrosis disease-causing *PirAB* VP toxins aggravate vibriosis? *Emerging Microbes & Infections*, 9(1), 1919–1932. <https://doi.org/10.1080/22221751.2020.1811778>
- Turner, J. W., Good, B., Cole, D., & Lipp, E. K. (2009). Plankton composition and environmental factors contribute to *Vibrio* seasonality. *The ISME Journal*, 3(9), Article 9. <https://doi.org/10.1038/ismej.2009.50>
- Uddin, M. E., Akter, T., Sultana, P., Sultana, P., Hasan, M. I., Lubna, M. A., Monem, H. A., Parvez, M. A. K., Nahar, S., & Khan, M. S. (2018). Isolation, identification and antimicrobial susceptibility profile analysis of *Vibrio cholerae* O1 from stool samples of Bangladesh. *Advances in Microbiology*, 8(3), Article 3. <https://doi.org/10.4236/aim.2018.83013>
- Van der Merwe, D. (2015). Cyanobacterial (blue-green algae) toxins. *Handbook of Toxicology of Chemical Warfare Agents*, 421–429. <https://doi.org/10.1016/b978-0-12-800159-2.00031-2>
- Venkateswaran, K. (1999). *VIBRIO* | Standard cultural methods and molecular detection techniques in foods. *Encyclopedia of Food Microbiology*, 2248–2258. Retrieved from <https://www.sciencedirect.com/science/article/pii/B0122270703016652>
- Ventola, C. L. (2015). The antibiotic resistance crisis. *Pharmacy and Therapeutics*, 40(4), 277–283. PMID: 25859123; PMCID: PMC4378521. Retrieved from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378521/>
- Verma, J., Bag, S., Saha, B., Kumar, P., Ghosh, T. S., Dayal, M., Senapati, T., Mehra, S., Dey, P., Desigamani, A., Kumar, D., Rana, P., Kumar, B., Maiti, T. K., Sharma, N. C., Bhadra, R. K., Mutreja, A., Nair, G. B., Ramamurthy, T., & Das, B. (2019). Genomic plasticity associated with antimicrobial resistance in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America*, 116(13), 6226–6231. <https://doi.org/10.1073/pnas.1900141116>

- Verma, P., & Chattopadhyay, K. (2021). Current perspective on the membrane-damaging action of thermostable direct hemolysin, an atypical bacterial pore-forming toxin. *Frontiers in Molecular Biosciences*, 8. <https://www.frontiersin.org/articles/10.3389/fmolb.2021.717147>
- Vieira, R. H. S. F., Costa, R. A., Menezes, F. G. R., Silva, G. C., Theophilo, G. N. D., Rodrigues, D. P., & Maggioni, R. (2011). Kanagawa-negative, *tdh*- and *trh*-positive *Vibrio parahaemolyticus* isolated from fresh oysters marketed in Fortaleza, Brazil. *Current Microbiology*, 63(2), 126–130. <https://doi.org/10.1007/s00284-011-9945-x>
- Wan Nurhafizah, W. I., Lee, L., Laith, A. R., Nadirah, M., Danish-Daniel, Muhd., & Zainathan, S., & Najiah, M. (2021). Virulence properties and pathogenicity of multidrug-resistant *Vibrio harveyi* associated with luminescent vibriosis in Pacific white shrimp, *Penaeus vannamei*. *Journal of Invertebrate Pathology*, 186(14), 107594. <https://doi.org/10.1016/j.jip.2021.107594>
- Wang, Y., Barton, M., Elliott, L., Li, X., Abraham, S., O'Dea, M., & Munro, J. (2017). Bacteriophage therapy for the control of *Vibrio harveyi* in greenlip abalone (*Haliotis laevis*). *Aquaculture*, 473, 251–258. <https://doi.org/10.1016/j.aquaculture.2017.01.003>
- Ward, C. E. (2014). Investigating the interactions between cyanobacteria and *Vibrio parahaemolyticus*. *Honors Theses and Capstones*, 27. Retrieved from <https://scholars.unh.edu/cgi/viewcontent.cgi?article=1169&context=honors>
- Watzin, M. C., Miller, E. B., Shambaugh, A. D., & Kreider, M. A. (2006). Application of the WHO alert level framework to cyanobacterial monitoring of Lake Champlain, Vermont. *Environmental Toxicology*, 21(3), 278–288. <https://doi.org/10.1002/tox.20181>
- West, P. A., & Lee. (1982). Ecology of *Vibrio* species, including *Vibrio cholerae*, in natural waters of Kent, England. *Journal of Applied Bacteriology*, 52(3), 435–448. <https://doi.org/10.1111/j.1365-2672.1982.tb05074.x>

- Whitton, B. A., & Potts, M. (2012). Introduction to the cyanobacteria. *Ecology of Cyanobacteria II*, 1–13. https://doi.org/10.1007/978-94-007-3855-3_1
- Wiles, T. J., & Mulvey, M. A. (2013). The RTX pore-forming toxin α -hemolysin of uropathogenic *Escherichia coli*: Progress and perspectives. *Future Microbiology*, 8, 73–84. <https://doi.org/10.2217/fmb.12.131>
- World Wildlife Fund. (2022). Farmed shrimp overview. *World Wildlife Fund*. Retrieved from <https://www.worldwildlife.org/industries/farmed-shrimp>
- Wu, Q., Vaziri, A. Z., Omid, N., Hassan Kaviar, V., Maleki, A., Khadivar, P., & Kouhsari, E. (2022). Antimicrobial resistance among clinical *Vibrio cholerae* non-O1/non-O139 isolates: Systematic review and meta-analysis. *Pathogens and Global Health*, 0(0), 1–10. <https://doi.org/10.1080/20477724.2022.2114620>
- Wyban, J., Walsh, W., & Godin, D. (1995). Temperature effects on growth, feeding rate and feed conversion of the Pacific white shrimp (*Penaeus vannamei*). *Aquaculture*, 138, 267–279. [https://doi.org/10.1016/0044-8486\(95\)00032-1](https://doi.org/10.1016/0044-8486(95)00032-1)
- Xi, C., Zhang, Y., Marrs, C. F., Ye, W., Simon, C. G., Foxman, B., & Nriagu, J. O. (2009). Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Applied and Environmental Microbiology*, 75(17), 5714–5718. <https://doi.org/10.1128/aem.00382-09>
- Xu, M., Wu, J., & Chen, L. (2019). Virulence, antimicrobial and heavy metal tolerance, and genetic diversity of *Vibrio cholerae* recovered from commonly consumed freshwater fish. *Environmental Science and Pollution Research International*, 26(26), 27338–27352. <https://doi.org/10.1007/s11356-019-05287-8>
- Yamazaki, K., Takashige Kashimoto, Kado, T., Yoshioka, K., & Ueno, S. (2022). Increased vascular permeability due to spread and invasion of *Vibrio vulnificus* in the wound infection exacerbates potentially fatal necrotizing disease. *Frontiers in Microbiology*, 13. <https://doi.org/10.3389/fmicb.2022.849600>
- Yano, Y., Hamano, K., Satomi, M., Tsutsui, I., Ban, M., & Aue-umneoy, D. (2014). Prevalence and antimicrobial susceptibility of *Vibrio* species related to food safety

- isolated from shrimp cultured at inland ponds in Thailand. *Food Control*, 38, 30–36. <https://doi.org/10.1016/j.foodcont.2013.09.019>
- Youngren-Grimes, B. L., Grimes, D. J., & Colwell, R. R. (1988). Growth of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* under strict anaerobic conditions. *Systematic and Applied Microbiology*, 11(1), 13–15. [https://doi.org/10.1016/s0723-2020\(88\)80042-0](https://doi.org/10.1016/s0723-2020(88)80042-0)
- Yu, G., Zhu, M., Chen Youxin, & Li, R. (2015). *Polyphasic characterization of four species of Pseudanabaena (Oscillatoriales, Cyanobacteria) from China*. ResearchGate; Magnolia Press. https://www.researchgate.net/publication/282279865_Polyphasic_characterization_of_four_species_of_Pseudanabaena_Oscillatoriales_Cyanobacteria_from_China_and_insights_into_polyphyletic_divergence_within_the_Pseudanabaena_genus
- Yu, Y.-B., Jae Sue Choi, Kang, J.-C., Kim, H.-J., & Kim, J. (2022). Shrimp bacterial and parasitic disease listed in the OIE: A review. *Microbial Pathogenesis*, 166, 105545–105545. <https://doi.org/10.1016/j.micpath.2022.105545>
- Zanichelli, V., Monnier, A. A., Gyssens, I. C., Adriaenssens, N., Versporten, A., Pulcini, C., Le Maréchal, M., Tebano, G., Vlahović-Palčevski, V., Benic, M. S., Milanic, R., Harbarth, S., Hulscher, M. E., & Huttner, B. (2018). Variation in antibiotic use among and within different settings: A systematic review. *Journal of Antimicrobial Therapy*, 73(6), vi17–vi29. <https://doi.org/10.1093/jac/dky115>
- Zhang, H. (2017). *Cause and Effects of Soil Acidity*. 2. Retrieved from <https://extension.okstate.edu/fact-sheets/cause-and-effects-of-soil-acidity.html>
- Zhang, X., Sun, J., Chen, F., Qi, H., Chen, L., Sung, Y. Y., Huang, Y., Lv, A., & Hu, X. (2021). Phenotypic and genomic characterization of a *Vibrio parahaemolyticus* strain causing disease in *Penaeus vannamei* provides insights into its niche adaptation and pathogenic mechanism. *Microbial genomics*, 7(5), 000549. <https://doi.org/10.1099/mgen.0.000549>

- Zhang, X.-H., Lin, H., Wang, W., & Austin, B. (2018). Significance of *Vibrio* species in the marine organic carbon cycle—A review. *Science China Earth Sciences*, 61(10), 1357–1368. <https://doi.org/10.1007/s11430-017-9229-x>
- Zhang, Y. B., Li, Y., & Sun, X. L. (2011). Antibiotic resistance of bacteria isolated from shrimp hatcheries and cultural ponds on Donghai Island, China. *Marine Pollution Bulletin*, 62(11), 2299–2307. <https://doi.org/10.1016/j.marpolbul.2011.08.048>
- Zhang, Y. B., Li, Y., & Sun, X. L. (2011). Antibiotic resistance of bacteria isolated from shrimp hatcheries and cultural ponds on Donghai Island, China. *Marine Pollution Bulletin*, 62(11), 2299–2307. <https://doi.org/10.1016/j.marpolbul.2011.08.048>
- Zheng, Q., Hu, Y., Zhang, S., Noll, L., Böckle, T., Dietrich, M., Herbold, C. W., Eichorst, S. A., Woebken, D., Richter, A., & Wanek, W. (2019). Soil multifunctionality is affected by the soil environment and by microbial community composition and diversity. *Soil Biology and Biochemistry*, 136, 107521. <https://doi.org/10.1016/j.soilbio.2019.107521>
- Zhu, S., Kojima, S., & Homma, M. (2013). Structure, gene regulation and environmental response of flagella in *Vibrio*. *Frontiers in Microbiology*, 4. <https://doi.org/10.3389/fmicb.2013.00410>
- Zoqratt, M. Z. H. M., Eng, W. W. H., Thai, B. T., Austin, C. M., & Gan, H. M. (2018). Microbiome analysis of Pacific white shrimp gut and rearing water from Malaysia and Vietnam: Implications for aquaculture research and management. *PeerJ*, 6, e5826. <https://doi.org/10.7717/peerj.5826>
- Zorriehzahra, M. J., & Banaederakhshan, R. (2015). Early mortality syndrome (EMS) as new emerging threat in shrimp industry. *Advances in Animal and Veterinary Science*, 3(2s), 64-72. <http://dx.doi.org/10.14737/journal.aavs/2015/3.2s.64.72>

APPENDICES

Journal Publications

1. **Dayang Najwa, A. B.**, Elekson, N., & Dalene, L. (2023). The prevalence of *Vibrio cholerae* and *Vibrio parahaemolyticus* virulence genes and multiple antibiotics resistant (MAR) assessment from local shrimp farm in Sarawak. *Borneo Journal of Resource Science and Technology*, 13(1), 93-107.
<https://doi.org/10.33736/bjrst.5375.2023>
2. **Dayang Najwa, A. B.**, Elekson, N., Dalene, L., & Sing Tung, T. (2023). *Vibrio species and cyanobacteria: Understanding their association in local shrimp farm using canonical correspondence analysis (CCA)*. Manuscript Submitted for Publication
3. Dalene, L., Elekson, N., Dalene, L., & **Dayang Najwa, A. B.** (2023). *Prevalence of Vibrio parahaemolyticus, Vibrio cholera, and Vibrio alginolyticus in White-leg shrimp (Litopenaeus vannamei) farm in Sarawak in one production cycle*. Under review
4. Elekson, N., Sabrina, H., Dalene, L., Eddy, B., Nurul, F. R., Nasra, P., Grace, B., Amirah, Z. J., Nur, D. Z., **Dayang Najwa, A. B.**, Manju, S., & Tunung, R. (2022). *Assessment of Pseudomonas aeruginosa biofilm-forming capacities from drinking water in water vending machine*. *Food Research*, 6(3), 76-83.
[http://dx.doi.org/10.26656/fr.2017.6\(3\).324](http://dx.doi.org/10.26656/fr.2017.6(3).324)



Shrimp post-larvae.



Acclimation process before post-larvae stocking.



Aeration process by using machine to promote continuous flow of oxygen.



Automatic feeder attached to the end of the bridge to equally dispense shrimp feed.

Pond A: MPN Raw Data

Number of samplings	Sampling site triplicates	Number of positive tubes			MPN/10 mL	95% Confidence Range
		0.01	0.00 1	0.00 01		
Sampling 1	A	3	3	3	>11000	4200-40000
	B	0	0	0	<30	0-95
	C	0	0	0	<30	0-95
Sampling 2	A	0	0	0	<30	0-95
	B	3	3	3	>11000	4200-40000
	C	0	0	0	<30	0-95
Sampling 3	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000
Sampling 4	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000
Sampling 5	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000
Sampling 6	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000

Pond B: MPN Raw Data

Number of samplings	Sampling site triplicates	Number of positive tubes			MPN/10 mL	95% Confidence Range
		0.01	0.001	0.0001		
Sampling 1	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000
Sampling 2	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000
Sampling 3	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000
Sampling 4	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000
Sampling 5	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000
Sampling 6	A	3	3	3	>11000	4200-40000
	B	3	3	3	>11000	4200-40000
	C	3	3	3	>11000	4200-40000

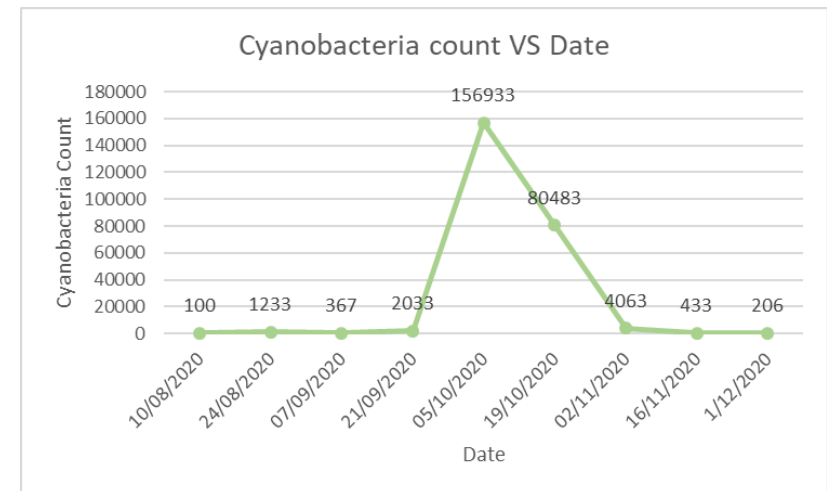
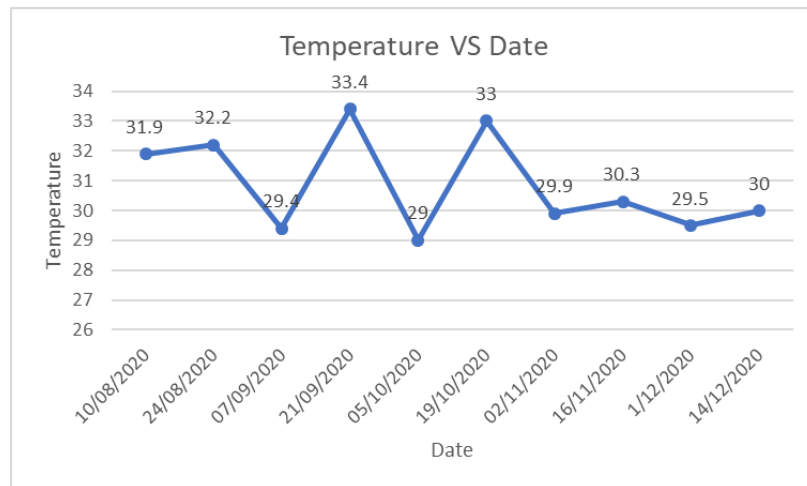
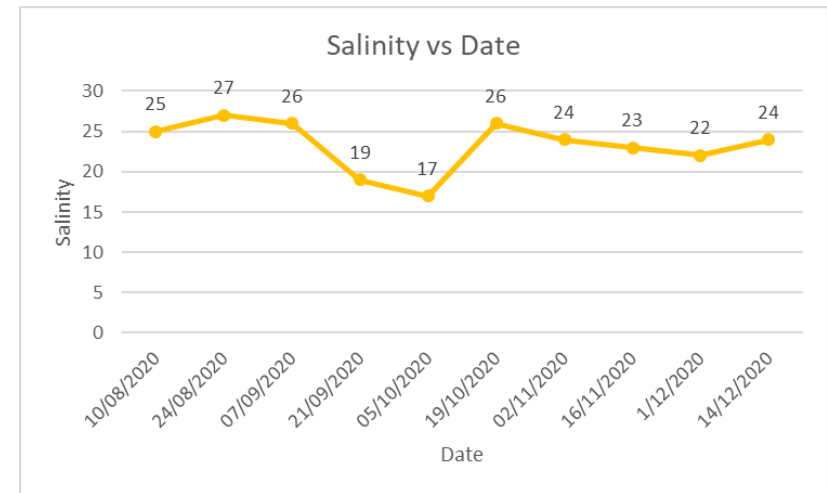
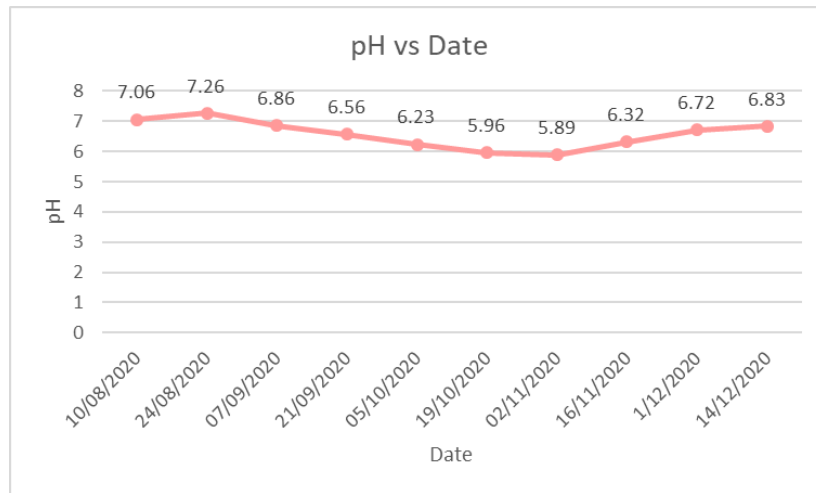
Effluent: MPN Raw Data

Number of samplings	Number of positive tubes			MPN/10 mL	95% Confident Range
	0.0 1	0.001	0.000 1		
Sampling 1	0	0	0	<30	0-95
Sampling 2	3	3	3	>11000	4200-40000
Sampling 3	3	3	3	>11000	4200-40000
Sampling 4	3	3	3	>11000	4200-40000
Sampling 5	3	3	3	>11000	4200-40000
Sampling 6	3	3	3	>11000	4200-40000
Sampling 7	3	3	3	>11000	4200-40000
Sampling 8	3	3	3	>11000	4200-40000
Sampling 9	3	3	3	>11000	4200-40000
Sampling 10	3	3	3	>11000	4200-40000

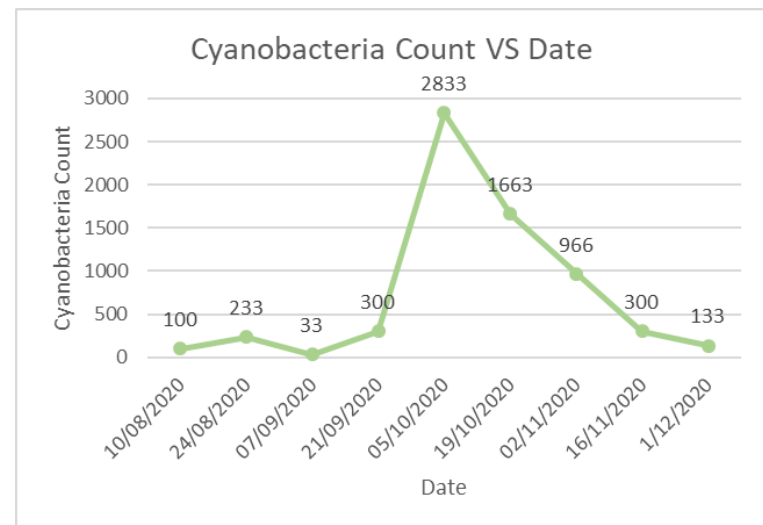
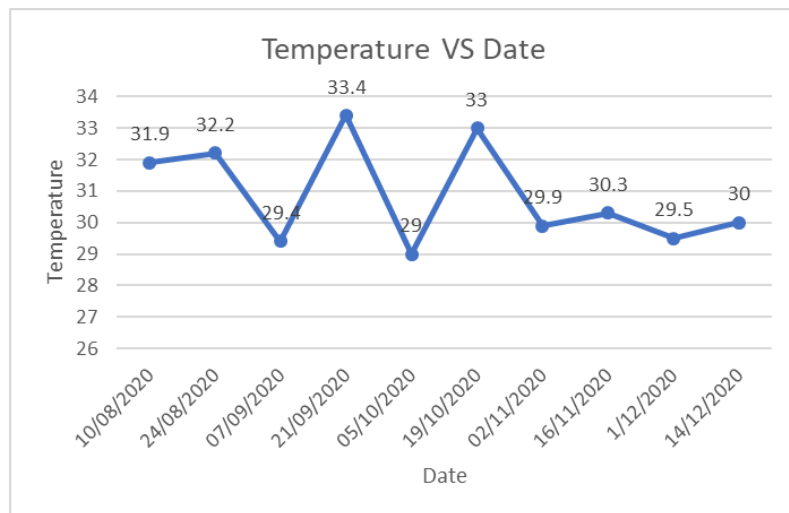
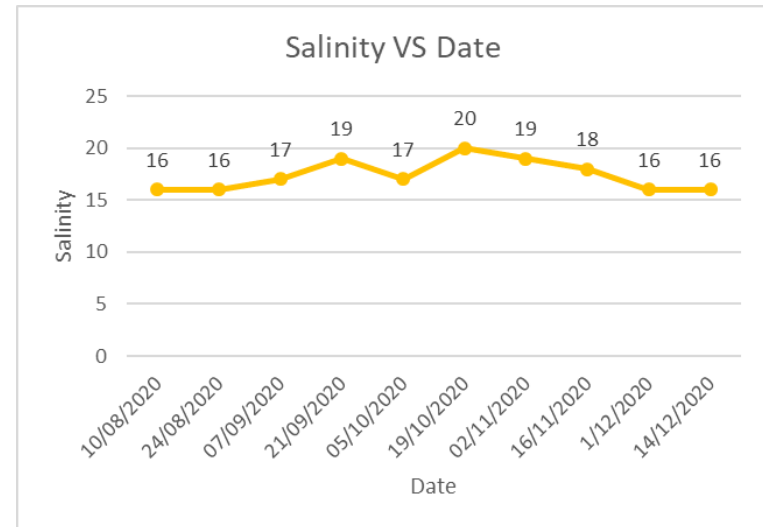
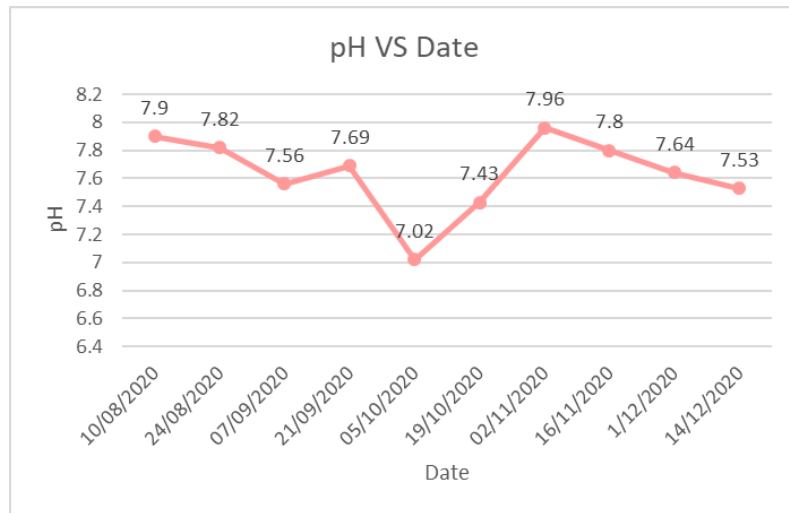
Influent: MPN Raw Data

Number of samplings	Number of positive tubes			MPN/10 mL	95% Confident Range
	0.01	0.001	0.000 1		
Sampling 1	2	1	0	150	37-420
Sampling 2	2	2	2	350	87-940
Sampling 3	3	3	3	>11000	4200-40000
Sampling 4	3	3	3	>11000	4200-40000
Sampling 5	3	3	3	>11000	4200-40000
Sampling 6	3	3	3	>11000	4200-40000
Sampling 7	3	3	3	>11000	4200-40000
Sampling 8	3	3	3	>11000	4200-40000
Sampling 9	3	3	3	>11000	4200-40000
Sampling 10	3	3	3	>11000	4200-40000

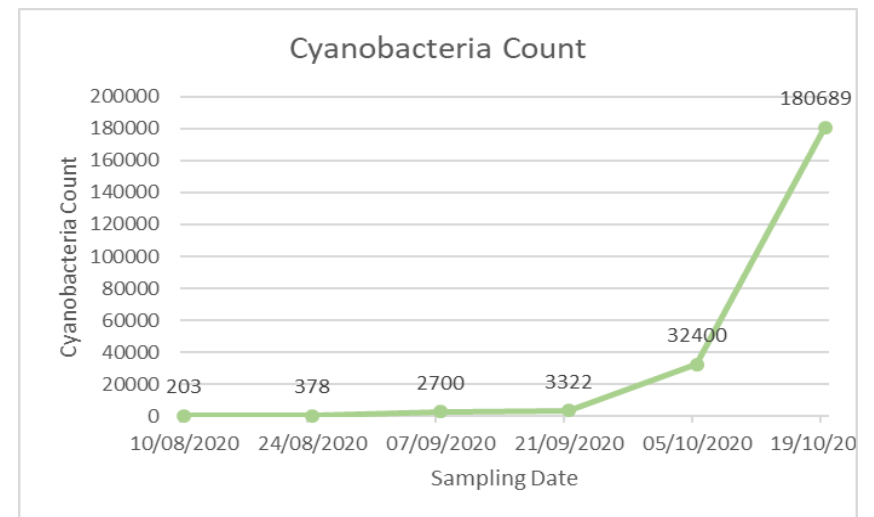
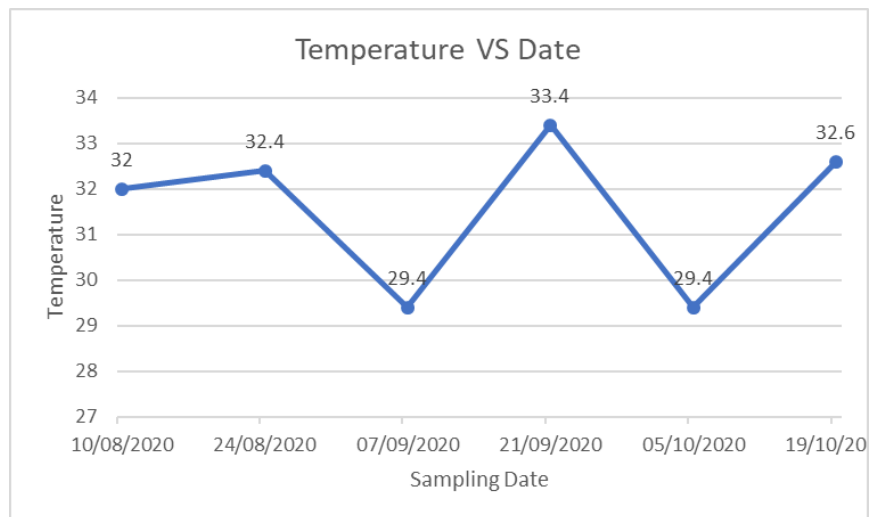
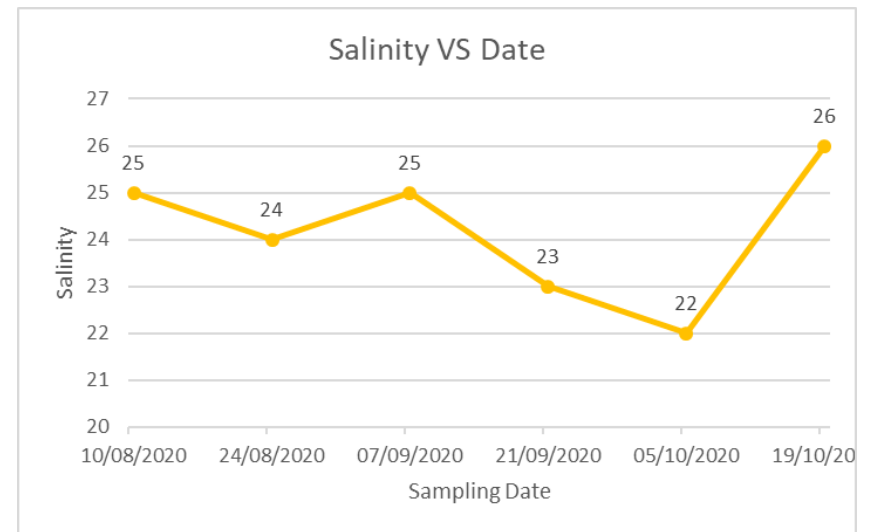
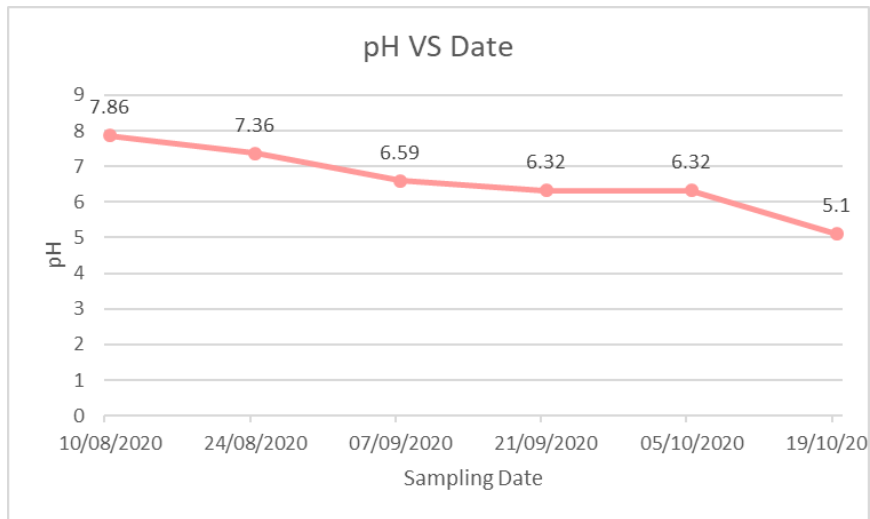
Effluent: Environmental Parameters and Cyanobacteria Cell Counting (Pre-log)



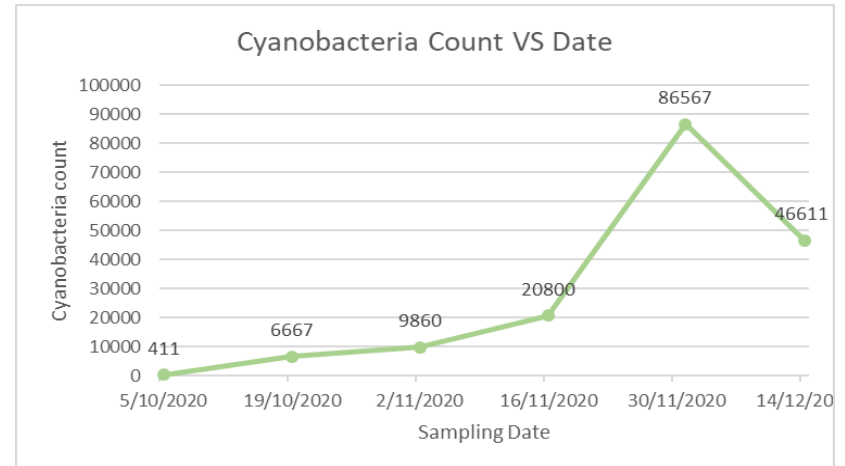
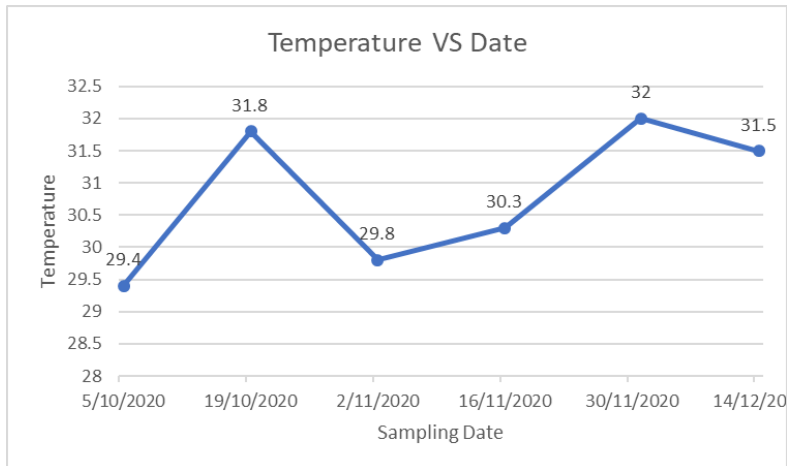
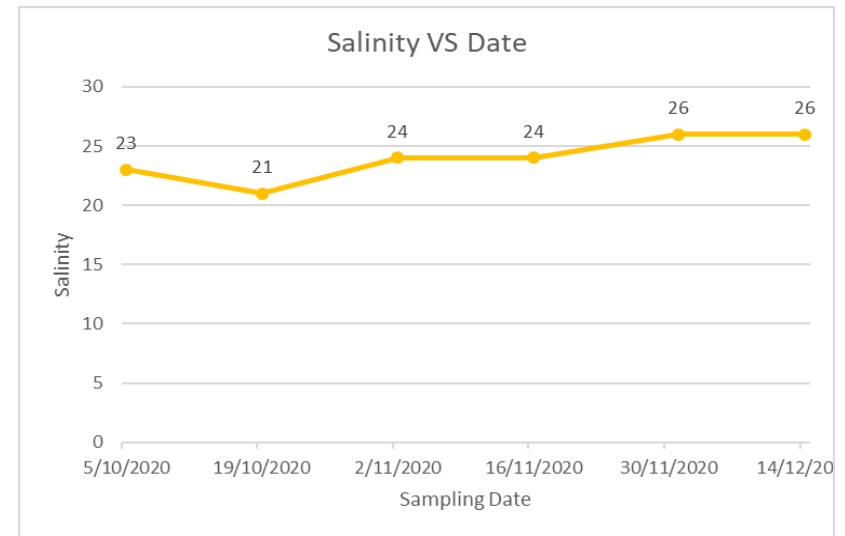
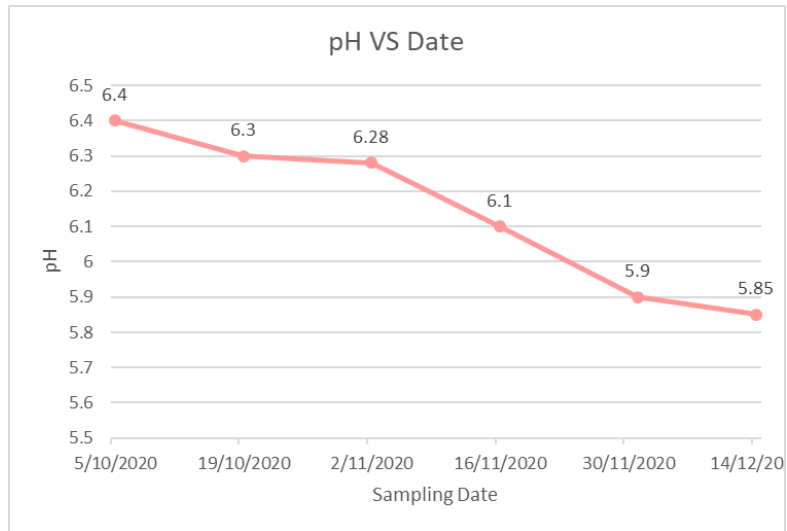
Influent: Environmental Parameters and Cyanobacteria Cell Counting (Pre-log)



Pond B: Environmental Parameters and Cyanobacteria Cell Counting (Pre-log)



Pond A: Environmental Parameters and Cyanobacteria Cell Counting (Pre-log)



V. cholerae Antibiotic Susceptible Isolates (Henceforth, 1 = Present)

Antibiotic	Code	Conc (µg)	VC0 1	VC0 2	VC0 3	VC0 4	VC0 5	VC0 6	VC0 7	VC0 8	VC0 9	VC1 0	Total	Percentage
Amikacin	AK	30	1	1	1						1		4	40%
Amoxycillin/clavulanic acid	AMC	30			1	1	1	1	1	1	1	1	8	80%
Ampicillin	AMP	10					1		1		1	1	4	40%
Chloramphenicol	C	30			1	1	1	1	1	1	1	1	8	80%
Ceftazidime	CAZ	30	1	1	1				1	1	1	1	7	70%
Ciprofloxacin	CIP	5	1	1	1	1	1			1	1		7	70%
Ceftriaxone	CRO	30							1	1	1	1	4	40%
Imipenem	IPM	10	1	1	1	1	1	1	1	1	1	1	10	100%
Kanamycin	K	30	1	1	1								3	30%
Cephalothin	KF	30					1						1	10%
Meropenem	MEM	10		1	1			1	1	1	1	1	7	70%
Neomycin	N	30	1	1	1								3	30%
Nalidixic Acid	NA	30	1						1	1	1	1	5	50%
Norfloxacin	NOR	10	1	1	1	1	1	1	1	1	1	1	10	100%
Rifampicin	RD	5	1	1	1	1	1					1	6	60%
Sulphamethoxazole	RL	25											0	0%
Streptomycin	S	10	1										1	10%
Compound Sulphonamide	S3	300	1	1	1					1			4	40%
Sulphamethoxazole – Trimetoprim	SXT	25		1	1	1	1	1	1	1		1	8	80%
Tetracycline	TE	30	1	1	1	1	1	1	1	1	1	1	10	100%

V. cholerae Antibiotic Intermediate Isolates

Antibiotic	Code	Conc (µg)	VC0 1	VC0 2	VC0 3	VC0 4	VC0 5	VC0 6	VC0 7	VC0 8	VC0 9	VC1 0	Total	Percentage
Amikacin	AK	30							1	1		1	3	30%
Amoxycillin/clavulanic acid	AMC	30											0	0%
Ampicillin	AMP	10								1			1	10%
Chloramphenicol	C	30	1	1									2	20%
Ceftazidime	CAZ	30											0	0%
Ciprofloxacin	CIP	5						1	1			1	3	30%
Ceftriaxone	CRO	30											0	0%
Imipenem	IPM	10											0	0%
Kanamycin	K	30						1	1	1	1	1	5	50%
Cephalothin	KF	30										1	1	10%
Meropenem	MEM	10				1	1						2	20%
Neomycin	N	30											0	0%
Nalidixic Acid	NA	30											0	0%
Norfloxacin	NOR	10											0	0%
Rifampicin	RD	5											0	0%
Sulphamethoxazole	RL	25											0	0%
Streptomycin	S	10		1	1								2	20%
Compound Sulphonamide	S3	300						1	1		1	1	4	40%
Sulphamethoxazole – Trimetoprim	SXT	25											0	0%
Tetracycline	TE	30											0	0%

V. cholerae Antibiotic Resistant Isolates

Antibiotic	Code	Conc (µg)	VC0 1	VC0 2	VC0 3	VC0 4	VC0 5	VC0 6	VC0 7	VC0 8	VC0 9	VC1 0	Total	Percent age
Amikacin	AK	30				1	1	1					3	30%
Amoxycillin/clavulanic acid	AMC	30	1	1									2	20%
Ampicillin	AMP	10	1	1	1	1		1					5	50%
Chloramphenicol	C	30											0	0%
Ceftazidime	CAZ	30				1	1	1					3	30%
Ciprofloxacin	CIP	5											0	0%
Ceftriaxone	CRO	30	1	1	1	1	1	1					6	60%
Imipenem	IPM	10											0	0%
Kanamycin	K	30				1	1						2	20%
Cephalothin	KF	30	1	1	1	1		1	1	1	1		8	80%
Meropenem	MEM	10	1										1	10%
Neomycin	N	30				1	1	1	1	1	1	1	7	70%
Nalidixic Acid	NA	30		1	1	1	1	1					5	50%
Norfloxacin	NOR	10											0	0%
Rifampicin	RD	5						1	1	1	1		4	40%
Sulphamethoxazole	RL	25	1	1	1	1	1	1	1	1	1	1	10	100%
Streptomycin	S	10				1	1	1	1	1	1	1	7	70%
Compound Sulphonamide	S3	300				1	1						2	20%
Sulphame thoxazole – Trimetoprim	SXT	25	1								1		2	20%
Tetracycline	TE	30											0	0%

V. parahaemolyticus Antibiotic Susceptible Isolates

Antibiotic	Code	Conc (µg)	VP0 1	VP0 2	VP0 3	VP0 4	VP0 5	VP0 6	VP0 7	VP0 8	VP0 9	VP1 0	Total	Percentage
Amikacin	AK	30	1	1	1	1	1	1	1	1	1	1	10	100%
Amoxycillin/clavulanic acid	AMC	30		1	1	1	1	1	1	1	1	1	9	90%
Ampicillin	AMP	10		1	1	1	1	1		1			6	60%
Chloramphenicol	C	30	1	1	1	1	1	1	1	1	1	1	10	100%
Ceftazidime	CAZ	30	1	1	1	1	1	1	1	1	1	1	10	100%
Ciprofloxacin	CIP	5	1	1	1	1	1	1	1	1	1	1	10	100%
Ceftriaxone	CRO	30	1	1	1	1	1	1	1	1	1	1	10	100%
Imipenem	IPM	10		1	1	1	1	1					5	50%
Kanamycin	K	30	1	1	1	1	1	1		1	1	1	9	90%
Cephalothin	KF	30											0	0%
Meropenem	MEM	10	1		1	1	1	1	1	1	1		8	80%
Neomycin	N	30	1	1	1	1	1		1	1			7	70%
Nalidixic Acid	NA	30	1	1	1	1	1	1	1	1	1	1	10	100%
Norfloxacin	NOR	10	1	1		1	1	1	1	1	1	1	9	90%
Rifampicin	RD	5	1	1	1	1	1			1	1	1	8	80%
Sulphamethoxazole	RL	25											0	0%
Streptomycin	S	10											0	0%
Compound Sulphonamide	S3	300	1										1	10%
Sulphamethoxazole – Trimetoprim	SXT	25	1	1	1	1	1	1	1	1	1	1	10	100%
Tetracycline	TE	30		1	1	1	1	1	1	1	1	1	9	90%

V. parahaemolyticus Antibiotic Intermediate Isolates

Antibiotic	Code	Conc (µg)	VP0 1	VP0 2	VP0 3	VP0 4	VP0 5	VP0 6	VP0 7	VP0 8	VP0 9	VP1 0	Total	Percent age
Amikacin	AK	30											0	0%
Amoxycillin/clavulanic acid	AM C	30											0	0%
Ampicillin	AMP	10											0	0%
Chloramphenicol	C	30											0	0%
Ceftazidime	CAZ	30											0	0%
Ciprofloxacin	CIP	5											0	0%
Ceftriaxone	CRO	30											0	0%
Imipenem	IPM	10							1	1	1	1	4	40%
Kanamycin	K	30							1				1	10%
Cephalothin	KF	30											0	0%
Meropenem	ME M	10										1	1	10%
Neomycin	N	30											0	0%
Nalidixic Acid	NA	30											0	0%
Norfloxacin	NOR	10											0	0%
Rifampicin	RD	5											0	0%
Sulphamethoxazole	RL	25											0	0%
Streptomycin	S	10											0	0%
Compound Sulphonamide	S3	300			1								1	10%
Sulphame thoxazole – Trimetoprim	SXT	25											0	0%
Tetracycline	TE	30											0	0%

V. parahaemolyticus Antibiotic Resistant Isolates

Antibiotic	Code	Conc (µg)	VP0 1	VP0 2	VP0 3	VP0 4	VP0 5	VP0 6	VP0 7	VP0 8	VP0 9	VP1 0	Tota l	Percentag e
Amikacin	AK	30											0	0%
Amoxycillin/clavulanic acid	AMC	30	1								1		2	20%
Ampicillin	AMP	10	1						1		1	1	4	40%
Chloramphenicol	C	30											0	0%
Ceftazidime	CAZ	30											0	0%
Ciprofloxacin	CIP	5											0	0%
Ceftriaxone	CRO	30											0	0%
Imipenem	IPM	10	1										1	10%
Kanamycin	K	30											0	0%
Cephalothin	KF	30	1	1	1	1	1	1	1	1	1	1	10	100%
Meropenem	MEM	10		1									1	10%
Neomycin	N	30											0	0%
Nalidixic Acid	NA	30											0	0%
Norfloxacin	NOR	10			1								1	10%
Rifampicin	RD	5						1	1				2	20%
Sulphamethoxazole	RL	25	1	1	1	1	1	1	1	1	1	1	10	100%
Streptomycin	S	10	1	1	1	1	1	1	1	1	1	1	10	100%
Compound Sulphonamide	S3	300		1		1	1	1	1	1			6	60%
Sulphamethoxazole – Trimetoprim	SXT	25											0	0%
Tetracycline	TE	30	1										1	10%