

**THE EFFECTS OF SALINITY AND
SCREWPINE (*Pandanus tectorius*) LEAF
EXTRACT ON *Vibrio* DENSITY IN AXENIC AND
NON-AXENIC BRINE SHRIMP (*Artemia
franciscana*) CULTURES**

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Extract on *Vibrio* Density in Axenic and Non-axenic Brine Shrimp
(*Artemia franciscana*) Cultures**

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**Thesis Submitted in Fulfilment of the Requirement for the Master of
Science in the Institute of Climate Adaptation and Marine Biotechnology
Universiti Malaysia Terengganu**

AUG 2025

DEDICATION

Dedicated this thesis to:

My supervisor, Prof. Yeong Yik Sung, Ph.D

My co-supervisor, Assoc. Prof. Chen Shuaijun

My father and mother, as well as all the relatives and teachers who have supported me throughout my academic and life journeys.

For all their endless guidance, dedication, and sacrifice.

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu in fulfillment of the requirements for the degree of Master of Science

The Effects of Salinity and Screwpine (*Pandanus tectorius*) Leaf Extract on *Vibrio* Density in Axenic and Non-axenic Brine Shrimp (*Artemia franciscana*) Cultures

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AUG 2025

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**School/Institute : Institute of Climate Adaptation and Marine
Biotechnology**

The brine shrimp is a valuable live feed organism used in aquaculture. However, *Artemia* can easily spread pathogens such as *Vibrio* to other aquatic organisms, resulting in disease outbreaks and major decreases in aquaculture productivity. This thesis investigated a more cost-effective and ecologically friendly alternative to standard antibacterial agents for eradicating or reducing *Vibrio* load in *Artemia*. This study examined the effects of salinity and methanolic *Pandanus tectorius* leaf extract (PLE) on *Vibrio* load in *Artemia* and their rearing water. Experiments were carried out over a 48-hour period using various salinities (30, 40, 50, and up to 200 g/L). Under axenic conditions, *Artemia* nauplii were challenged with *Vibrio parahaemolyticus* and *Vibrio campbellii* at 10^7 and 10^8 cells/mL, respectively. After 48 hours, the *Vibrio* loads were determined. Total sterilization, as evidenced by the absence of *Vibrio* colonies on TCBS agar, was obtained at salinities more than 120 g/L. PCR indicated that no *Vibrio* was discovered in the rearing water at salinities greater than 140 g/L under non-axenic, low-density bacterial conditions. However, under high-density bacterial conditions, viable *Vibrio* was discovered at salinities as high as 200 g/L. Notably, exposure to salinities more than 70 g/L combined with 1 g/L PLE completely eradicated *Vibrio* from both rearing water and

nauplii. For cost savings, replacing 1 g/L of PLE with 7.14 g/L of freeze-dried *P. tectorius* leaf powder at 80 g/L salinity resulted in a similar *Vibrio*-free results. In adult *Artemia*, the combined salinity and *P. tectorius* treatment did not completely eliminate *Vibrio*, but it did greatly reduce it. This study effectively established a simple, cost-effective strategy for creating *Vibrio*-free nauplii and reducing *Vibrio* levels in adult *Artemia*, so contributing to the prevention of vibriosis outbreaks in *Artemia* cultures and the larger aquaculture environment. Future study should concentrate on improving the approach for establishing *Vibrio*-free adult *Artemia* and determining the long-term impact of these therapies on their health and reproductive performance.

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu
sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

**Kesan Saliniti dan Ekstrak Daun Pandan (*Pandanus tectorius*) terhadap densiti
Vibrio dalam kultur axenik dan bukan axenik udang artemia (*Artemia
franciscana*)**

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Penyelia : Professor Yeong Yik Sung, Ph.D

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Marin

Udang garam (brine shrimp) adalah organisma makanan hidup yang bernilai dan digunakan dalam akuakultur. Walau bagaimanapun, *Artemia* boleh dengan mudah menyebarkan patogen seperti *Vibrio* kepada organisma akuatik lain, menyebabkan wabak penyakit dan penurunan besar dalam produktiviti akuakultur. Tesis ini mengkaji alternatif yang lebih kos efektif dan mesra alam berbanding agen antibakteria standard untuk membasmi atau mengurangkan beban *Vibrio* dalam *Artemia*. Kajian ini meneliti kesan kepekatan garam dan ekstrak daun *Pandanus tectorius* metanolik (PLE) terhadap beban *Vibrio* dalam *Artemia* dan air pembiakannya. Eksperimen dijalankan selama 48 jam menggunakan pelbagai kepekatan garam (30, 40, 50, dan sehingga 200 g/L). Dalam keadaan aseptik, *Artemia* nauplii dicabar dengan *Vibrio parahaemolyticus* dan *Vibrio campbellii* pada 10^7 dan 10^8 sel/mL masing-masing. Selepas 48 jam, beban *Vibrio* ditentukan. Pensterilan total, yang dibuktikan dengan ketiadaan koloni *Vibrio* pada agar TCBS, diperoleh pada kepekatan garam lebih daripada 120 g/L. Ujian PCR menunjukkan tiada *Vibrio* ditemui dalam air pembiakan pada kepekatan garam melebihi 140 g/L dalam keadaan bukan aseptik dengan kepekatan bakteria rendah. Walau

bagaimanapun, dalam keadaan kepekatan bakteria tinggi, *Vibrio* hidup ditemui pada kepekatan garam setinggi 200 g/L. Menariknya, pendedahan kepada kepekatan garam lebih daripada 70 g/L yang digabungkan dengan 1 g/L PLE sepenuhnya membasmi *Vibrio* dari air pembiakan dan nauplii. Untuk penjimatan kos, penggantian 1 g/L PLE dengan 7.14 g/L serbuk daun *P. tectorius* beku kering pada kepekatan garam 80 g/L menghasilkan keputusan bebas *Vibrio* yang serupa. Dalam *Artemia* dewasa, rawatan gabungan kepekatan garam dan *P. tectorius* tidak membasmi *Vibrio* sepenuhnya, tetapi ia mengurangkannya dengan ketara. Kajian ini berjaya mewujudkan strategi yang mudah dan kos efektif untuk menghasilkan nauplii bebas *Vibrio* dan mengurangkan tahap *Vibrio* dalam *Artemia* dewasa, sekaligus menyumbang kepada pencegahan wabak vibriosis dalam kultur *Artemia* dan persekitaran akuakultur yang lebih luas. Kajian masa depan harus memfokuskan pada memperbaiki pendekatan untuk mewujudkan *Artemia* dewasa bebas *Vibrio* dan menentukan impak jangka panjang terapi ini terhadap kesihatan dan prestasi pembiakan mereka.

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Thanks to my family, consistent support is the solid backing for me to move forward. In the future, I will carry this gratitude and bravely embark on a new journey.

APPROVALS

I certify that an Examination Committee has met on 7th August 2025 to conduct the final examination of Zhou Xinwei, on his Master thesis entitled “**The Effects of Salinity and Screwpine (*Pandanus tectorius*) Leaf Extract on *Vibrio* Density in Axenic and Non-axenic Brine Shrimp (*Artemia franciscana*) Cultures**” in accordance with the regulations approved by the Senate of Universiti Malaysia Terengganu. The Committee recommends that the candidate be awarded the relevant degree. The members of the Examination Committee are as follows:

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This thesis has been accepted by the Senate of Universiti Malaysia Terengganu in fulfilment of the requirement for the degree of Master of Science.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UMT or other institutions.

ZHOU XINWEI

Date:

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LIST OF ABBREVIATIONS

°C	Celsius degree
CFU	Colony forming units
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
FASW	Filtered autoclaved sea water
g	Gram
>	Greater-than
≥	Greater-than-equal
h	Hour
lx	Illuminance intensity: lux
<	Less-than
≤	Less-than-equal
L	Litre
m:v	Mass-to-volume ratio
μL	Microliter
μL	Microliter
μm	Micrometre
μmol	Micromole
mbar	Millibar
mg	Milligram
mL	Milliliter
min	Minute
ng	Nanogram
PKC	Palm kernel cake-based emulsion

PLE	Pandanus tectorius leaf extract
%	Percentage
×g	Relative Centrifugal Force: gravitational acceleration
s	Second
NaOH	Sodium hydroxide
NaClO	Sodium hypochlorite
Na ₂ S ₂ O ₃	Sodium thiosulfate
<i>spp.</i>	Species
TCBS	Thiosulfate Citrate Bile Sucrose
TFTC	Too few to count
TNTC	Too numerous to count
v:v	Volume-to-volume ratio

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Aquaculture is an essential source of seafood, ensuring global food security (Bouwmeester et al., 2020). Aquaculture began as early as 4000 years ago in Egypt (Chimits, 1957) and over 2000 years ago in China (Lu & Li, 2006). Due to the inability of traditional fishing to meet the growth of the human population, aquaculture has received greater attention (Costa-Pierce, 2008). However, aquatic organisms are prone to disease outbreaks in the aquaculture process. The economic losses caused by the outbreak of diseases and the costs invested in disease control are enormous. These disease outbreaks have also directly or indirectly affected food security.

The brine shrimp *Artemia* is an euryhaline crustacean, widely used as a live food species in aquaculture (Henry et al., 2019; Madkour et al., 2023). *Artemia* has a high nutritional value and serves as primary food for fish and shrimp, helping them maintain good immunity and health (Mali et al., 2023). With increasing recognition of the value of *Artemia*, natural harvesting methods have become scarce, prompting a surge in artificial aquaculture of *Artemia*. However, in the process of artificial aquaculture, factors such as crowding, nutrient deficiency, and hunger ultimately heighten the susceptibility of aquatic animals to bacterial infections (Quesada et al., 2013). The invasion of *Vibrio* can consequently lead to *Artemia* infection with vibriosis or load *Vibrio*. Using live feed presents biosafety risks to aquaculture, as it may inadvertently introduce bacterial pathogens (Høj et al., 2009). Raised *Artemia* stands out as the most commonly used option among the live feeds employed.

Artemia can transmit vibriosis to other aquatic organisms that feed on *Artemia*, resulting in significant economic losses (Roy et al., 2022).

Vibrio genus, a Gram-negative bacterium, is widely distributed in river water, seawater, and marine animals. Some strains of *Vibrio* have the certain potential to induce *Vibrio* infections into aquatic products, consequently posing a significant threat to aquaculture and human health (Letchumanan et al., 2014). *Vibrio campbellii* stands as one of the most significant pathogens in shrimp or fish farming, inflicting considerable economic losses on aquaculture (Phuoc et al., 2008). *Vibrio anguillarum* and *Vibrio parahaemolyticus* are infectious strains of *Artemia* embryonic cells (Duan et al., 2022).

1.2 Problem statement and justification

V. parahaemolyticus is a pathogenic bacterial species for many shrimp species including *Artemia* (Kumaran & Citarasu, 2016), as with *V. campbellii* (Haldar et al., 2011). Finding effective strategies to inhibit these pathogenic *Vibrios* is crucial, especially for the large-scale cultivation of *Artemia* and the aquatic organisms that rely on *Artemia* as a live feed. In current aquaculture practices, antibiotics play a crucial role as a tool for preventing outbreaks of infectious diseases and mitigating disease-related economic losses (Laganà et al., 2011). However, prolonged antibiotic usage can result in the development of bacterial resistance, rendering it an unsustainable long-term solution. Moreover, antibiotics entering the human body through the food chain can pose risks to human health (Loo et al., 2020). Therefore, alternative strategies are being pursued to suppress *Vibrio* infection during *Artemia* farming. Zheng et al.'s study discovered that essential oils can efficiently inhibit *V. campbellii*, enhance the survival percentage of *Artemia* (Zheng et al., 2021), and offer potential strategies for controlling vibriosis in aquaculture processes. Probiotic protection methods have also been attempted for the prevention of vibriosis in *Artemia*. Fernandes et al. showed that lactic acid bacteria can reduce the incidence rate of vibriosis caused by *V. parahaemolyticus* (Fernandes et al., 2023). Additionally, there are studies demonstrating that genetic technology can serve as a

strategy for inhibiting *Vibrio* and controlling *Vibrio* infection in *Artemia* (Novriadi et al., 2023).

1.3 Significance of the study

Inhibiting the growth of *Vibrio* and ensuring the survival percentage of *Artemia* is a major challenge in *Artemia* farming. However, many methods to inhibit *Vibrio* are complex and inevitably incur higher costs. This study aims to identify the simplest and most cost-effective approach to achieve this goal. By controlling salinity, which aligns with the wide salinity tolerance of *Artemia*, *Vibrio* can be suppressed, consequently enhancing the yield of *Artemia*. In addition, the components in *P. tectorius* leaves have effective antibacterial effects. Develop a method for combining salinity with *P. tectorius* leaves or their extracts to achieve *Vibrio*-free *Artemia*. Provide effective and low-cost methods for controlling vibriosis in *Artemia* farming, as well as eliminate *Vibrio* for the production of *Vibrio*-free *Artemia*.

1.4 Research Objectives

This study focuses on finding an optimal salinity exposure condition to reduce or eliminate *Vibrio* colonization while ensuring a good survival in *Artemia*. Therefore this study aimed:

- 1) To determine the effect of salinity on the *Vibrio* load of *Artemia* during axenic culture and non-axenic culture
- 2) To determine the combined effect of salinity and *P. tectorius* leaves extract on the *Vibrio* load of *Artemia*

CHAPTER 2

LITERATURE REVIEW

2.1 Brine shrimp *Artemia*

2.1.1 Basic information and biological characteristics of *Artemia*

Artemia or commonly known as brine shrimp, is a primitive *Arthropod* that measures between 8 and 12 millimeters (Azra et al., 2022). *Artemia* is a genus in which the majority of species reproduce sexually, while a very small number reproduce through parthenogenesis. *Artemia* reaches maturity after approximately 15 molts and takes 8 to 20 days (Camara, 2020; Treece, 2000). Under suitable environmental conditions, the maturity time depends on the variety of *Artemia* and the feed used. Adult female *Artemia* develops fertilized eggs after mating. The development of fertilized eggs into free-swimming nauplii or diapause cysts, that depends on environmental conditions. In unsuitable conditions, fertilized eggs develop into cysts, such as high salinity, low dissolved oxygen levels, and other adverse factors (Støttrup & McEvoy, 2008). *Artemia* has phototaxis and is more active in lightness. *Artemia* is distributed in many parts of the world, and different regions have adapted to different unfavorable conditions such as high temperature, low oxygen levels, complex ion composition, dryness, and a large amount of ultraviolet radiation (Camara, 2020). Their common characteristic is high salinity adaptability. The salinity adaptation range of *Artemia* spans from 10 g/L (Abatzopoulos et al., 2006) to 340 g/L (Post & Youssef, 1977). González Castro et al. also reported that the growth and survival rates of *Artemia* populations along the

Pacific coast of Mexico were negatively correlated with salinity, with optimal survival occurring between 100 and 120 g/L (Castro-Mejía et al., 2011).

Artemia is a non-selective filter feeding organism. They can feed on a variety of foods, such as protozoa, microalgae, bacteria, and small-size debris (Toi et al., 2013). All the particle sizes that the nauplii can feed on range from 6.8 to 27.5 μm , with the optimal size being around 16.0 μm (Chaoruangrit, Plodsomboon, Rogers, & Sanoamuang, 2017). The particle size that adult *Artemia* can feed on cannot exceed 50 μm (Nevejan et al., 2018). *Artemia* passively filters and enriches particles, and any biological particles within the aforementioned size range can serve as their food (Madkour, Dawood, & Sewilam, 2023). *Artemia* nauplii appears 20 hours after hatching (Støttrup & McEvoy, 2008). They start non-selective filter feeding in their second stage of development (instar II or metanauplius), which occurs 8 hours after hatching (Interaminense et al., 2014).

2.1.2 The development and value of *Artemia*

Artemia was first introduced in the 18th century and has been widely used in many biological fields (Sorgeloos, 1980). From 1933 to 1939, Seale, Gross, and Rollefson discovered that 0.4 mm *Artemia* nauplii was the main food source for fish in its early stages. This discovery stimulated the production of *Artemia* at that time (Sorgeloos, 1980). In the early 1950s, commercial *Artemia* cysts were first harvested from San Francisco Bay (SFB) beaches in California and the Great Salt Lake (GSL) in Utah, and sold at low prices to the aquarium pet trade (Islam, Ahmed, Shehzad, & Lee, 2019). In the mid-1970s, the demand for cysts sharply increased due to the growing demand for *Artemia* nauplii among juvenile fish (Lavens & Sorgeloos, 2000). The production of cysts has rapidly increased from a few tons per year to nearly 3000 tons per year (Figure 2.1.2) (Litvinenko, Litvinenko, Boiko, & Kutsanov, 2015). The figure shows the main producers of *Artemia* in the world, including Great Salt Lake (Utah, USA), China, Russia, Kazakhstan, Uzbekistan, Vietnam, and other salt ponds and lakes in the world with their production of *Artemia* cysts (Litvinenko et al., 2015).

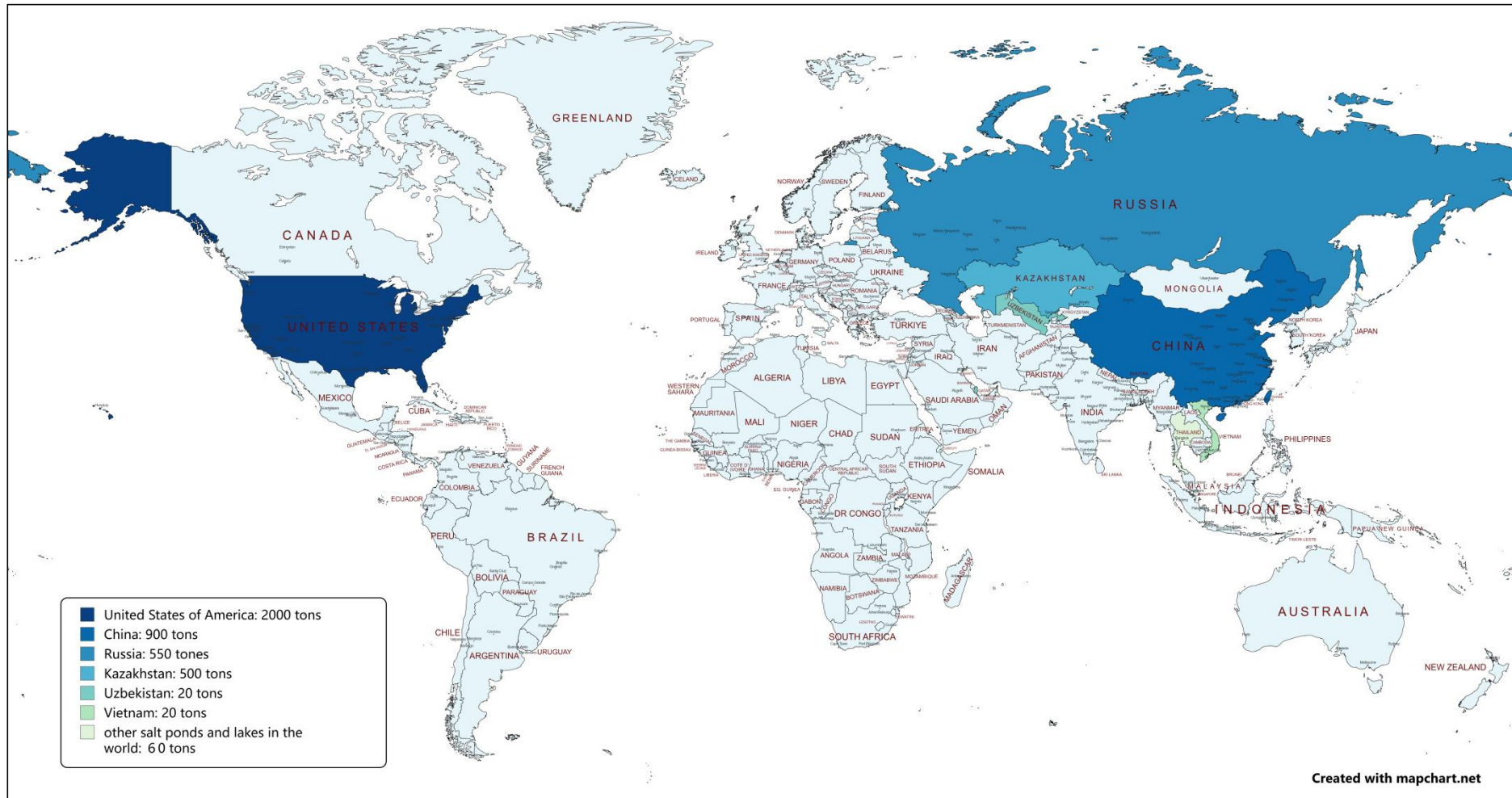


Figure 2.1.2 Main countries produce *Artemia* cysts.

Live foods are the key source of nutrition for farmed fish larvae and are essential for feeding marine altricial fish larvae (Madkour et al., 2023). Zooplankton is usually a natural food source for fish and shrimp larvae, but it is either economically unfeasible or technically difficult to achieve (Ayón et al., 2008). *Artemia* is an excellent live food that can meet the nutritional needs of fish larvae in the early stages of growth. They are the most convenient live food in aquaculture, and only require a simple hatching process to obtain nauplii (Sorgeloos, 1980). When using *Artemia* during the rearing of fish larvae, it greatly reduces the mortality rate of many fish and increases their growth rate (Madkour et al., 2023). *Artemia franciscana* nauplii can meet the feeding needs of some marine species, such as marine fish, sea anemones, crustaceans, seahorses, soft corals, clownfish, and cephalopods (Lim et al., 2000). Since the industrialization of *Artemia*, researchers have devoted decades of effort to increasing their production. However, the current production still cannot meet the increasing demand for *Artemia* by humans.

2.2 *Vibrio* genus

2.2.1 Basic information and biological characteristics of *Vibrio*

Vibrio genus is a common, Gram-negative, rod-shaped bacterial group. Widely distributed in fresh-water, estuarine, and marine environments (Craig Baker-Austin et al., 2017). *Vibrio spp.* prefer warm, brackish water, and their abundance in natural environments often reflects environmental temperature (Craig Baker-Austin et al., 2018). *Vibrio spp.* grow the fastest among bacteria and respond almost immediately to favorable environmental conditions such as temperature, salinity, and dissolved oxygen (Abioye et al., 2021; Craig Baker-Austin et al., 2020). There is a larger number of species of *Vibrio spp.*, currently recognized as 147 species and 4 subspecies, most of which are non pathogenic species, some of which are symbionts (Sampaio et al., 2022). Less than 12 species are human pathogens (such as *Vibrio cholera*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, etc.), and other species infected marine animals such as fish, shrimp, corals, and crustaceans (Sampaio et al., 2022).

2.2.2 The hazards and detection methods of *Vibrio spp.*

One of the biggest threats to sustainable aquaculture is existing and emerging pathogens (Kumar et al., 2021). Vibriosis is the main bacterial infection in marine and brackish water aquaculture systems (Ina-Salwany et al., 2019). The outbreak of vibriosis in aquatic animals is mainly caused by closely related *Vibrio spp.*, including *V. harveyi*, *V. campbellii*, *V. owensii*, *V. alginolyticus*, *V. parahaemolyticus*, etc (Mohamad et al., 2019). The Centers for Disease Control and Prevention in the United States estimates that there are 8028 cases of *Vibrio* infections and 57 deaths annually (Mead et al., 1999). In the human population, the transmission of *Vibrio* infection is mainly through the consumption of raw or undercooked shellfish (J. G. Morris & Black, 1985). This leads to acute gastroenteritis characterized by diarrhea, vomiting, and abdominal cramps (Jay, 2000). To ensure food health and safety, it is very important to control *Vibrio* during the aquaculture process.

Thiosulfate Citrate Bile Sucrose (TCBS) agar is a selective agar medium commonly used for the selective culture of *Vibrio spp.* (Lotz et al., 1983), and is an important medium for *Vibrio* research. TCBS agar has high pH and salinity, which can inhibit other bacteria and stimulate the growth of *Vibrio spp.* *Vibrio spp.* colonies growing on TCBS agar plates usually with different colors, such as yellow for *V. harveyi*, green for *V. campbellii*, and blue-green for *V. parahaemolyticus*. However, the color of bacterial colonies is influenced by various factors and can only be used as a preliminary judgment basis. Specific identification needs to be verified in conjunction with other methods, such as specific polymerase chain reaction (PCR) and identification sequencing. Although TCBS agar is a selective medium, it still allows non-*Vibrio spp.* to grow (G. K. Morris et al., 1976), so it cannot be used as the only means of isolating *Vibrio spp.*

2.3 *Pandanus tectorius*

2.3.1 Basic information and biological characteristics of *P. tectorius*

Pandanus tectorius is a plant native to humid and subhumid tropical lowlands, planted at an altitude of 600 meters, but rarely seen in the wild above an altitude of 20 meters (Sudhakara, 2006). Their morphology is related to age: they undergo a juvenile semi-prostrate period in the first 4-9 years and then undergo 5-12 years of upright trunk growth before flowering begins (Sudhakara, 2006). They can grow up to 15 meters high and are a robust, branched, and multi-stemmed plants (Thomson et al., 2006). The *P. tectorius* leaves are arranged in a spiral pattern in three rows, sword-shaped, with spines and ribs at the edges, and flagella at the top (Baba et al., 2016) (Figure 2.3.1).



Figure 2.3.1 Thicket of *P. tectorius* and zoomed-in leaves.

2.3.2 The development and value of *P. tectorius*

The use of *P. tectorius* by humans began in the field of traditional medicine when they were used as medicinal plants to combat diseases. People in some impoverished areas still use this inexpensive, easily accessible, and highly effective medicinal plant. With the development of modern science and technology, people

have conducted more thorough research on *P. tectorius* and discovered that it has a wide range of medicinal values.

Infectious diseases are on the rise globally and require effective body defense mechanisms to be controlled through immune regulation processes (Rehan, 2022). To prevent the spread of pathogenic microorganisms, especially those that affect aquaculture, antibacterial medicines are essential (Chan, 2008). Compared to antibiotics and other antibacterial medicines, using plant or herbal extracts is safer for aquatic organisms and causes less damage to the environment (Anirudhan et al., 2021). In the research of Anirudhan et al., it was shown that *P. tectorius* extract are efficient antibacterial drug that can effectively enhance shrimp's resistance to *V. parahaemolyticus*. Additionally, infection by pathogenic microorganisms can trigger inflammatory reactions in the body. The extract of *P. tectorius* leaves has a potential sedative effect, which can alleviate the inflammatory response of mouse paws (Del Mundo et al., 2020). The ethanol extract of the *P. tectorius* fruit has a very effective antidiabetic activity. After 14 days of oral administration, the blood sugar of mice was significantly reduced, and there was no oral side effect (Wu et al., 2014). In addition to the medicinal value mentioned above, *P. tectorius* also has many proven active functions, such as anti-hypercholesterolemic and anti-atherosclerotic potencies, antioxidant and antibacterial activity, antiviral activity, etc (Andriani et al., 2020; Den Hartogh & Tsiani, 2019; Nhu, 2015). Compared to the fruit, *P. tectorius* leaves have more biological activity, such as anti-tuberculosis, antibacterial, anti-inflammatory, hepatoprotective, central nervous system inhibitor, antidiarrheal, anticonvulsant, diuretic, and neuroprotective activity (Baba et al., 2016).

CHAPTER 3

METHODOLOGY

3.1 *Vibrio* culture

Two pathogenic *Vibrio* strains were selected for this study: *Vibrio campbellii* (LMG21363) and *Vibrio parahaemolyticus* (CW8T2). The isolated *Vibrio* stock, previously stored in 20% glycerol at -80°C, was aseptically inoculated into Marine Broth (Difco™ Marine Broth 2216, USA) at a ratio of 1:2000 (v:v) and incubated overnight at 28°C. The *Vibrio* suspension was transferred to falcon tubes and centrifuged at 1,0000×g for 10 min. The supernatant was discarded and the *Vibrio* cells were resuspended with filtered autoclaved sea water (FASW). The density of *Vibrio* cells were measured by spectrophotometer at 600 nm. Assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/mL (Yik Sung et al., 2007).

3.2 Methanolic *Pandanus tectorius* leaf extract (PLE)

In Setiu Wetlands, Terengganu, Malaysia, *Pandanus tectorius* leaves were collected in the early morning and transported to local laboratory (Institute of Climate Adaptation and Marine Biotechnology, University Malaysia Terengganu,) as soon as possible (Figure 3.2 a). The leaves were washed and cut into small pieces, then frozen overnight at -80°C. The next day, the fragmented leaves were freeze-dried (Figure 3.2 b) and ground into fine powder (Figure 3.2 c), stored at -20°C. The fine powder was extracted with pure methanol in a ratio of 1:10 (m:v) at room temperature for 48 hours. The leaf residue is filtered and removed by Whatsman No. 1 filter paper. The filtration process was conducted under a

ventilation hood. The methanol solution containing PLE (*Pandanus tectorius* leaf extract) was concentrated with a rotary evaporator (BUCHI R-300, Switzerland) at a pressure of 337 mbar and a temperature of 42°C. Until no methanol droplets dropped in the collection bottle, the oily brown-green PLE was collected in a brown bottle and stored at 4°C. In this study, the process of extracting PLE was based on the method developed by Anirudhan et al. (Anirudhan et al., 2023). The extraction rate of PLE from freeze-dried powder of *P. tectorius* leaves reached 14%.

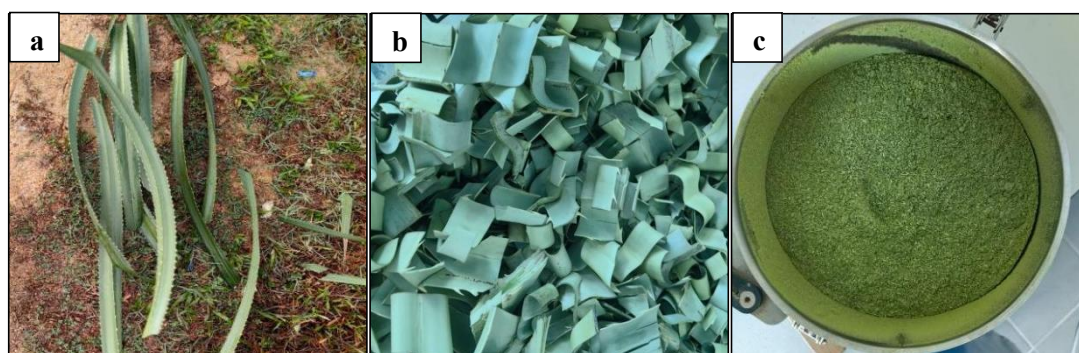


Figure 3.2 Images of *P. tectorius* leaves. **a)** Fresh leaves. **b)** Cut and freeze-dried leaves. **c)** Ground leaf powder.

The PLE is difficult to completely dissolve in seawater, so it was pre-dissolved in Dimethyl sulfoxide (DMSO) before use. The ratio of PLE to DMSO is 2:3 (v:v), and the PLE was also weighed. After calculation, the final concentration of the PLE solution is expressed in “mg/mL” or “g/L” to facilitate its use and calculations in subsequent experiments.

3.3 *Artemia* hatching and hatchery culture

3.3.1 Axenic hatching

The experimental material for this study is *Artemia franciscana*. The cysts from the Great Salt Lake, USA (Ocean Star International, INC). The axenic *Artemia* larvae were produced by hatching cysts axenically following Baruah et al. (Kartik Baruah et al., 2009). 0.5 g of *Artemia* cysts were hydrated in 89 mL distilled water

for 60 min. Subsequently, 3.3 mL NaOH (32%) was added to provide an alkaline condition for the decapsulation process. Then 50 ml NaClO (10%) was added to remove the capsule, and 0.22 μ m filtered aeration was provided was ventilated during this process. All operations were carried out under the biosafety cabinet, and all necessary tools were autoclaved at 121°C for 20 min before use. The decapsulation was stopped after 3 min by adding 50 mL Na₂S₂O₃ (10 g/L). The aeration was then stopped and the decapsulated cysts underwent a thorough rinse with seawater using a sterile sieve with 50 μ m pore size. At this point, the disinfection process of the cysts has ended, and sterile cysts have been obtained. Subsequently, the cleansed cysts were carefully transferred to a sterile 500 mL screw-cap bottle containing 500 mL of seawater. All seawater was previously filtered by 0.22 μ m hydrophilic membrane and autoclaved (filtered autoclaved seawater, FASW). To prevent cyst sedimentation and provide oxygen for the later-hatched nauplii, the filtered air was pumped into the FASW through the cap. Cysts were exposed to constant incandescent light (2000 lx), with the temperature maintained at 28°C for 28 hours. Hatching water was spread onto Marine agar and incubated for 5 days to test the success of the axenic hatching process (K. Baruah et al., 2010). If colonies grow, the hatched nauplii cannot be used for subsequent experiments.

3.3.2 Non-axenic hatching and bacteria

Without undergoing disinfection and decapsulation, the dried cysts were directly placed in seawater for hatching. The hatching conditions were identical to those used for axenic hatching, with the key difference being that all tools were unsterilized. The seawater used for hatching was ordinary seawater (the range of salinity is 32-34 g/L), not FASW, and the aeration was also unfiltered. This setup simulated the introduction of bacteria by the hatchery during hatching, ensuring that all bacteria in the hatching environment originated from the cysts and the external environment.

3.3.3 *Artemia* hatching and adult culture in hatchery condition

In the *Artemia* hatchery (Universiti Malaysia Terengganu), 10 g cysts were placed in a hatching tower with 10 L of seawater. The hatching process involved 28 hours of aeration and exposure to 2000 lx of light. Stop aeration and light exposure, and let the shell settle for five minutes. Hatched cyst shells and unhatched cysts sank to the bottom, while empty cysts floated on the surface, and nauplii stayed suspended in the water. The suspended nauplii were collected from the outlet and filtered through a 50 µm sieve. They were quickly weighed and subsequently transferred to a culture tank with a density of 1 g of nauplii (wet weight) per ton of seawater. Each tank (1 ton) was fed with 20 L PKC Nutri+® per day (Iryani et al., 2020), divided into two doses. Adult *Artemia* was obtained by feeding until the 8th to 10th day.

3.4 Experimental design

3.4.1 Experiment 1: Exposure experiment on axenic *Artemia* nauplii

Prepared filtered autoclaved salt water with different salinities ranging from 30, 60, 70 to 150 g/L in advance. Sea salt was directly added to natural seawater, and the salinity was adjusted to target levels. Then the salt water was filtered by 0.22 µm hydrophilic membrane and autoclaved. Thirty (30) axenic nauplii were placed in a 50 mL sterile falcon tube containing 30 mL salt water (1 nauplius/mL). The nauplii were exposed to 10^7 and 10^8 cells/mL of *Vibrio* in a 28°C environment under the light. The salinity exposure experiments were conducted according to the following 6 different conditions:

- 1) Axenic *Artemia* nauplii and *V.campbellii* (10^7 cells/mL)
- 2) Axenic *Artemia* nauplii and *V.campbellii* (10^8 cells/mL)
- 3) Axenic *Artemia* nauplii and *V.parahaemolyticus* (10^7 cells/mL)
- 4) Axenic *Artemia* nauplii and *V.parahaemolyticus* (10^8 cells/mL)
- 5) Axenic *Artemia* nauplii only, without *Vibrio*
- 6) *V.parahaemolyticus* (10^7 cells/mL) only, without *Artemia*

During the exposure, the falcon tubes were shake slightly to ensure sufficient dissolved oxygen in the salt water. After 48 hours of exposure, the survival of *Artemia* nauplii and the inhibition percentage of *Vibrio* were detected for the treatment groups 1 to 4 mentioned above. In treatment group 5, which contained only *Artemia* nauplii, the survival percentage of nauplii was detected after 48 hours. In treatment group 6, which contained only *V.parahaemolyticus*, the inhibition percentage of *Vibrio* was detected after 48 hours and 72 hours. Three colonies were randomly selected from TCBS agar in all different salinity treatment groups of the 48 h salinity exposure of axenic *Artemia*. The inhibition percentage of *Vibrio* was detected by TCBS agar, and the detection method is described in the “**3.5 Detection of inhibition of bacteria and survival of *Artemia***” section later on. In addition, three colonies were randomly selected from those formed on TCBS agar in treatment groups 3, 4, and 6. Identification sequencing was performed on these three colonies to verify that the *Vibrio* introduced in the exposure experiment was indeed *V.parahaemolyticus*. The methods for isolating strains and identification sequencing are described in the “**Identification sequencing**” section later on.

3.4.2 Experiment 2: Exposure experiment on non-axenic *Artemia* nauplii

Prepared non-sterile 500 mL bottles and 500 mL brackish water with different salinities ranging from 30 to 200 g/L in advance. Non-axenic nauplii were roughly counted and placed into prepared brackish water (about 1 nauplius/mL). Exposed in 28°C temperature for 48 hours, continuously exposed to non-sterile air and under light (2000 lx). The exposure condition for transferring nauplii to new seawater (or saltwater) is called a low-density bacterial condition, where the density of marine bacteria in the rearing water did not exceed 10^7 cells/mL. Starting from 0 hours, bacterial density detection (using Marine agar) and *Vibrio* density detection (using TCBS agar) in rearing water were conducted every 12 hours. At 48 h, the rearing water of different salinities ranging from 30, 60, 70 to 150 g/L (11 groups) were collected for *Vibrio* genus PCR (Polymerase chain reaction) detection. In addition, three colonies were randomly selected from TCBS agar in each of the following four different salinity treatments of the 48 h. Those four different salinity treatments were 30, 70, 100 and 120 g/L. A total of twelve strains were isolated for

identification sequencing. This “low-density bacterial condition” section is only for exploring changes in bacterial count, providing methodological support for the later “high-density bacterial condition” section, and therefore does not require nauplii survival testing.

In order to test the effectiveness of salinity in inhibiting *Vibrio*, a new exposure method was designed and a new antibacterial component (PLE) was introduced. After non-axenic hatching, salt (or concentrated saline) was added directly to the hatching water to adjust the different salinities ranging from 30, 50, 60 to 200 g/L. In addition to nauplii, shells, unhatched cysts, and a larger number of bacteria were contained in the hatching water. Exposed environment such as light, temperature, and aeration is the same as in the previous text. The exposure condition for not transferring nauplii to new seawater (or saltwater) is called a high-density bacterial condition, where the density of marine bacteria in the rearing water exceeds 10^7 cells/mL. The salinity exposure experiments were conducted according to the following four different conditions:

- 1) Addition of salt
- 2) Addition of salt and PLE (1g/L)
- 3) Addition of salt and DMSO (1g/L)
- 4) Addition of salt and freeze-dried powder of *P. tectorius* leaves (7.14 g/L)

In treatment groups 1 and 2, the survival percentage of nauplii and the inhibition percentage of *Vibrio* were detected at 12, 24, and 48 hours. The detection of *Vibrio* inhibition percentage here is divided into two types: in rearing water and in *Artemia*. In treatment group 1, *Vibrio* genes in rearing water were detected by PCR at exposure 48 hours. To eliminate the interference of DNA from dead *Vibrio*, the live *Vibrio* genes in rearing water and *Artemia* were also detected by PCR at exposure 48 hours in treatment groups 1 to 4.

The working concentration of PLE in treatment group 2 is 1 g/L. Based on Anirudhan et al.'s findings that 1-6 g/L PLE effectively inhibits *V. parahaemolyticus* and enhances *Litopenaeus vannamei* survival (Anirudhan et al., 2023), I initially

tested 1 g/L PLE, which demonstrated adequate *Vibrio* inhibition. As PLE was dissolved in DMSO, 1 g/L DMSO was added as treatment group 3 to verify whether DMSO affects *Vibrio*. To enhance economic benefits in practical applications, directly replacing PLE with freeze-dried powder of *P. tectorius* leaves can save costs. Therefore, treatment group 4 was designed, and the amount of freeze-dried powder was calculated as 7.14 g/L based on the extraction rate of PLE (14%). The calculation process is illustrated in the formula below. The working concentration of PLE (1 g/L) and leaf powder (7.14 g/L) mentioned above did not affect nauplii survival. A 50 µm sieve completely wrapped the freeze-dried powder, looked like a tea bag in appearance. At the same time as the exposure began, the “tea bag” was soaked in rearing water. It should be noted that PLE is a methanol extract of leaf, whereas direct use of leaf powder represents an aqueous extraction. Thus, when comparing their antibacterial effects, 1 g/L PLE does not equvalate to 7.14 g/L leaf powder in terms of bioactive compound content.

$$m_{leaves} = \frac{m_{PLE}}{Extraction\ rate} = \frac{1\ g}{14\%} = 7.14\ g$$

3.4.3 Experiment 3: Exposure experiment on adult *Artemia*

Adult *Artemia* was cultured in 1-ton seawater tanks. Due to the susceptibility of *Artemia* cultivated in seawater (around 30 g/L salinity) to diseases, a tank with good health of *Artemia* was selected for the experiment. The *Artemia* here should be uniform in size, exhibit normal swimming and mating behavior, and show no signs of mass mortality. The rearing water and *Artemia* were transferred together to 3 L aquariums. As the experimental design required *Vibrio* elimination within a short timeframe, no pre-exposure salinity adaptation was performed on adult *Artemia*. The inclusion of salinity acclimation would not only extend the process duration but also increase operational costs. Therefore, salt was added directly to the aquariums to adjust the different salinities ranging from 30, 100, 120 to 200 g/L. Exposed environment such as light, temperature, and aeration followed the daily climate of hatchery. No feeding was given during the exposure. The exposure experiments were conducted according to the following two different conditions:

- 1) Addition of salt
- 2) Addition of salt and freeze-dried powder of *P. tectorius* leaves (7.14 g/L)

In treatment groups 1 to 2, the live *Vibrio* genes in rearing water and adult *Artemia* were detected by PCR at 48 hours of exposure. Subsequently, in treatment group 2, three PCR products were selected for identification sequencing. The three PCR products were obtained from a rearing water sample (120 g/L salinity) and two *Artemia* samples (30 and 120 g/L salinities).

3.5 Detection of survival of *Artemia* and inhibition of bacteria

In Experiment 1, there was a small and fixed number of nauplii in the exposure treatments (in tubes). All of the live nauplii in each tube were counted directly with the naked eyes and the survival were calculated (illustrated in the formula below). However, in Experiment 2, there were a large number of uncountable nauplii in the exposure treatments (in bottles). In each bottle, 500 µL of uniformly mixed nauplii and rearing water were taken out together. All nauplii and surviving nauplii in these 500 µL were counted and the survival percentage was calculated. This step was repeated 3 times, and the average was recorded as the survival percentage of nauplii in one bottle.

$$\text{Survival percentage (\%)} = \frac{\text{Number of live nauplii}}{\text{Total number of nauplii}} \times 100\%$$

To detect the inhibition of bacteria or *Vibrio*, the rearing water samples were diluted in FASW. The dilution method is as follows (Herigstad, Hamilton, & Heersink, 2001): 100 µL of the rearing water sample was pipetted into a dilution tube containing 900 µL of FASW. This tube was vortexed completely. After vortexing, 100 µL of this volume was removed and placed into a second dilution tube containing 900 µL of FASW. This series dilution process was repeated exactly until there was sufficient diluting of the sample. The diluted samples were spread onto Marine agar plates or TCBS (Thiosulfate Citrate Bile Sucrose) agar (GranuCult® prime) plates (López-Torres & Lizárraga-Partida, 2001). The colony forming units

(CFU) on plates were counted and the inhibition percentage was calculated after 12-24 h of incubation at 28°C. The plates with colony counts of <30 CFU and >300 CFU were not counted, denoted by TFTC (too few to count) and TNTC (too numerous to count). In this study, the following two points need to be distinguished:

1) Detection of *Vibrio* inhibition percentage (%): The dilution factor of all samples was fixed at 1000 times (3 series dilutions), and 50 µL of the last diluted sample solution was added to TCBS agar plates.

2) Detection of bacterial or *Vibrio* density (CFU/mL): The dilution factor of the samples was not fixed, and each sample was diluted to the appropriate dilution factor to record the overall changes in bacterial or *Vibrio* count.

Fifty (50) nauplii or 10 adult of *Artemias* as an *Artemia* sample. The *Artemia* sample was placed in a sterile 1.5 mL microfuge tube, and the rearing water was removed using a pipette and rinsed once with FASW. The *Artemia* was crushed thoroughly in the 1.5 mL microfuge tube using a sterile plastic pestle. FASW was added to the 1 mL scale line, and the remaining *Artemia* tissue on the pestle was also washed and collected. At this point, the *Artemia* sample has been diluted 10 times (1 series dilution), and can be further diluted as mentioned earlier.

3.6 Polymerase chain reaction (PCR) for detection of *Vibrio* genus

3.6.1 Collection of metagenomic DNA from rearing water and DNA extraction

The rearing water was selected and filtered by a sterile sieve with 50 µm pore size to remove the *Artemia*. Each rearing water sample was accurately measured at 200 mL and centrifuged at 4°C, 3,2300×g for 20 min. All of the solid and cells was completely collected and used for metagenomic DNA extraction. The DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega Corporation, USA), according to the manufacturers' instructions. Finally, each DNA sample was dissolved in 50 µL double distilled water. The concentration of DNA samples was

detected by NanoPhotometer™ (N60, Implent™, Germany). In subsequent PCR amplification, all of the DNA samples were diluted to the same concentration (0.72 ng/μL) with double distilled water before being used as templates. At the same time, negative control (double distilled water as the DNA template) and positive control (*V. parahaemolyticus* genomic DNA as the DNA template) were established.

3.6.2 Collection of live bacterial DNA from rearing water or *Artemia*

In the “Detection of inhibition of bacteria and survival of *Artemia*” section, TCBS agar plates after colony counting were used for live bacterial DNA collection. 5 milliliters of FASW were added to the TCBS agar plates, scraped off with a flat spoon, and mixed with all the bacterial colonies. The bacterial suspension was centrifuged at 1,4000×g for 3 min. The bacterial cells obtained by rinsing with autoclaved distilled water and centrifuging again can be used for DNA extraction.

3.6.3 *Vibrio* genus PCR: primer and PCR conditions

A pair of specific primer for *Vibrio* genus was found from Kim et al.’s research (H. J. Kim, Ryu, Lee, Kim, & Kim, 2015). The pair of primer was designated as VG C2694352 F46 (5'- GTC ARA TTG AAA ARC ART TYG GTA AAG G -3') and VG C2694352 R734 (5'- ACY TTR ATR CGN GTT TCR TTR CC -3'), resulting in amplification of a 689-bp DNA fragment. Polymerase chain reaction (PCR) amplifications were carried out with 12.5 μL of MyTaq Mix, 2× (Bioline, UK), 0.72 ng/μL of template DNA, 0.6 μmol/L of primer and double distilled water in a final reaction volume of 25 μL. PCR amplification was performed in T100™ Thermal Cycler (621BR07105, Bio-Rad, Singapore) with an initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 30 s, finishing with a final extension at 72°C for 10 min and storage at 4°C after that. Amplified products and HyperLadder™ 1kb (Bioline, UK) (Figure 3.6.3) were electrophoresed on 1% agarose gel in 1× TBE buffer (Promega, USA), stained

with SYBR™ Safe DNA Gel Stain (Invitrogen, USA), photographed with Gel Imaging System Model: Omega Lum W (APLEGEN™ Gel Company, USA).

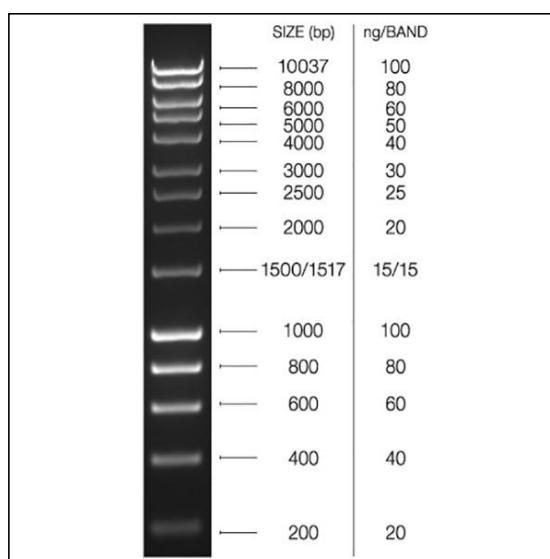


Figure 3.6.3 Standard bands of HyperLadder™ 1kb after electrophoresis.

3.7 Identification sequencing

Single bacterial colonies were selected randomly from TCBS agar plates and the strains were sequenced. Each strain was purified by streaking them two times on TCBS agar (López-Torres & Lizárraga-Partida, 2001) and axenically inoculated in Marine Broth by incubation overnight at 28°C. The strains were collected by centrifugation and genomic DNA was extracted.

The DNA samples of strains were sent to Apical Scientific (Selangor, Malaysia) for further DNA identification sequencing. The bacterial full-length 16S gene, ~1500 bp, was amplified using universal primers 27F and 1492R. The PCR products were purified by standard PCR clean-up method. The purified PCR products were subjected to bidirectional sequencing with universal primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

3.8 Overall experimental design

To clarify the research methodology, the following flowchart outlines the overall process.

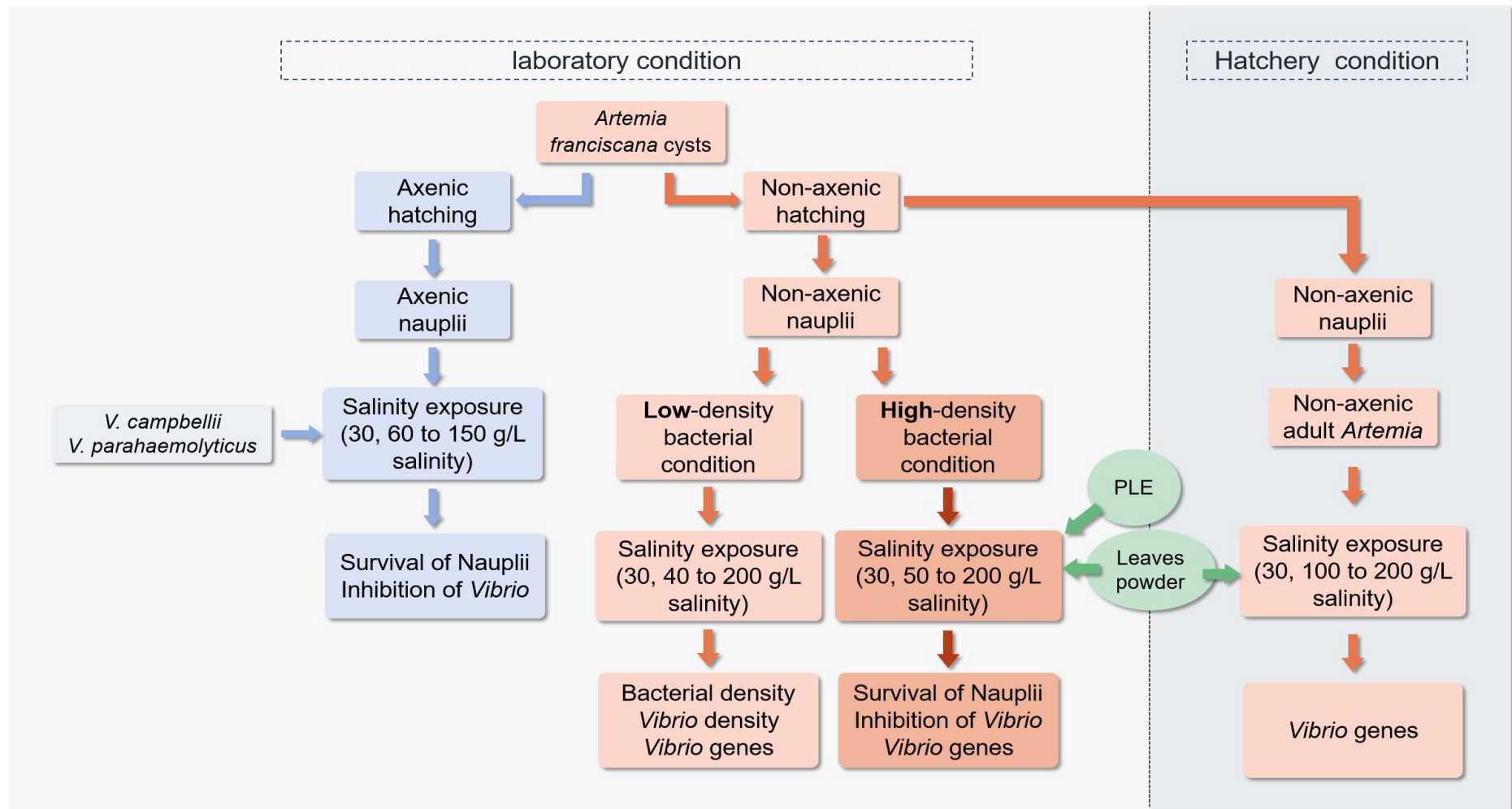


Figure 3.8 Flowchart of overall experimental design.

CHAPTER 4

RESULTS

4.1 Experiment 1: Exposure experiment on axenic *Artemia* nauplii

4.1.1 Impact of salinity exposure on the survival of nauplii

After 48 hours of exposure, in the *V. campbellii* exposure treatment, the survival of *Artemia* nauplii showed a regular change with increasing salinities. Both lower and higher salinities decreased the survival of nauplii (Figure 4.1.1 a). The highest survival were 93.3% and 98.9%, observed in treatments with initial *V. campbellii* concentrations of 10^7 and 10^8 cells/mL, corresponding to salinities of 80 and 90 g/L, respectively. In the *V. parahaemolyticus* exposure treatment, the survival of nauplii did not show a regular change with increasing salinities (Figure 4.1.1 b). Except for the control group (30 g/L salinity), the highest survival were 58.9% and 80.0%, observed in treatments with initial *V. parahaemolyticus* concentrations of 10^7 and 10^8 cells/mL, corresponding to salinities of 90 and 80 g/L, respectively. In the exposure treatment without *Vibrio*, the increase in salinity was inversely proportional to the survival of nauplii (Figure 4.1.1 c). It can be seen that higher salinity also negatively impacts the survival of nauplii. The increase in salinity resulted in higher mortality of *Artemia* naupli, and the lethal concentration at 50% (LC₅₀) was determined to be 113 g/L through nonlinear regression analysis. In addition, the error bars in the graph show that the standard deviation of the survival increases at higher salinities. This indicates that the survival of nauplii become unstable under higher salinity.

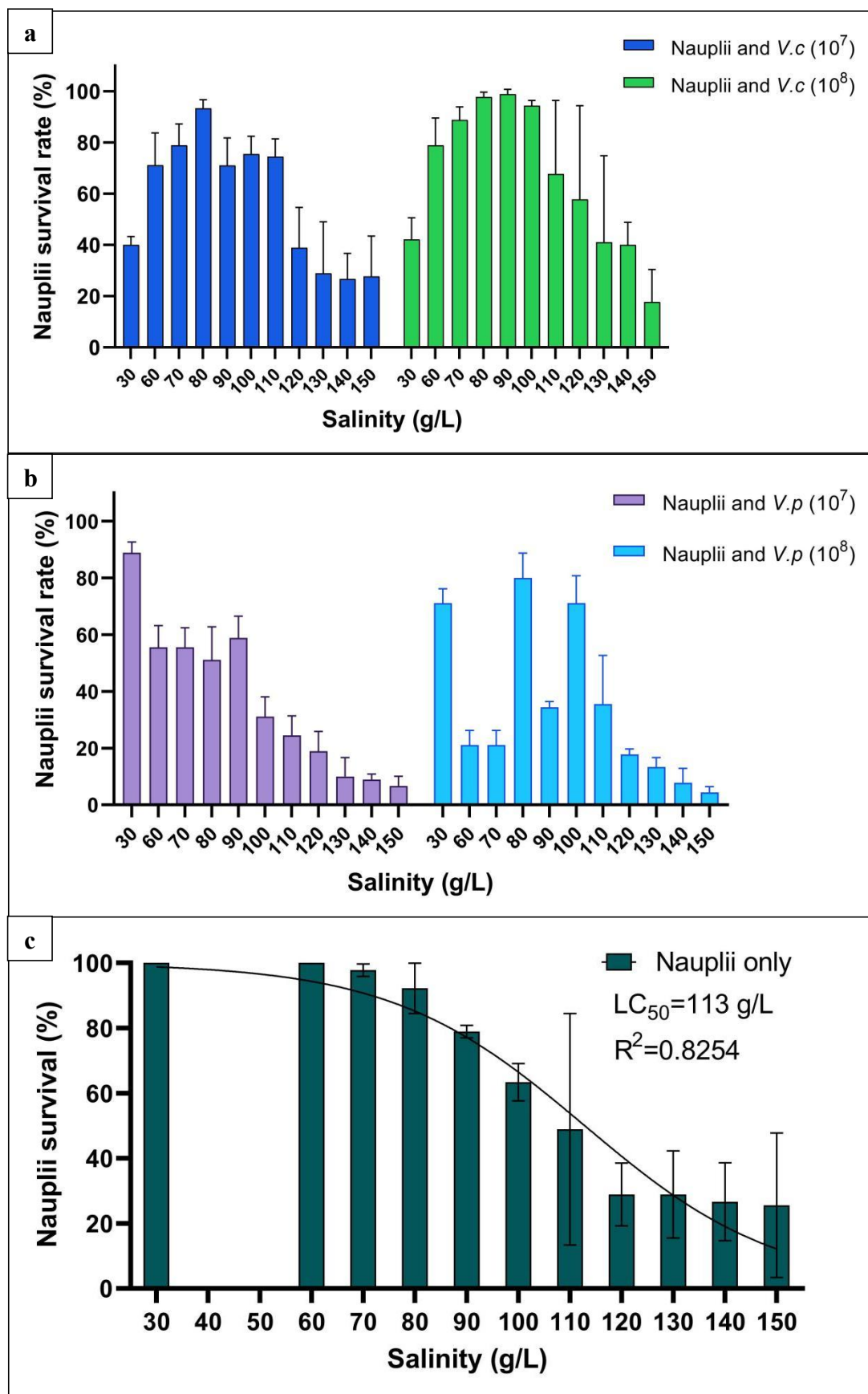


Figure 4.1.1 The survival of nauplii in each exposure treatment. **a)** Exposure with *V. campbellii*. **b)** Exposure with *V. parahaemolyticus*. **c)** Exposure without *Vibrio*.

4.1.2 Impact of salinity exposure on the inhibition percentage of *Vibrio*

The colonies were counted, the inhibition percentages of *Vibrio* were calculated using the initial concentration (10^7 and 10^8 cells/mL) as the control. Figure 4.1.2 (a and b) shows *Vibrio* was inhibited in environments with axenic nauplii and high salinity. In the *V. campbellii* exposure treatment, the inhibition percentages reached 100% at salinity 90 and 110 g/L, as observed in treatments with initial *V. campbellii* concentrations of 10^7 and 10^8 cells/mL (Figure 4.1.2 a). In the *V. parahaemolyticus* exposure treatment, the inhibition percentages reached 100% at salinity 80 and 120 g/L, observed in treatments with initial *V. parahaemolyticus* concentrations of 10^7 and 10^8 cells/mL (Figure 4.1.2 b). When the *V. parahaemolyticus* was exposed for 48 and 72 h in a condition without *Artemia*, both inhibition percentages reached 100% at salinity 70 g/L.

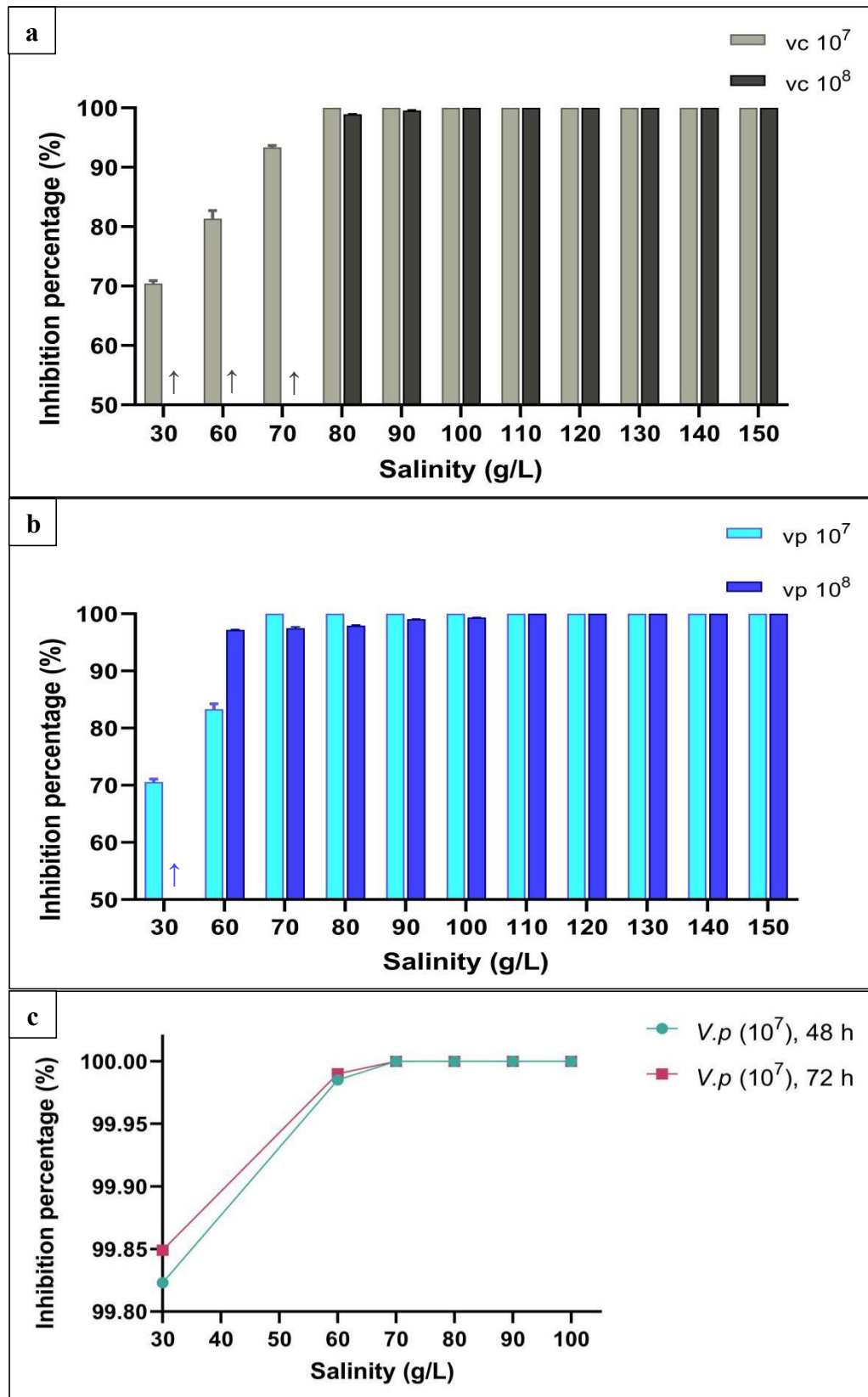


Figure 4.1.2 The inhibition percentage of *Vibrio* in each exposure treatment. **a)** Exposure with *V. campbellii*. **b)** Exposure with *V. parahaemolyticus*. **a)** and **b)** Some salinity groups in the figure do not show bars in the histogram because the corresponding colony counts in the treatment group were TNTC and could not be quantified. **c)** Exposure without *Artemia*.

4.1.3 Identification sequencing for verification

After overnight incubation, the rearing water formed colonies of the same size, shape, and green color on TCBS agar plates (Figure 4.1.3). To ensure the accuracy of the experiment, three green colonies were randomly selected for identification sequencing for reverse confirmation. Table 4.1.3 shows the molecular identification results of the 3 strains. The three strains were all *V. parahaemolyticus*, thus verifying the accuracy of the experiment in reverse.

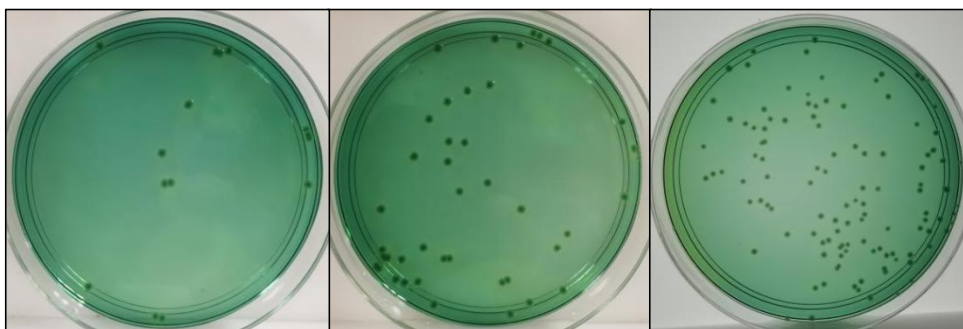


Figure 4.1.3 Green colonies grown on TCBS agar plates.

Table 4.1.3 Identification results for 3 randomly selected strains.

Sample no.	Bacterial identity	Similarity(%)
1	<i>Vibrio parahaemolyticus</i>	99.40
2	<i>Vibrio parahaemolyticus</i>	99.40
3	<i>Vibrio parahaemolyticus</i>	99.44

4.2 Experiment 2: Exposure experiment on non-axenic *Artemia* nauplii (low-density bacterial condition)

4.2.1 Impact of salinity exposure on the densities of bacteria and *Vibrio*

Within 48 hours after the exposure experiment started, the densities of bacteria and *Vibrio* were detected a total of five times (time points 0, 12, 24, 36 and

48 h) from rearing water. The colonies were counted from Marine agar and TCBS agar plates. The results from the marine agar plates showed changes in the density of all marine bacteria in the rearing water, while the TCBS agar plates showed the changes in the density of *Vibrio* in the same water. Figure 4.2.1 (a) shows the results from Marine agar, when salinity is more than 80 g/L, the overall number of bacteria decreases with increasing salinity. From 30 to 100 g/L salinity, the bacterial explosions happened at 24 hours in each salinity. However, from 110 to 200 g/L salinity, the bacterial explosions did not occur. All of these indicate that bacterial proliferation is effectively inhibited by higher salinity in this non-axenic exposure condition.

Figure 4.2.1 (b) shows the results from TCBS agar, all the colonies grown on plates had different sizes and shapes, but they were all yellow (Figure 4.2.1 c). When salinity was ≤ 150 g/L, vertical analysis of each bar in the bar graph indicated that *Vibrio* density first increased and then decreased from 0 to 48 h. The *Vibrio* density at 48 hours remained higher than at 0 hours. This indicates that the density of *Vibrio* was not inhibited by salinity when the salinity was ≤ 150 g/L. Horizontal analysis shows that the *Vibrio* were inhibited by high salinity above 150 g/L. Until the salinity reached 200 g/L, colonies still grew on the TCBS agar plates at various times. Salinity as high as 200 g/L still could not kill all the *Vibrio* within 48 h.

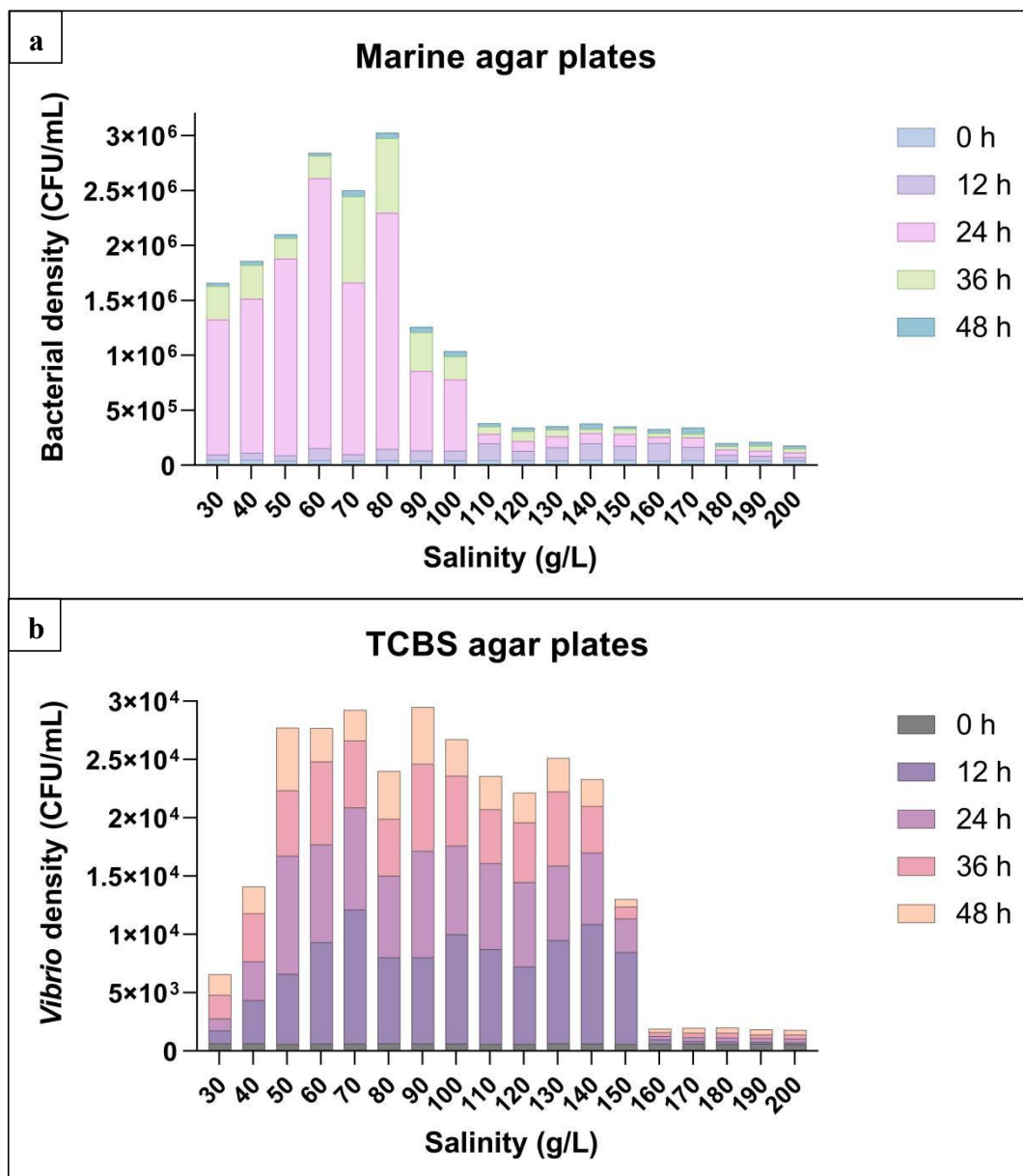


Figure 4.2.1 a) Density detection of bacteria by Marine agar plate. **b)** Density detection of *Vibrio* by TCBS agar plate.

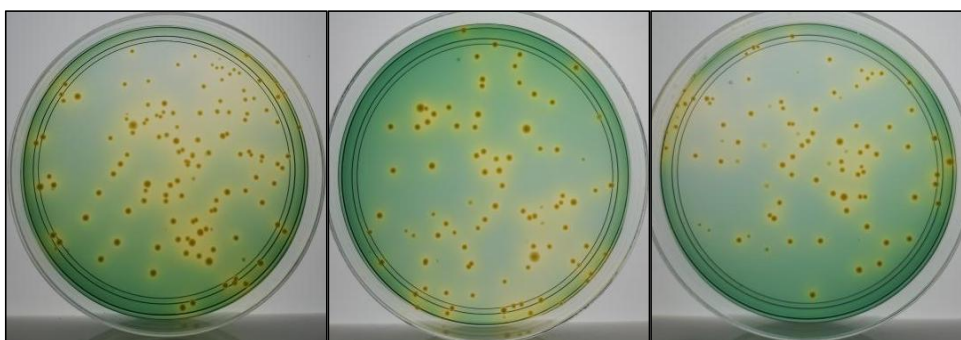


Figure 4.2.1 c Yellow colonies grown on TCBS agar plates.

4.2.2 PCR results for metagenomic DNA from rearing water

The metagenomic DNA from rearing water in 30, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150 g/L salinity treatment groups were detected (Figure 4.2.2). It is obvious that the three replication of the control group (30 g/L) all showed relatively bright 689bp sized bands. Therefore, it is proven that *Vibrio* was indeed introduced into the hatching process and subsequently into the rearing water. Since the concentration of all DNA templates was made uniform before PCR detection the brightness of the bands can reflect the differences in the concentration of *Vibrio* genes, thereby reflecting the differences in *Vibrio* density. When the salinity is greater than 80 g/L, the brightness of the bands decreases significantly. When the salinity reached 140 g/L or higher, the bands completely disappeared. This indicates that *Vibrio* was inhibited between salinity 90 and 130 g/L, and the inhibition was more significant at salinity 140 g/L or higher.

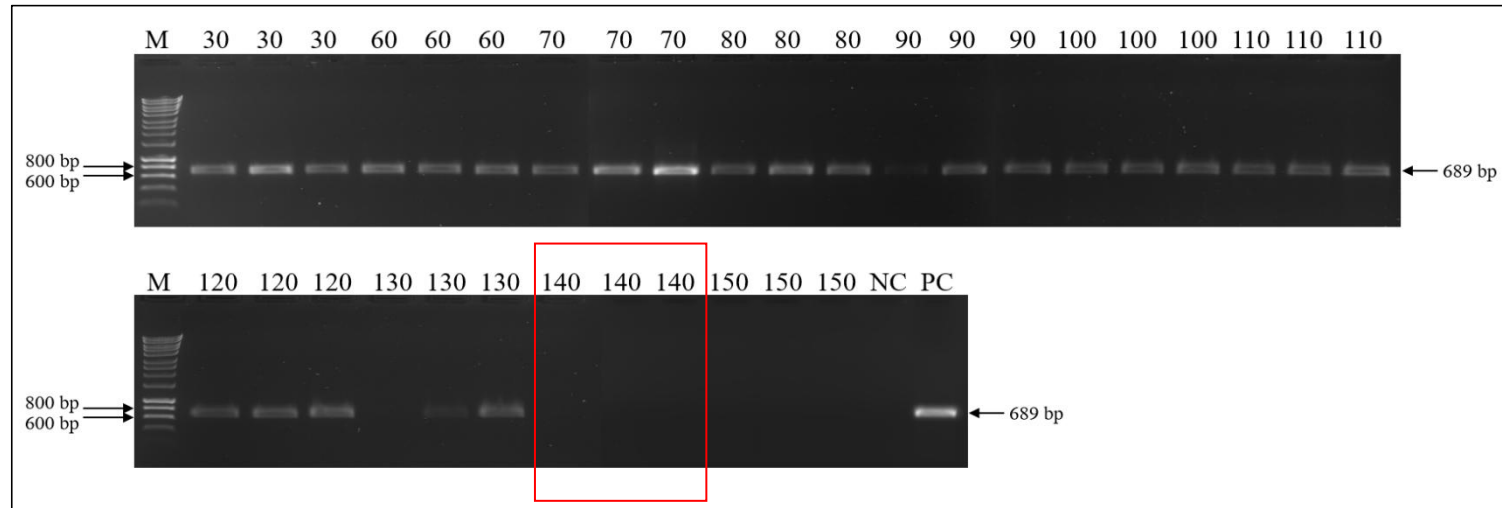


Figure 4.2.2 *Vibrio* genus PCR results of metagenomic DNA. Lane M: 1000-bp DNA ladder. Lane 30, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150: salinity treatments 30 g/L, 60 g/L, 70 g/L, 80 g/L, 90 g/L, 100 g/L, 110 g/L, 120 g/L, 130 g/L, 140 g/L and 150 g/L. Lane NC: negative control. Lane PC: positive control.

4.2.3 Identification sequencing

There were no bands when salinity ≥ 140 g/L (Figure 4.2.2), but colonies were still counted on TCBS agar plates (Figure 4.2.1 b). It was once suspected that TCBS agar did not specifically supply the growth of *Vibrio*. To verify the speculation, twelve yellow colonies were randomly selected from 30, 70, 100 and 120 g/L salinity treatments for identification sequencing. Table 4.2.3 (a) shows the molecular identification results of the 12 strains, they were all *Exiguobacterium spp.* This indicates that the yellow colonies on TCBS agar plates were most of *Exiguobacterium spp.* However, the proportion of *Vibrio* was too low, to the extent that there were no *Vibrio* in these 12 randomly selected colonies. Now it is confirmed, that TCBS agar was not specifically supplied for the growth of *Vibrio*, and *Exiguobacterium spp.* is one of the exceptions. In addition, the genomic DNA of *Exiguobacterium spp.* was subjected to *Vibrio* genus PCR and no 689-bp band was obtained (Figure 4.2.3). This proves that the *Exiguobacterium* DNA does not cause false-positive results in *Vibrio* genus PCR, and also proves the specificity of this pair of primers.

Table 4.2.3 a) Identification results for 12 randomly selected strains.

Sample no.	Salinity (g/L)	Bacterial identity	Similarity(%)
1	30	<i>Exiguobacterium arabatum</i>	100.00
2	30	<i>Exiguobacterium arabatum</i>	100.00
3	30	<i>Exiguobacterium arabatum</i>	100.00
4	70	<i>Exiguobacterium arabatum</i>	100.00
5	70	<i>Exiguobacterium arabatum</i>	100.00
6	70	<i>Exiguobacterium arabatum</i>	100.00
7	100	<i>Exiguobacterium arabatum</i>	100.00
8	100	<i>Exiguobacterium arabatum</i>	99.93
9	100	<i>Exiguobacterium arabatum</i>	100.00
10	120	<i>Exiguobacterium arabatum</i>	99.71
11	120	<i>Exiguobacterium aestuarii</i>	99.71
12	120	<i>Exiguobacterium arabatum</i>	100.00

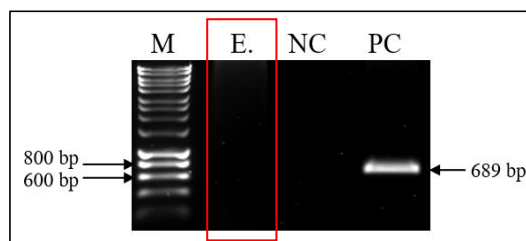


Figure 4.2.3 PCR detection for *Exiguobacterium spp.* by *Vibrio* genus primer. Lane M: 1000-bp DNA ladder. Lane E.: *Exiguobacterium spp.* Lane NC: negative control. Lane PC: positive control.

Due to the sequencing results before (Table 4.2.3 a), there was no *Vibrio* in rearing water at low-density bacterial conditions. Subsequently, exposure experiments with high-density bacterial conditions were designed. Before the exposure experiment began, the hatching water was diluted and spread onto TCBS agar plates. After overnight incubation, 14 colonies were randomly selected for identification sequencing. The sequencing results in Table 4.2.3 (b) show that all 14 randomly selected colonies are *Vibrio*, indicating that there are indeed many live *Vibrio* in the hatching water.

Table 4.2.3 b) Identification results for 14 randomly selected strains from hatching water.

Sample no.	Salinity (g/L)	Bacterial identity
1	30	<i>Vibrio mediterranei</i>
2	30	<i>Vibrio tubiashii</i>
3	30	<i>Vibrio mediterranei</i>
4	30	<i>Vibrio mediterranei</i>
5	30	<i>Vibrio mediterranei</i>
6	30	<i>Vibrio mediterranei</i>
7	30	<i>Vibrio mediterranei</i>
8	30	<i>Vibrio mediterranei</i>
9	30	<i>Vibrio mediterranei</i>
10	30	<i>Vibrio paucivorans</i>
11	30	<i>Vibrio paucivorans</i>
12	30	<i>Vibrio mediterranei</i>
13	30	<i>Vibrio mediterranei</i>
14	30	<i>Vibrio mediterranei</i>

4.3 Experiment 2: Exposure experiment on non-axenic *Artemia* nauplii (high-density bacterial condition)

4.3.1 Impact of salinity exposure on the survival of nauplii

In high-density bacterial conditions, the survival of nauplii was detected at 12, 24, and 48 hours with salinities ranging from 30, 50 to 200 g/L. Figure 4.3.1 shows that after 48 hours of exposure, the treatment of 80 g/L salinity resulted in the highest survival percentage of nauplii, reaching 92.4%. In addition, excessive salinity reduced the survival of nauplii. But strangely, the survival percentage of nauplii in all salinity treatment groups was greater than 50%, slightly different from the results in Figure 4.1.1.

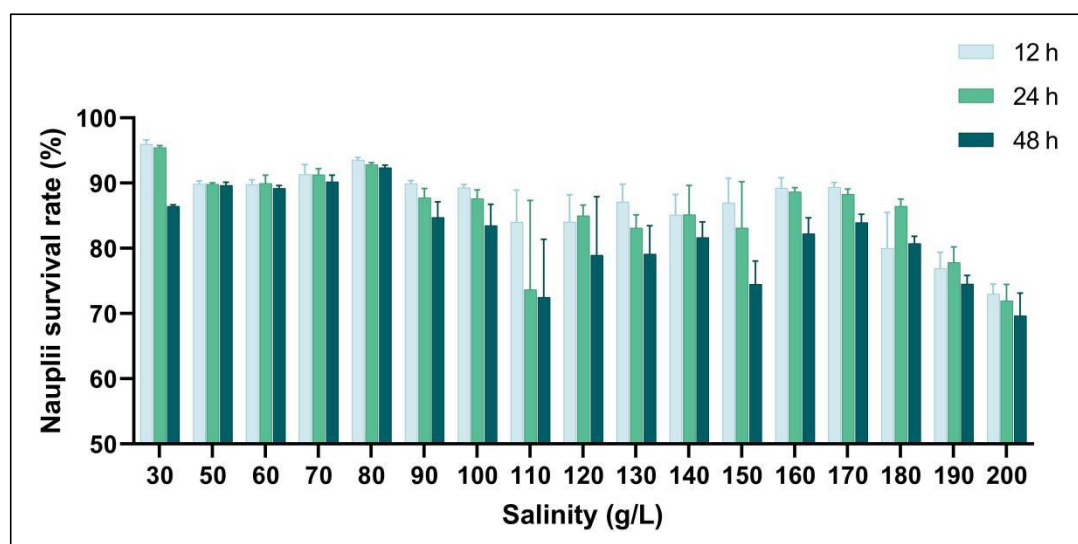


Figure 4.3.1 Survival of non-axenic nauplii in high-density bacterial condition.

4.3.2 Impact of salinity exposure on the inhibition percentage of bacteria

Due to the fixed dilution factor, some TCBS agar plates may have colony counts greater than 300 CFU (TNTC). The inhibition percentage was calculated after fixing all TNTC to 300 CFU. When calculating the inhibition percentage, use the colony count from the 30 g/L salinity treatment at each time point as the control. As shown in Figure 4.3.2, at the same salinity (30 to 100 g/L salinity treatment), the

bacterial inhibition percentage decreases over time. This indicates that bacteria reproduce fast in those salinity conditions. In those salinity conditions, a large difference in inhibition percentage between adjacent time points, and a weaker inhibitory effect on bacterial growth.

After 48 h exposure experiment, the inhibition percentage of bacteria in rearing water tended to stabilize and reached a high level (>95.0%) at 110 to 200 g/L (Figure 4.3.2 a). The highest inhibition percentage at 48 hours was 98.8%, corresponding to salinities of 160 and 170 g/L. As shown in Figure 4.3.2 (b), the inhibition percentage of bacteria in nauplii tended to stabilize and reached a high level (>95.0%) at 120 to 160 g/L. The highest inhibition percentage at 48 hours was 97.6%, corresponding to 140 g/L salinity.

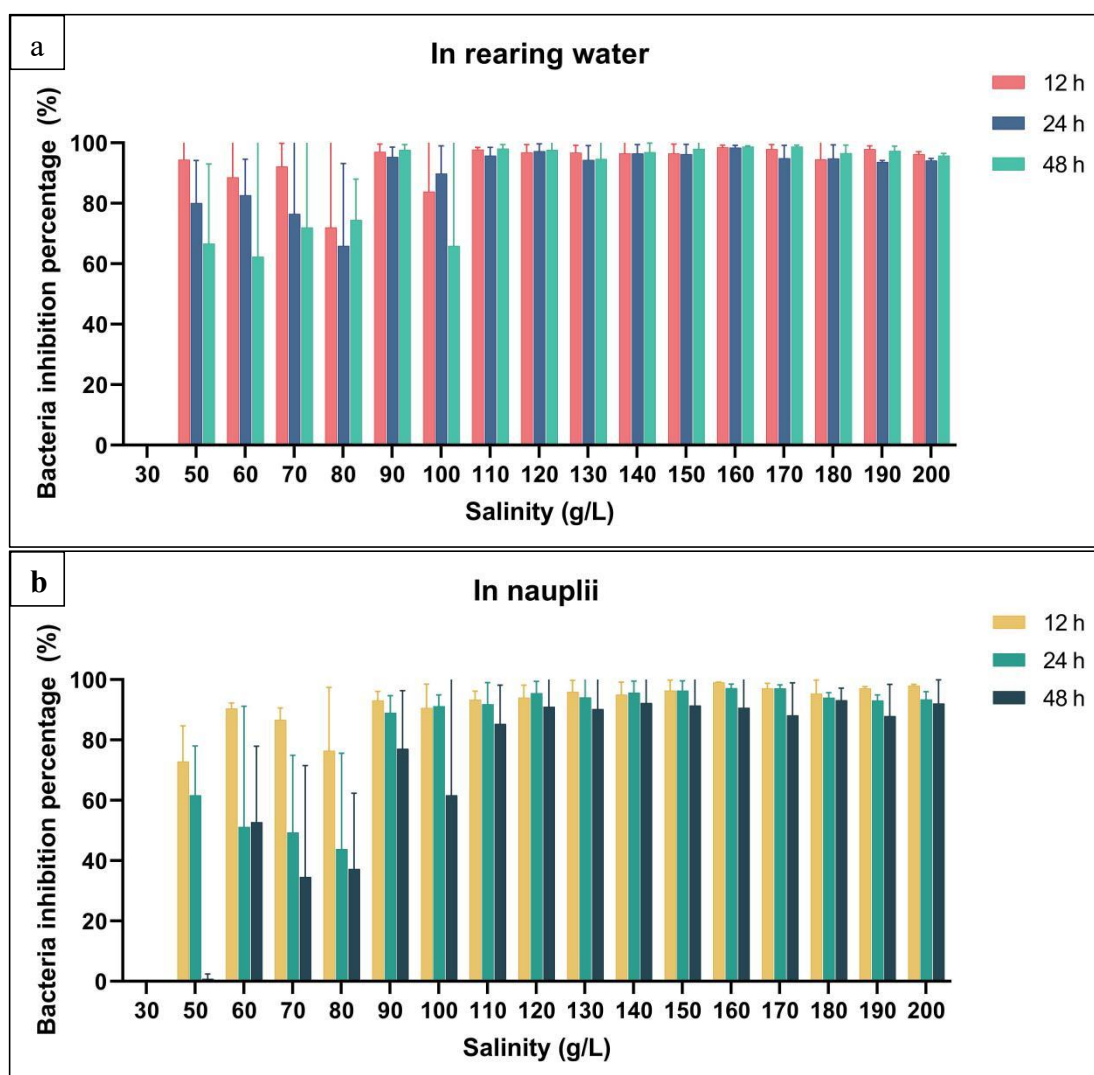


Figure 4.3.2 The inhibition percentage of bacteria from salinity exposure. **a)** From rearing water samples. **b)** From nauplii samples.

4.3.3 PCR results for metagenomic DNA and live bacterial DNA from salinity exposure treatment

After 48 hours of salinity exposure, the *Vibrio* genes was detected by PCR at salinity ranging from 30, 50, 60 to 200 g/L. As shown in Figure 4.3.3, the bands weaken and almost disappear with increasing salinity. This indicates that in the high-density bacterial condition, high salinity conditions effectively inhibited *Vibrio*. However, the PCR results of rearing water samples (Figure 4.3.3 a and b) can show that even at a salinity of up to 200 g/L, *Vibrio* was still able to survive in rearing water. However, in nauplii samples, there were no bands at 200 g/L salinity (Figure 4.3.3 c). This indicates that all *Vibrio* inside the nauplii's body were eliminated at the salinity exposure of 200 g/L for 48 hours. Compared to the results in Figure 4.1.1, the survival percentage of nauplii is less than 50% at 150 g/L salinity. If the salinity reached 200 g/L, the survival percentage of nauplii will be even lower. In addition, use a large amount of salt will increase the cost in the production process of *Vibrio*-free nauplii. Therefore, PLE was introduced in the following experiments.

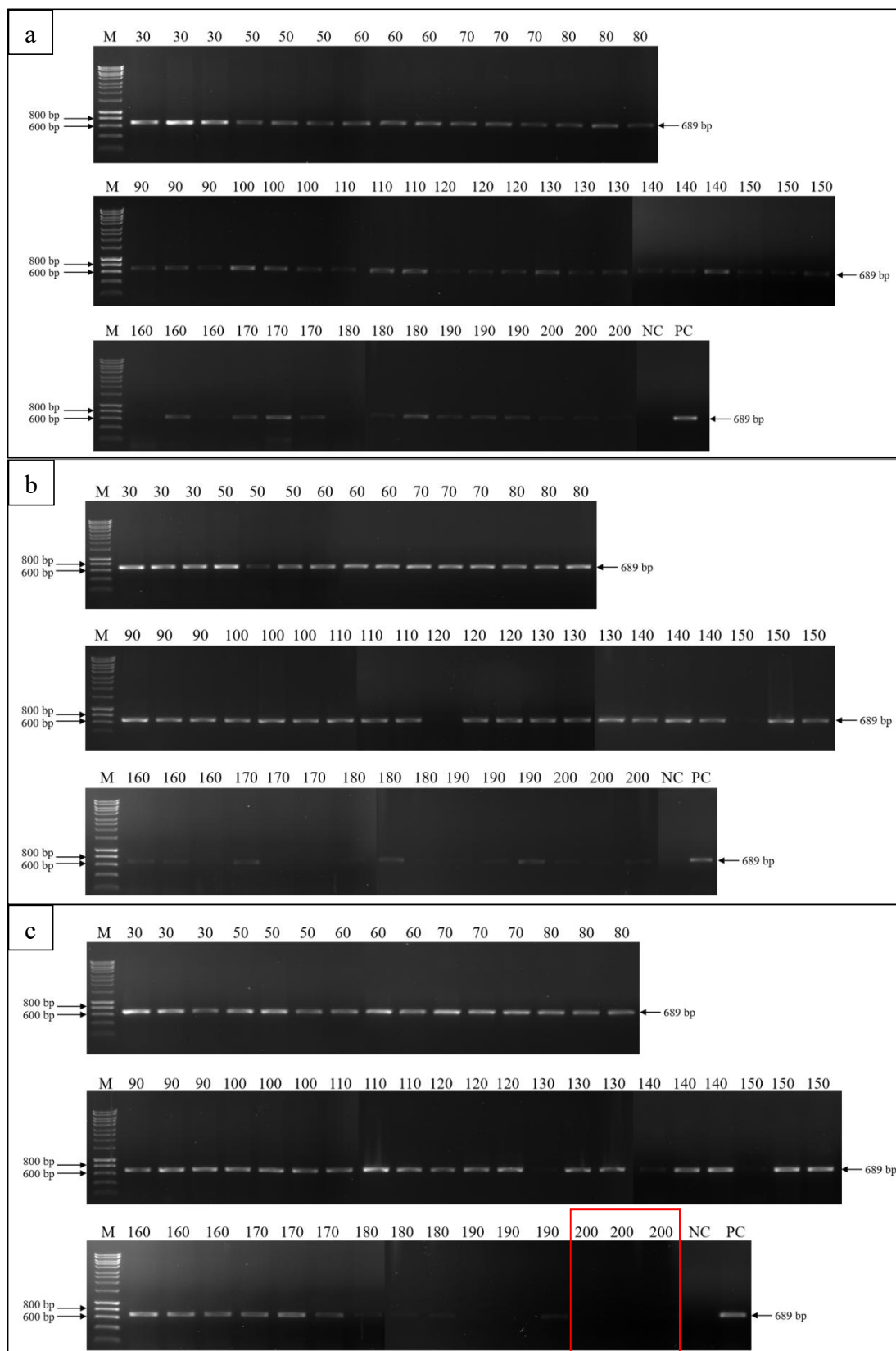


Figure 4.3.3 *Vibrio* genus PCR results from salinity exposure treatments. Lane M: 1000-bp DNA ladder. Lane 30, 50, 60 to 200: salinity exposure treatments, the salinities ranging from 30, 50, 60 to 200 g/L. Lane NC: negative control. Lane PC: positive control. **a)** Metagenomic DNA samples in rearing water. **b)** Live bacterial DNA samples in rearing water. **c)** Live bacterial DNA samples in nauplii.

4.3.4 Impact of salinity and PLE exposure on the inhibition percentage of bacteria

Nauplii were exposed to the condition that combines salinity and PLE. The salinities ranged from 30, 50, 60 to 100 g/L. As shown in Figure 4.3.4 (a and b), the effect of inhibition reached its peak at 48 hours. Among them, in rearing water, the highest inhibition percentage of bacteria reached 99.2% at 90 g/L salinity (Figure 4.3.4 a). In nauplii, the highest inhibition percentage of bacteria reached 98.6% at 90 g/L salinity (Figure 4.3.4 b).

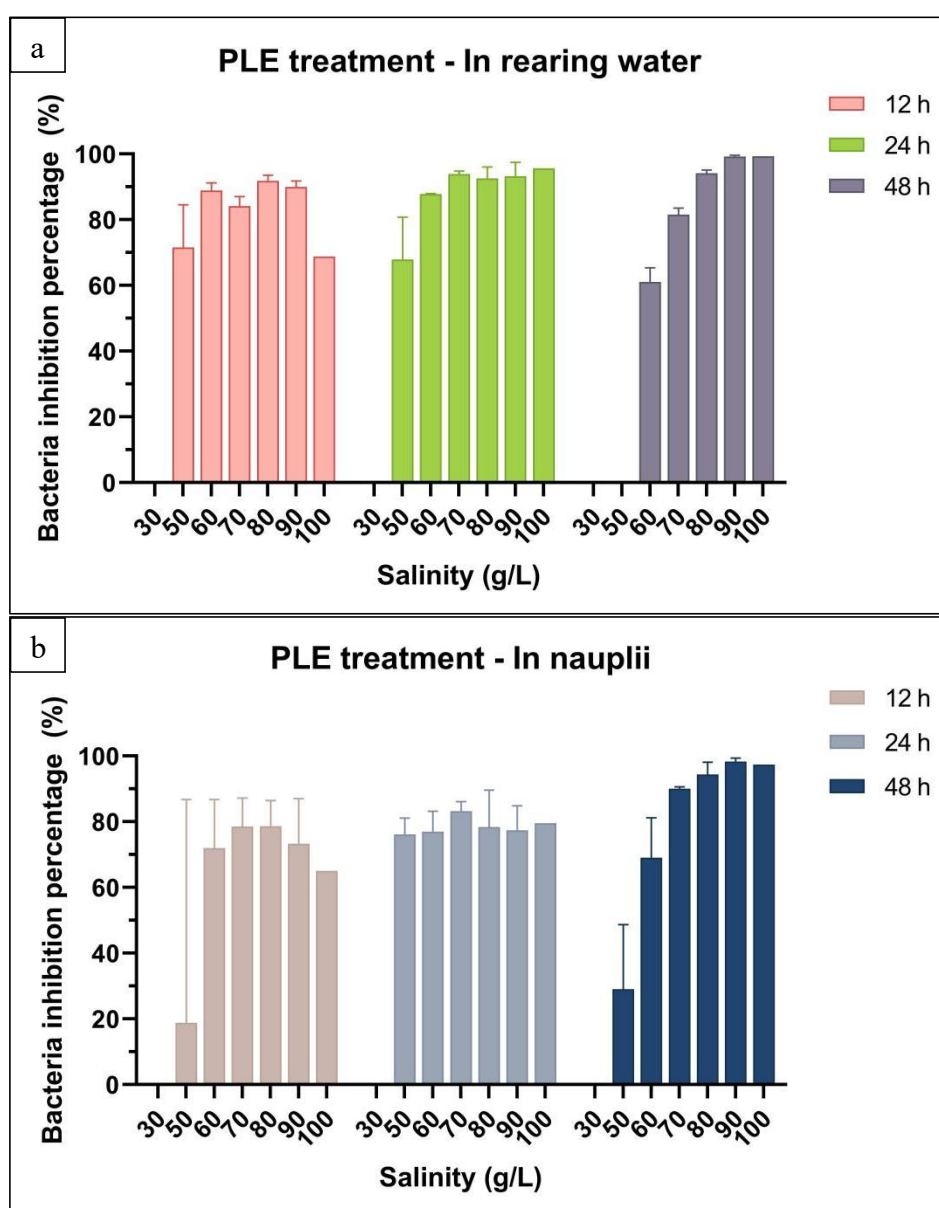


Figure 4.3.4 a) The inhibition percentage of bacteria in rearing water from salinity and PLE exposure. **b)** The inhibition percentage of bacteria in nauplii from salinity and PLE exposure.

4.3.5 PCR results for live bacterial DNA from salinity and PLE exposure treatment

After 48 hours of salinity exposure combined with PLE, the *Vibrio* genes were detected by PCR at salinity ranging from 30, 50, 60 to 100 g/L. The weak bands are shown in Figure 4.3.5 (a and b). Compared with Figure 4.3.3 (b and c), even at a salinity of 30 g/L, there are weaker bands in Figure 4.3.5 (a and b), which indicates that 1 g/L of PLE has a very effective ability to inhibit *Vibrio*. Surprisingly, no bands were observed in the rearing water samples and nauplii samples at 60 and 70 g/L salinity, respectively. This means that in the condition of 1 g/L PLE, the salinity condition only requires 70 g/L to achieve *Vibrio*-free rearing water and *Vibrio*-free nauplii. To verify that DMSO cannot inhibit *Vibrio*, 1 g/L DMSO was added instead of PLE to salinity treatments. The salinities ranged from 30, 50, 60 to 80 g/L. In Figure 4.3.5 (c and d), bright bands were observed for all salinity treatments. That is to say, each salinity treatment added with DMSO had live *Vibrio* present in the rearing water and nauplii. Therefore, it has been proven that the effective ingredient for eliminating *Vibrio* is PLE rather than DMSO.

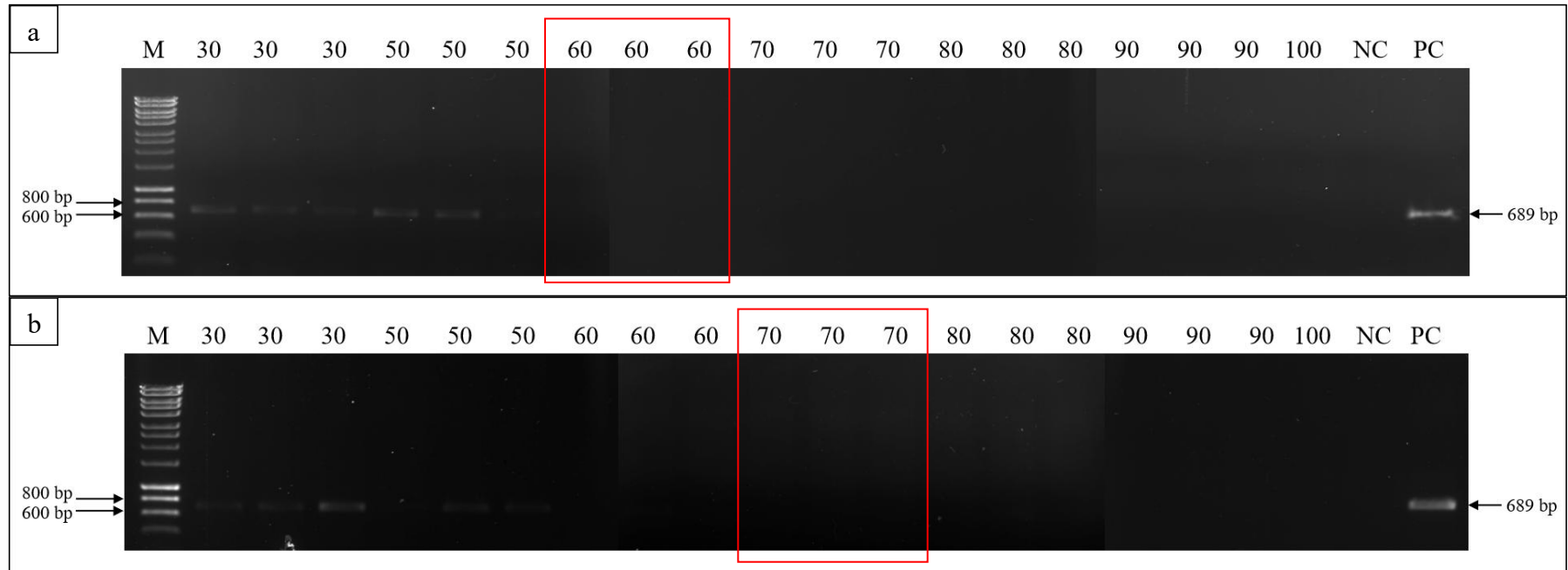


Figure 4.3.5 *Vibrio* genus PCR results from salinity combined with PLE exposure treatments. Lane M: 1000-bp DNA ladder. Lane 30, 50, 60 to 100: salinity exposure treatments, the salinities ranging from 30, 50, 60 to 100 g/L. Lane NC: negative control. Lane PC: positive control. **a)** Live bacterial DNA samples in rearing water. **b)** Live bacterial DNA samples in nauplii.

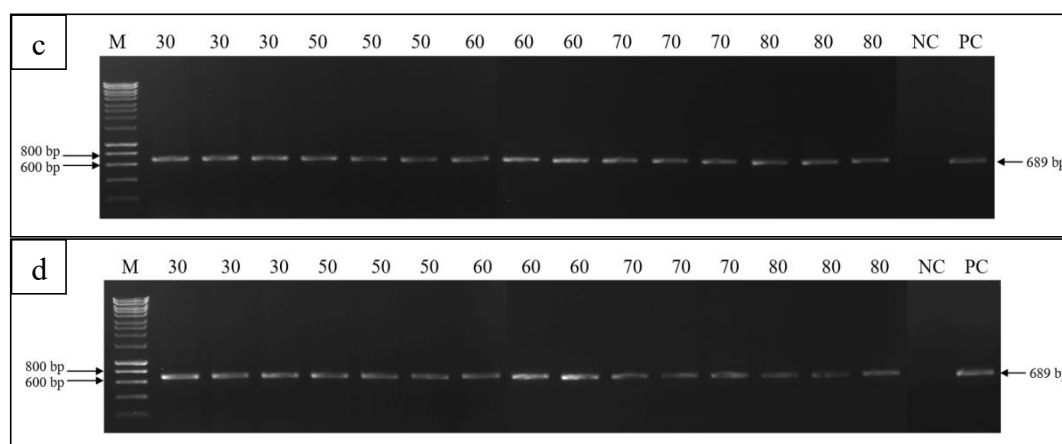


Figure 4.3.5 *Vibrio* genus PCR results from DMSO treatments. Lane M: 1000-bp DNA ladder. Lane 30, 50, 60 to 80: salinity exposure treatments, the salinities ranging from 30, 50, 60 to 80 g/L. Lane NC: negative control. Lane PC: positive control. **c)** Live bacterial DNA samples in rearing water. **d)** Live bacterial DNA samples in nauplii.

4.3.6 PCR results for live bacterial DNA from salinity and *P. tectorius* leaves exposure treatment

To reduce the production cost, 1 g/L PLE was replaced by 7.14 g/L freeze-dried leaf powder. Figure 4.3.6 (a and b) shows that there are no visible bands in both of rearing water samples and the nauplii samples at 80 g/L salinity. This means that 7.14 g/L freeze-dried leaf powder combined with 80 g/L salinity exposure for 48 hours to achieve *Vibrio*-free rearing water and *Vibrio*-free nauplii. Due to the incomplete dissolution of active ingredients in *P. tectorius* leaves in rearing water, the salinity in this context is higher than that under PLE conditions (70 g/L). Subsequently, exposure experiments were conducted using 80 g/L combined with 7.14 g/L of leaf powder in a hatchery environment, *Vibrio*-free rearing water and *Vibrio*-free nauplii were also obtained (Figure 4.3.6 c and d).

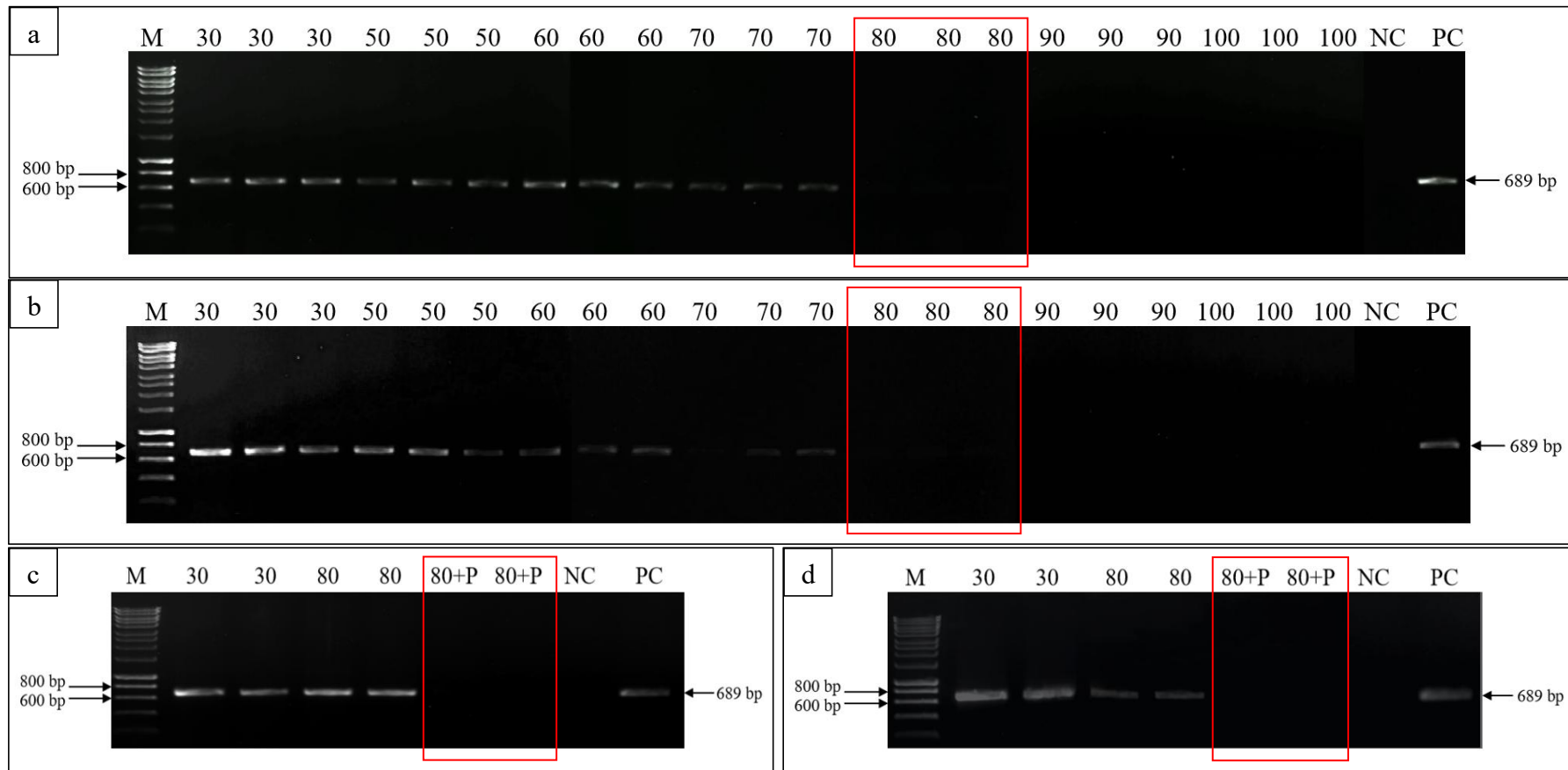


Figure 4.3.6 *Vibrio* genus PCR results from *P. tectorius* leaves treatments. Lane M: 1000-bp DNA ladder. Lane 30, 50, 60 to 100: salinity exposure treatments, the salinities ranging from 30, 50, 60 to 100 g/L. Lane 80+P: 80 g/L salinity + *P. tectorius* leaves. Lane NC: negative control. Lane PC: positive control. **a)** Live bacterial DNA samples in rearing water, in laboratory environment. **b)** Live bacterial DNA samples in nauplii, in laboratory environment. **c)** Live bacterial DNA samples in rearing water, in hatchery environment. **d)** Live bacterial DNA samples in nauplii, in hatchery environment.

4.4 Experiment 3: Exposure experiment on adult *Artemia*

Exposure of adult *Artemia* using a combination of 80 g/L salinity combined with 7.14 g/L of *P. tectorius* leaves could not eliminate *Vibrio* completely in hatchery condition. Therefore, adult *Artemia* were exposed to higher salinity and combined with *P. tectorius* leaves. Firstly, the adult *Artemia* were exposed in salinity conditions for 48 hours, with the salinities ranging from 30, 100, 120, and 200 g/L. After 24 hours of exposure, all adult *Artemia* died at salinities of 160, 180, and 200 g/L. After 48 hours of exposure, adult *Artemia* are all survived at salinities of 30, 100 and 120 g/L. PCR detected live *Vibrio* in rearing water and found bands for each salinity level (Figure 4.4 a). Next, adult *Artemia* were exposed to conditions of 30, 100, and 120 g/L salinity combined with *P. tectorius* leaves. After 24 hours of exposure, the rearing water became turbid and *Artemia* died at 30 g/L salinity, while the state of *Artemia* at other salinities remained normal. After 48 hours of exposure, *Artemia* survived at only 100 g/L salinity. PCR detected live *Vibrio* in rearing water and still found bands for each salinity level (Figure 4.4 b). Finally, it was proven that the method of combining salinity with *P. tectorius* leaves cannot achieve *Vibrio*-free adult *Artemia*.

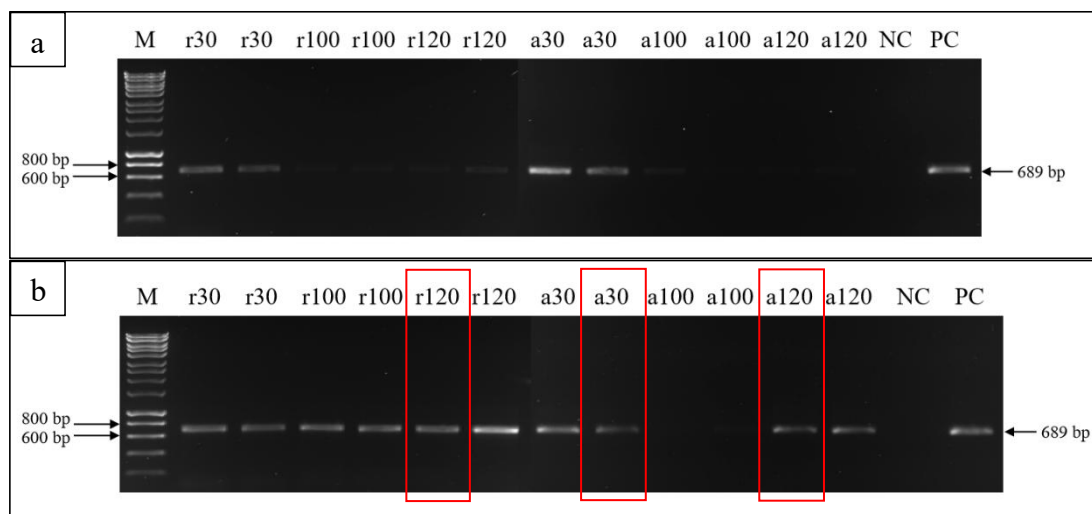


Figure 4.4 *Vibrio* genus PCR results from *P. tectorius* leaves treatments. Lane M: 1000-bp DNA ladder. Lane r30, r100 and r120: rearing water samples from salinity exposure treatments, the salinities ranging from 30, 100 and 120 g/L. Lane a30, a100 and a120: adult *Artemia* samples from salinity exposure treatments, the salinities ranging from 30, 100 and 120 g/L. Lane NC: negative control. Lane PC: positive

control. **a)** High salinity exposure treatments only. **b)** High salinity combined *P. tectorius* leaves exposure treatments.

Three *Vibrio* genus PCR products from above Figure 4.4 (b) were selected for identification sequencing. The results showed that these amplified DNA fragments (689-bp) were indeed derived from *Vibrio spp.*(Table 4.4). prove again the specificity and reliability of this pair of primers for *Vibrio* genus PCR.

Table 4.4 Identification results for 3 PCR products.

Sample name	Bacterial identity	Similarity(%)
W120	<i>Vibrio alginolyticus</i>	98.59
A30	<i>Vibrio harveyi</i>	97.82
A120	<i>Vibrio rotiferianus</i>	97.20

CHAPTER 5

DISCUSSION

5.1 *Artemia* prepared for exposure experiments

Time control is crucial throughout the entire *Artemia* preparation process. 28 hours are required from the beginning of hatching to just before the exposure begins, at which time the *Artemia* larvae reach instar II. If the hatching time is shorter than 28 hours, *Vibrio* will not be taken in nauplii during the early stage (instar I) of exposure, which is equivalent to shortening the time for *Vibrio* challenge. If the hatching time is exceeded, low-density bacteria will be unable to meet the feeding needs of nauplii, resulting in a reduced survival after 48 hours of exposure. Additionally, during the experiment, it was found that the exposure time exceeding 48 hours could lead to the death of nauplii due to lack of food. Therefore, in this study, the cysts hatching must stop 28 hours after beginning, then the salinity exposure begins and lasts for 48 hours.

In order to reduce the influence of other factors on the experiment, adult *Artemia* must be hatched and cultured at standard salinity (30 g/L). However, there are more harmful bacteria in the rearing water at this salinity, which greatly reduces the survival of *Artemia*. Therefore, more *Artemia* should be cultured and separated into more tanks. To ensure sufficient experimental materials for subsequent exposure experiments. Select adult *Artemia* from the tanks with high survival percentage and active states for subsequent exposure experiments.

5.2 Inhibition percentage of bacteria

High salinity inhibited the *Vibrio* growth rate but increased the viability and bacterial membrane integrity (L. H. Kim & Chong, 2017). Figure 4.1.2 indicates that high salinity can indeed inhibit the growth of *Vibrio*, which is consistent with the research conclusion of Kim et al. In the exposure with *V. parahaemolyticus*, initiated with a concentration of 10^7 cells/mL, the treatment without nauplii (Figure 4.1.2 c) showed a lower salinity threshold (70 g/L) required for achieving 100% inhibition of *Vibrio*. Compared to the treatment *Vibrio* with nauplii (Figure 4.1.2 b), a higher salinity threshold (80 g/L) is required for achieving 100% inhibition of *Vibrio*. The reason for these results is that higher salinity increases the activity of *Vibrio* and makes the cell membrane more robust. In addition, when *Vibrio* was ingested by nauplii, it was also indirectly protected by nauplii.

Compared to nauplii, adult *Artemia* has a longer gut, can accommodate more *Vibrio*, and have a longer residence time for *Vibrio*. Therefore, adult *Artemia* protects *Vibrio* for a longer period. Thus, *Vibrio* inside adult *Artemia* is more difficult to eliminate than *Vibrio* inside nauplii. This viewpoint can be proven by comparing Figure 4.3.6 (c and d) and Figure 4.4 (a and b). In the condition of salinity combined with *P. tectorius* leaves, only 80 g/L salinity was required to achieve *Vibrio*-free nauplii, but 120 g/L salinity cannot achieve *Vibrio*-free adult *Artemia*. Without salt tolerance acclimation, adult *Artemia* died when salinity exceeded 120 g/L due to their inability to adapt to the sudden salinity change. This was a limitation in this study. In future research, methods need to be found to attain *Vibrio*-free adult *Artemia*. A reliable idea is proposed here. In future research, it can be attempted to culture *Artemia* with a salinity of 80 g/L until adulthood. This can greatly reduce the load of *Vibrio* on *Artemia*. Subsequently, use the method of combining salinity (120 g/L) with *P. tectorius* leaves (7.14 g/L) to achieve *Vibrio*-free adult *Artemia*.

5.3 Factors affecting the survival of *Artemia*

While inhibiting the growth of *Vibrio*, it is more important to consider the survival of *Artemia*. Under standard salinity conditions (30 g/L), *Artemia* is

susceptible to the pathogenic effects of *Vibrio*, resulting in a reduction in their survival. However, the salinity exposure results of axenic nauplii without *Vibrio* (Figure 4.1.1 c) indicates that the survival of *Artemia* is not only affected by the pathogenic *Vibrio*, but also by salinity. The higher salinity reduces the survival of *Artemia* (Dana & Lenz, 1986). Additionally, from the error bars can be seen that the state of nauplii itself is very unstable in higher salinity conditions. It is speculated that this is due to the sudden transfer of nauplii hatched at normal salinity to a high salinity environment, resulting in drastic changes in salinity. And this drastic change in salinity was even more severe for adult *Artemia*. Adult *Artemia* died within 48 hours of exposure to 140 g/L salinity condition. Joseph et al. also made similar findings in their research. Acute changes in salinity cause physiological reactions, affecting the metabolic performance and immune ability of shrimp against *Vibrio*, thereby increasing susceptibility to infection, moreover higher salinity enhances the pathogenicity of *Vibrio* (Joseph & Philip, 2020).

When the salinity is 80 g/L, both the nauplii challenged with *V. campbellii* and *V. parahaemolyticus* can achieve higher survival (Figure 4.1.1 a and b). The same results were obtained in the exposure experiment of non-axenic nauplii (Figure 4.3.1). Selecting this specific salinity level of 80 g/L can optimize the survival of nauplii in open aquaculture systems, even in the presence of potential *Vibrio* infections. Additionally, at this salinity level, the survival of nauplii in the presence of *Vibrio* at a concentration of 10^8 cells/mL is higher compared to that of *Vibrio* at a concentration of 10^7 cells/mL. It is speculated that this phenomenon occurs because *Artemia* exhibit continuous and non-selective filter-feeding behavior, and higher concentrations of *Vibrio* create abundant food sources for them, thus supporting their survival. Comparing Figure 4.1.1 and Figure 4.3.1, the same phenomenon was also observed, confirming this speculation. The survival percentage of the latter in all salinity treatments was greater than 50%, higher than that of the former.

5.4 Two times improvements in the detection method of *Vibrio*

The salinity exposure of non-axenic nauplii simulated the breeding environment under non-experimental conditions. Figure 4.2.1 (b) indicates that only when the salinity exceeds 150 g/L can the bacteria introduced through open hatching be inhibited, and complete elimination cannot be achieved. However, this is different from the results in salinity exposure of axenic nauplii (Figure 4.1.2). It is suspected that the colonies counted on TCBS agar plates in Figure 4.2.1 (b) may not be *Vibrio*. In other words, not all the yellow colonies growing on TCBS agar plates are *Vibrio*; there may also be salt-tolerant bacteria growing beside them. Therefore, rearing water was collected for *Vibrio* genus specific amplification, to detect the presence of *Vibrio* in each salinity treatment group. As shown in Figure 4.2.2, when the salinity >130 g/L, the bands of 689-bp size cannot be seen. This proves that not all those colonies were *Vibrio*, the previous speculation has been confirmed.

However, the results in salinity exposure of axenic nauplii (Figure 4.1.2) show that 100% elimination of *Vibrio* can be achieved at 120 g/L salinity. This is inconsistent with the results shown in Figure 4.2.2. This is because PCR detection can only detect the presence of *Vibrio* genes, but cannot indicate whether the source of *Vibrio* genes (*Vibrio* cells) is alive. In other words, during the 48 hours of exposure, the *Vibrio* that has been suppressed or even killed can still be detected by PCR. To ensure that there were no live *Vibrio* when salinity >130 g/L, a simple experiment was designed for double confirmation. In the salinity exposure of the non-axenic nauplii experiment (low-density bacterial condition), after 48 hours, the rearing water of the 30 and 140 g/L salinity treatment group was spread on TCBS agar plates. After overnight incubation, all the colonies that grew were scraped off, mixed, and collected for DNA extraction and PCR amplification (follow the methodology of “**3.6.2 Collection of live bacterial DNA from rearing water or *Artemia***”). The PCR result (Figure 5.4) shows that there is indeed viable *Vibrio* in the rearing water under normal conditions (30 g/L salinity), while there is no viable *Vibrio* at under 140 g/L salinity conditions. This simple method cleverly avoids the interference of non-reproductive *Vibrio* on PCR detection.

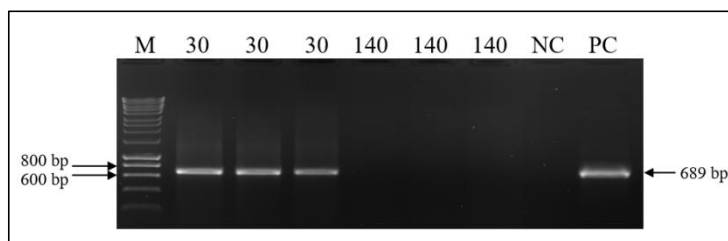


Figure 5.4 *Vibrio* genus PCR results for mixed genomic DNA of all bacteria from TCBS agar plates. Lane M: 1000-bp DNA ladder. Lane 30 and 140: salinity treatments 30 g/L and 140 g/L. Lane NC: negative control. Lane PC: positive control.

5.5 Unexpectedly obtain *Exiguobacterium spp.*

In the results 4.2.3 (a) for identification sequencing, no *Vibrio spp.* was detected, but all were *Exiguobacterium spp.* (*E. arabatum* and *E. aestuarii*). Bacteria of the *Exiguobacterium* genus are Gram-positive facultative anaerobes, widely distributed in multiple harsh habitats, including cold and hot environments with temperatures ranging from -12 to 55 °C (Rodrigues & Tiedje, 2007) and some strains exhibit salt tolerance (Cao et al., 2020). *Exiguobacterium spp.* have been isolated from harsh environments, including ancient Siberian permafrost, Greenland glacial ice, hot springs at Yellowstone National Park, and soils in temperate and tropical regions (Vishnivetskaya et al., 2009). Lopez-Cortes et al. have isolated two strains of *Exiguobacterium spp.* in the body of *Artemia franciscana*, which were derived from cysts (Lopez-Cortes, Schumann, Pukall, & Stackebrandt, 2006). *E. arabatum* is a probiotic isolated by Cong et al. from the intestine of healthy *Penaeus vannamei* (Cong et al., 2017). *E. aestuarii* is a probiotic isolated by Kim et al. from the reservoir, which can remove waste generated during shrimp farming and maintain a good environment for shrimp (S. Kim et al., 2022). There is such a large proportion of *Exiguobacterium spp.* on the TCBS agar plates, but a very small proportion of *Vibrio spp.* It is likely that these *Exiguobacterium spp.* acted as probiotics, suppressing the growth of *Vibrio*. By harnessing *Exiguobacterium spp.* as probiotics in combination with an optimal salinity level of 80 g/L within an open *Artemia* hatchery, it is possible to enhance the inhibition of *Vibrio*, thereby maximizing the survival percentage of *Artemia*.

5.6 The impact *P. tectorius* on *Artemia*

PLE not only has a direct inhibitory effect on *Vibrio* but also functions to regulate animal immunity (Rehan, 2022). In this study, PLE showed an overall improvement in the resistance of *Artemia* to *Vibrio*. In results 4.4 (**Experiment 3: Exposure experiment on adult *Artemia***), when the salinity was 30 g/L, the *Artemia* in the treatment group with *P. tectorius* leaves died within 24 hours of exposure, whereas the *Artemia* in the treatment group with salt alone still survived after 48 hours of exposure. However, Anirudhan et al.'s study proved that *P. tectorius* fruit extract was harmless to shrimp (Anirudhan et al., 2021). Therefore, the likelihood of *P. tectorius* leaves being toxic to *Artemia* is very low. It is more likely that the decay of the leaves provides nutrients for *Vibrio*, which is actually beneficial for its growth. An exposure experiment was designed with three concentrations of *P. tectorius* leaves: 1 times concentration (7.14 g/L), 2 times concentration (14.28 g/L) and 3 times concentration (21.42 g/L) of leaves, all at 30 g/L salinity. The experimental subject is *Artemia* nauplii. As shown in Figure 5.6, each treatment group had a band of 689-bp, and even more leaves could not eliminate all *Vibrio*. After the increase of leaves, the quality of rearing water deteriorated, and more leaves provided more nutrients for bacteria in the rearing water. In summary, *P. tectorius* leaves are harmless to *Artemia*. Therefore, in the actual production of *Vibrio*-free nauplii, if *P. tectorius* leaves are used instead of PLE, it is important to note that the exposure time should not exceed 48 hours to avoid excessive leaf decay, which could lead to the death of nauplii.

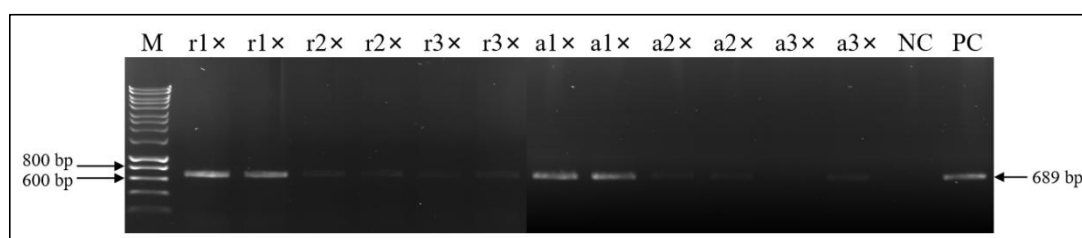


Figure 5.6 *Vibrio* genus PCR results for treatments with increased concentration of *P. tectorius* leaves. Lane M: 1000-bp DNA ladder. Lane r1 $\times$, r2 $\times$ and r3 $\times$: rearing water samples from treatments with 1, 2 and 3 times concentration of *P. tectorius* leaves. Lane a1 $\times$, a2 $\times$ and a3 $\times$: nauplii samples from treatments with 1, 2 and 3 times concentration of *P. tectorius* leaves. Lane NC: negative control. Lane PC: positive control.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

During the culture process of *Artemia* nauplii and adult, higher salinity indeed exhibits an inhibitory effect on *Vibrio*. Under normal salinity conditions, the pathogenicity of *Vibrio* causes the death of *Artemia*. And under high salinity, *Artemia* also perishes due to their inability to tolerate such conditions. *Vibrio* can be effectively inhibited and the survival percentage of nauplii and adult *Artemia* can be ensured only when exposed to optimal salinity of 80 g/L and 120 g/L for 48 hours. However this single salinity exposure method can only improve the quality of *Artemia* products and effectively reduce the load of *Vibrio* on *Artemia*. To achieve the *Vibrio*-free nauplii, it is necessary to expose them in an optimal condition, with a salinity of 80 g/L combined with 1 g/L of PLE (or 7.14 g/L of freeze-dried *P. tectorius* leaves powder) for 48 hours. In this study, no effective method was found for obtaining *Vibrio*-free adult *Artemia*, indicating that further exploration is needed in future research.

This study found that TCBS agar does not specifically support the growth of *Vibrio* genus. Therefore, the use of TCBS agar plate spreading cannot serve as a reliable method for identifying *Vibrio*. The *Vibrio* genus PCR can only detect the presence of *Vibrio*, but cannot determine the survival status of *Vibrio*. Both methods have certain limitations and need to be combined to verify the presence of live *Vibrio*. This combined detection method has high accuracy and can separate dead *Vibrio* most simply, providing an effective method for other research related to *Vibrio*.

This study also found that a large amount of *Exiguobacterium* spp. appeared with the hatching of *Artemia*. During *Artemia* open system culture, these bacterial

strains act as probiotics and provide a protective effect on *Artemia*. In a closed *Artemia* culture system, these bacterial strains may serve as alternative feed sources for *Artemia*.

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APPENDICES

APPENDIX 1

Experiment 1: Exposure experiment on axenic *Artemia nauplii***Colony count from TCBS agar plates in nauplii with *V. campbellii* treatment**

Salinity (g/L)	Colony count (CFU)					
	Initial density of 1×10^7			Initial density of 1×10^8		
30	297	300	290	TNTC	TNTC	TNTC
60	201	174	184	TNTC	TNTC	TNTC
70	69	63	67	TNTC	TNTC	TNTC
80	TFTC	TFTC	TFTC	114	110	98
90	0	0	0	55	30	52
100	0	0	0	TFTC	TFTC	TFTC
110	0	0	0	0	0	0
120	0	0	0	0	0	0
130	0	0	0	0	0	0
140	0	0	0	0	0	0
150	0	0	0	0	0	0

Colony count from TCBS agar plates in nauplii with *V. parahaemolyticus* treatment

Salinity (g/L)	Colony count (CFU)					
	Initial density of 1×10^7			Initial density of 1×10^8		
30	297	298	288	TNTC	TNTC	TNTC
60	171	156	174	272	287	291
70	TFTC	TFTC	TFTC	275	233	243
80	0	0	0	225	203	196
90	0	0	0	87	95	99
100	0	0	0	62	67	63
110	0	0	0	TFTC	TFTC	TFTC
120	0	0	0	0	0	0
130	0	0	0	0	0	0
140	0	0	0	0	0	0
150	0	0	0	0	0	0

APPENDIX 2

Experiment 2: Exposure experiment on non-axenic *Artemia* nauplii (high-density bacterial condition)

Colony count from TCBS agar plates in salinity exposure

Salinity (g/L)	Colony count (CFU)					
	Rearing water samples			Nauplii samples		
	12 h	24 h	48 h	12 h	24 h	48 h
30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
50	13	60	100	81	115	248
60	27	52	113	29	147	112
70	19	71	84	40	152	118
80	82	102	75	71	168	173
90	7	14	7	21	33	36
100	48	30	102	28	26	95
110	6	13	6	20	24	21
120	8	8	6	18	14	11
130	8	17	15	12	18	9
140	10	10	9	15	13	7
150	9	11	6	11	11	8
160	4	5	4	3	9	11
170	6	15	4	9	9	16
180	16	15	10	14	18	11
190	6	19	7	9	21	17
200	10	17	12	6	20	10

Colony count from TCBS agar plates in salinity combined with PLE exposure

Salinity (g/L)	Colony count (CFU)					
	Rearing water samples			Nauplii samples		
	12 h	24 h	48 h	12 h	24 h	48 h
30	122	251	TNTC	67	153	TNTC
50	33	77	300	48	36	177
60	13	31	117	17	35	76
70	19	16	55	13	25	25
80	10	18	18	14	32	15
90	12	16	2	16	34	4
100	40	11	2	21	35	6