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Investigating the Characteristics of Bacteria Isolated from the Sea Scallop (Placopecten magellanicus) Larvae and Tanks

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INVESTIGATING THE CHARACTERISTICS OF BACTERIA ISOLATED FROM SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) LARVAE AND TANKS

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An Abstract of the Thesis Presented
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Placopecten magellanicus, also known as the Atlantic deep-sea scallop, is a valuable marine species on North America's northeastern coast. Although adult scallops can be successfully bred in hatcheries, a perplexing and destructive event takes place during the last two weeks of larval development: a sudden and severe mortality event that causes a drastic decline in populations, with some reports suggesting that survival from egg to competent larva can sometimes be reduced to as low as 1-10% during a span of 48 hours. The precise reasons for larval mortality in sea scallops remain unclear. Prior studies have investigated infections in sea scallops. However, there still needs to be a more extensive investigation into the bacterial communities linked to wild and hatchery-reared deep-sea scallops and the biofilms present in hatchery tanks.

We looked at how the bacteria in tank biofilms in scallop hatcheries might affect the health of the larvae. Downeast Institute, Mook Sea Farm, and Darling Marine Center provided biofilm samples for tests in genomics and microbiology. Every 48 hours, larvae are fine-filtered from all tanks, drained and cleaned, and larvae are put into a fresh tank of filtered seawater. Three biofilm

swabs were taken from the bottom of the tank before cleaning and refilling after draining. The study started with samples from adult tanks. Swabs of the tank biofilms were used to grow certain bacteria on thiosulfate-citrate-bile salts-sucrose (TCBS) agar to check for pathogenic Vibrio spp. Plates were transferred to UMaine for gram and endospore staining, testing the bacteria's capacity to utilize sugar and the resistance of isolates to other antibiotics.

The prevalence of yellow isolates (54%) over green isolates (47%) in static tanks suggests the potential presence of different *Vibrio* species. While flow-through tanks hosted more similar isolate types, at 47% yellow and 50% green isolates. Clean tanks had more sucrose fermenting (51%) than non-sucrose fermenting (41%) isolates. Dirty static and flow-through tanks had higher number of colony growth and a higher prevalence of yellow isolates. Specifically, there were 55% yellow isolates and 30% green isolates in static and dirty tanks, whereas there were 47% yellow isolates and 47% green isolates in flow-through and dirty tanks. Hatchery and scallop farms do not currently use antibiotics in their production, but antibiotic residue from towns and human wastewater can persist in seawater and affect microbes in tanks. We subjected the isolates to antibiotics such as ampicillin, streptomycin, penicillin, and oxytetracycline as a preventive measure against bacterial infections. Streptomycin outperformed all other antibiotics against isolates from various tanks.

Keywords: Aquaculture, microbiome, host-associated microbiome, bacterial community, *Vibrio*

DEDICATION

This thesis is lovingly dedicated to the memory of my father, whose unwavering support and belief in my academic pursuits have been the bedrock of my journey. His lifelong dream was for one of his children to study abroad, and though he did not live to see it fulfilled, his vision has been a guiding light that led me to where I am today. His spirit has accompanied me every step of the way, and I carry his dreams and aspirations forward as my own.

To my mum, Omolara, and sisters, Dasola and Ajibola, whose love and encouragement have been my safe harbor, this achievement is as much yours as it is mine. You have both been pillars of strength and endless sources of motivation, embodying the essence of our family's bond and resilience.

To Stephanie, my dearest companion and ally, your love and steadfast support have been the gentle forces that propelled me forward through the challenging moments. Your unwavering faith in me and your heartfelt encouragement has helped me realize my potential and become the best version of myself. This journey has been brighter and more meaningful with you by my side.

This work stands as a testament to the collective hopes, sacrifices, and love of those mentioned above, and while words fall short of expressing the depth of my gratitude, let this dedication serve as a lasting emblem of my appreciation.

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CHAPTER ONE

1 INTRODUCTION

Oysters, mussels, clams, and scallops are examples of bivalve molluscs that make up a sizable portion of global fisheries production (FAO, 2004). Undoubtedly, the increase in human consumption of seafood will continue to be a global trend (FAO, 2022). A significant and necessary component of the diets of many people around the world is seafood, and as the world's population rises, these nations will continue to need to produce more seafood (FAO, 2022). The demand for fish products will rise in some nations because they view seafood as a vital and healthy component of a person's diet. Although finfish will continue to account for the majority of seafood demand, there will also be a growing need for mollusk production and harvest, notably of bivalves (Wang et al., 2017). Although the harvest of wild bivalve stocks will likely continue to be large, many of these stocks are likely now at or close to their maximum viable levels. In other cases, stocks may even be overharvested. The alternative to taking wild stock is aquaculture.

Bivalve aquaculture is a unique opportunity to spawn and raise larvae in small land-based facilities which do not require much land or infrastructure, which can be used to stock off-shore floating farms where they remain for several years without much maintenance and contribute to cleaning coastal water. The majority of the world's oceans include scallops, which live in a variety of climatic regions from the polar to the tropics (Brand, 1991; Peña, 2001). The climatic, oceanic, and biological characterization of the natural habitat determines how the environment impacts many species of scallops (Lodeiros et al., 2001; Minchin, 2003). The Atlantic deep-sea scallop, *Placopecten magellanicus*, is found along the eastern coast of the United States and Canada. Scallops are a diversified species of marine bivalve mollusks (family *Pectinidae*) with a global distribution in coastal seas (Tremblay et al., 2020). Due to their high reproductive efficiency and high market demand, scallops are an ideal candidate for hatchery and farm-based production.

The bacteria found in tank biofilms, which can affect water quality, nutrient cycling, and larvae health, are an important component of this microbial ecosystem. The bacterial communities in tanks can be sourced from and affected by seawater and the amount and type of filtering it undergoes before reaching the tanks, the water temperature and pH, the frequency of cleaning, stocking density of larvae, and the velocity of water movement in tanks. Most tank systems use either static (no circulation) or flow-through water dynamics. The main difference between static and flow-through tanks are their purpose and the presence or absence of continuous fluid flow Static tanks are primarily for housing new larvae, which are too small to be collected even on fine mesh, as well as storage and non-flow-related tasks. In contrast, flow-through tanks are designed for controlled, continuous fluid circulation, making them suitable for housing of adults, juveniles or veliger-stage larvae, as well as experiments and processes that require such flow (Helm et al., 2004). Therefore, it is crucial to investigate the activity of bacteria isolated from tank biofilms in these hatchery systems in order to comprehend the complex interactions that control larval development and health.

According to earlier studies (Utting & Millican, 1998; Andersen et al., 2011), scallops were traditionally caught in the wild and prepared for spawning. The fertilized eggs are then put in tanks after being spawned. When the larvae reach D-veliger-stage, they are moved to larger tanks and kept at 2 larvae per milliliter density. Every two days, the water is changed, and when the larvae are prepared to settle, the spat are put in settlement tanks (Helm et al., 2004).

Unfortunately, in Atlantic Sea scallop hatcheries the last two weeks of the larval maturation phase, veliger-stage, is plagued by massive animal death, going from 60 million scallop larvae down to several thousand individuals in a span of 48 hours (Beal, 2014). Survival of clutches to maturity remains very low, with an industry-standard rate around 1% (Downeast Institute, Maine, unpublished data; (Andersen et al., 2011). This drastic winnowing of larvae reduces the availability of cultured sea scallop spat for farmers, forcing scallop farms to rely almost exclusively on scallop spat collected from wild populations for stock. Infection control, and particularly biofilm control, is a primary concern for scallop hatcheries.

1.1 Justification for the study

Production facilities in Maine routinely check for the presence of *Vibrio* species in their tanks during "drain-downs," which involve emptying, cleaning, and refilling the tanks with filtered saltwater every two days. This regular testing provides an opportunity to obtain time-resolved *Vibrio* tracking without burdening larvae production specialists with lengthy or complicated protocols. To better understand the microbial dynamics in hatchery tank biofilms, we worked together in this pilot study with three sea scallop hatcheries in Maine.

1.2 Aims and objectives of the study

1.2.1 Aim

The main aim of this study is to investigate the activity of bacteria isolated from tank biofilms in a hatchery system for sea scallop, *Placopecten magellanicus*, larvae.

1.2.2 The objectives of the study are as follows:

i. Identify bacteria in scallop tank biofilms and compare static vs. flow-through tanks.

ii. Determine if static vs. flow-through tanks systems recruit different species of *Vibrio* bacteria.

iii. As a long-term goal, standardize management practices to improve scallop health and aquaculture success.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Sea Scallops

Sea scallops are benthic bivalve filter feeders (Drew, 1906). Their growth rate in the sea is relatively slow, taking five years to reach a shell height (SH) of 90 mm and for the males and females to reach sexual maturity. Generally, in the United States, sea scallops (*Placopecten magellanicus*) are considered marketable when they reach a shell height of about 60 millimeters (mm) or approximately 2.36 inches. This size typically indicates that the scallop is mature enough for commercial harvesting. However, the specific legal-size limit can differ by location and the management measures in place to ensure sustainable scallop populations. They can live to 20 years of age and reach a size of 200 mm (SH). Females are highly fecund, producing up to 270 million eggs annually (Langton et al., 1987). Fertilization occurs in the water column, where the larvae remain for about a month, depending on temperature. Larvae grow to about 254 microns in size, after which they settle to the bottom and metamorphose into spat (Culliney, 1974). Sea scallops can be found along the north-west coast of the Atlantic Ocean from Newfoundland and the north coast of the Gulf of St-Lawrence, Canada (Caddy, 1989; Squires, 1962) to Cape Hatteras in North Carolina, USA (Posgay, 1957). This species is found at depths of 1 m to 180 m (Gosner, 1978; Naidu et al., 1989; Dadswell, per. obs) on sand-gravel or gravel-rock mixed bottoms and occasionally on sandy, silty or rock bottom (Couturier et al., 1995). Sea scallops support a sizeable commercial drag fishery in Canada and the United States (Stokesbury, 2012). Major landings are from the Bay of Fundy, Georges Bank, and banks off New England. The adductor muscle (meat) is removed (shucked) from the commercially harvested scallops and sold as highly-priced seafood.

2.2 Anatomy of scallops

According to Branch et al. (2010), the shell of the Pecten species has two valves: the left valve is flattened, and the right (lower) valve is convex. The three layers of the valves are made of calcium carbonate. The

nacreous (inner), prismatic (middle), and periostacum (outer) layers are the names given to these strata. The umbo, or hinge, is where the shells are attached to one another (Helm et al. 2004). A scallop's shell can also be used to determine its age and reveal details about its environmental circumstances (Kilburn & Rippey 1982).

Table 2.1: Scallop Species and Hatchery Success Rates: A Comparative Overview

Species	**Successfully Raised in Hatcheries?**
Pacific Oyster	Yes
Eastern Oyster	Yes
Blue Mussel	Yes
Mediterranean Mussel	Yes
Atlantic Scallop	Yes
Bay Scallop	Yes
Yesso Scallop	No
Queen Scallop	No
Giant Scallop	No
Horse Mussel	No

A scallop's mantle serves a variety of purposes, such as secreting the shell and ligament, producing the swimming response through the velum, and having a respiratory purpose as shown in figure 1.1 below (Beninger & Le Pennec 2006). The internal, soft tissue anatomy of a hermaphroditic scallop is shown in Figure 1.1. In scallops, the gill cilia's beating regulates the entrance and outflow of water through inhalant and exhalant regions (Beninger & Le Pennec 2006).

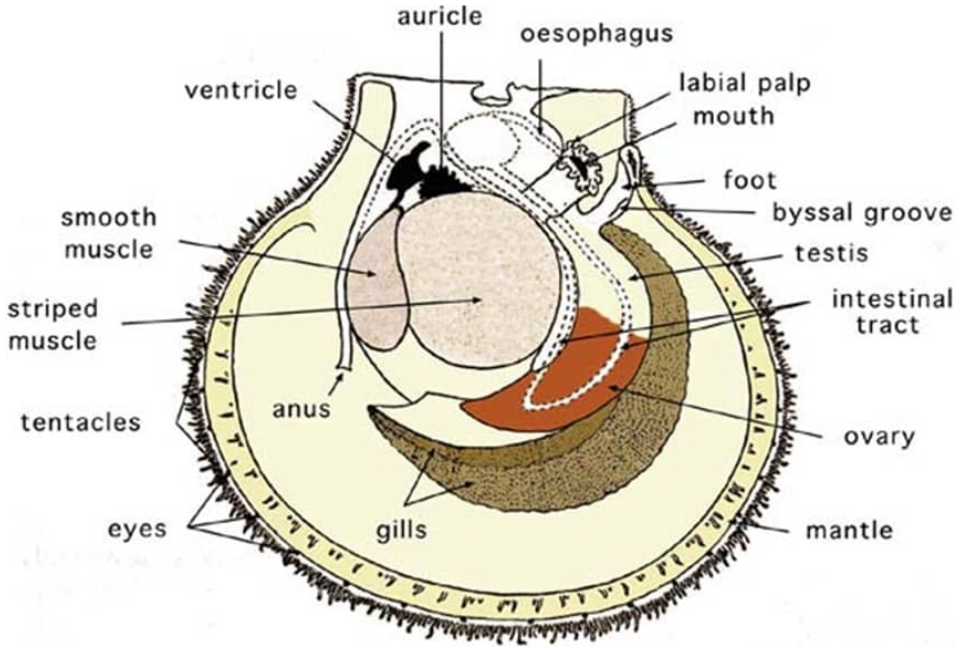

Figure 2.1: The internal, soft tissue anatomy of a hermaphroditic scallop (Helm et al., 2004)

2.3 Scallop aquaculture

Scallops are flavorful, not cannibalistic and hearty. Scallops are also a good aquaculture crop because of their high economic worth, quick growth rate, and early maturity. The most sought-after element of the scallop is the cream-colored adductor muscle. About 40 species of scallops are used for commercial purposes worldwide; 18 species produce the majority of the 2.8 million tonnes of live weight produced worldwide via capture fisheries and aquaculture (FAO, 2020). In nations like China, Chile, and Japan, scallop farming has largely supplanted capture fisheries as the demand for scallops rises (Ventilla, 1982; Guo et al., 1999; Stotz, 2000). The demand for scallops is rising even though they may be successfully farmed in a number of places (National Marine Fisheries Service, 2020). Nevertheless, more than 70% of the seafood consumed in the United States is imported, including the roughly 20% that comes from reimports (Gephart et al. 2019). Moreover, farming accounts for over 60% of the volume of seafood imported into the United States (National Marine Fisheries Service, 2020).

2.4 Introduction to Sea Scallop Hatchery Systems

Sea scallops are a highly prized seafood delicacy known for their sweet and tender meat. They are native to the North Atlantic Ocean and have been commercially harvested for centuries. However, overfishing and environmental changes have led to declines in wild scallop populations in some regions. To meet the increasing demand for this delectable mollusk while conserving wild stocks, sea scallop aquaculture has emerged as a promising solution. Sea scallop hatchery systems play a pivotal role in this endeavor, facilitating the controlled cultivation of scallops from their earliest larval stages to market size.

2.4.1 Sea scallop larval rearing and settlement

According to Helm et al. (2004), larvae can be raised in high-density static or flow-through systems, with the latter being the more popular choice. Determining the rate at which larvae are ingesting feed is essential since it is preferable to overfeed rather than underfeeding them (Helm et al., 2004). When raising larvae, environmental factors are crucial. In investigations involving the raising of larvae, light intensity is only occasionally examined. However, Krassoi et al. (1997) showed a correlation between aberrant development and high light intensities. The most critical factor in the growth and development of larvae is temperature, which is also frequently the key to getting the most significant outcomes in the initial phases of scallop larvae development (Roman et al., 1996; Le Pennec et al., 2003; Helm et al., 2004; Parsons & Robinson, 2006).

According to Cragg (2006), the development of larvae can be explained by their developmental phases, cleavage rate, and shell growth. Scallop larvae typically grow at a pace of 3 to 5 μm d-1 (Cragg, 2006); however, it is unclear if larvae grow more quickly in a lab setting than in the ocean. According to Cary et al. (1981), the rate at which larvae grow their shells typically indicates how efficiently the nutrition and feeding schedules are working. According to specific research, the number of enormous eggs produced by a given veliger indicates the size of the shell (Paulet et al., 1988). Self-fertilization of eggs can occur during spawning induction and even in the natural environment (Winkler & Estévez,

2003). Among the earliest signs of inbreeding depression in animals are a rise in deformities and decreased sustainability and fecundity (Concha et al., 2011). Research has demonstrated that Pacific Calico Scallop (*Argopecten circularis*) cross-fertilized eggs produce larvae that develop significantly more quickly than self-fertilized eggs (Ibarra et al., 1995). In *Pecten maximus*, inbreeding was linked to lower larval development rates (Beaumont, 1986). Fewer larvae were obtained from self-fertilized crossings in Tehuelche scallop (*Aequipecten tehuelchus*) than cross-matings (Navarte & Pascual, 2003). According to Lui et al. (2011), self-fertilized eggs produced reduced survival rates, juvenile development, and adult live weight in bay scallops from China compared to a mass-spawned group. On the other hand, Winkler & Estévez (2003) discovered that in Peruvian bay scallops (*A. purpuratus*), self-fertilized and cross-fertilized animals did not vary in larval and juvenile survival and growth. Moreover, self-fertilization did not affect larval growth or survivability in the Zigzag (*Euvola ziczac*) species (Betancourt et al., 1994).

When larvae reach the stage of pediveliger, they are prepared to settle and begin looking for a suitable substrate. High mortalities typically happen during settling due to the behavioral changes larvae undergo to find a suitable substrate. If settlement is induced too early and the larvae are not prepared to settle, high mortalities also happen (Culliney, 1974). Other factors, such as larval origin, rearing density, microalgae quality and quantity, and water conditions, have been previously described to induce significant mortalities in scallop larvae (Liu et al., 2006; Magnesen et al., 2006). A pathogenic strain of the bacteria *Vibrio splendidus* was the source of the massive mortalities of *A. purpuratus* larvae raised in commercial hatcheries (Rojas et al., 2015). One of the primary elements influencing *P. maximus* larval survival has also been identified as pathogenic bacteria (Marine, 2015). The rate of survival in larvae can also be influenced by the type of culture system employed; *A. purpuratus* larvae cultured in a closed aquaculture system acquired a higher percentage of larval survival than larvae cultured in a recirculating aquaculture system (Merino et al., 2009).

2.4.2 Challenges and Concerns in Sea Scallop Hatchery Systems

Sea scallop hatchery systems hold great promise for the aquaculture industry (FAO, 2016; Martin et al. 2021). However, they also face some challenges and concerns. Sea scallop larvae are delicate and susceptible to various stressors, including disease, suboptimal environmental conditions, and predation, leading to high mortality rates (Rudders et al. 2022). Maintaining consistent and high-quality microalgae supply for larval nutrition can be challenging and expensive and disease outbreaks can devastate hatchery populations, necessitating strict biosecurity measures to prevent contamination (Assefa & Abunna, 2018). In addition, inducing reliable spawning in adult scallops can be complex, and not all broodstock individuals may respond as desired (Sarkis & Lovatelli, 2007).

Scallop hatchery systems can have environmental impacts due to water use, effluent discharge, and habitat modification. Therefore, sustainable practices are crucial to minimize these effects (Martin et al. 2021).

2.5 Tank Biofilms and their Significance

Tank biofilms in the context of aquaculture refer to complex communities of microorganisms, including bacteria, algae, fungi, and other microorganisms, that adhere to surfaces within aquaculture tanks or systems. These biofilms play a significant role in the overall health and functionality of aquaculture ecosystems. Biofilms are structured communities of microorganisms that adhere to surfaces and are encased in a matrix of extracellular polymeric substances (EPS). Characteristics of biofilms include microbial diversity, spatial organization, and resistance to external stresses such as disinfectants and antibiotics. Biofilms can form on various substrates in aquaculture systems, including tank walls, pipes, and the surfaces of aquatic organisms (Cai & Arias, 2017).

2.5.1 Formation and Development of Tank Biofilms

Biofilm formation begins with the initial attachment of free-floating microorganisms to a substrate. These microorganisms then secrete EPS, forming a protective matrix that facilitates further

microbial attachment. As biofilms mature, they develop a complex three-dimensional structure with different layers of microorganisms, each serving specific functions within the community. Factors influencing biofilm formation and development include temperature, nutrient availability, water flow, and the presence of organic matter (Cai & Arias, 2017).

2.5.2 Importance of Biofilms in Aquaculture Systems

Biofilms contribute to the overall ecological balance of aquaculture systems and play a crucial role in nutrient cycling. They can help remove excess nutrients, such as ammonia and nitrate, through microbial processes. Biofilms contribute to water quality improvement by acting as biological filters, which is essential for the health of aquatic organisms. These microbial communities also serve as a food source for some aquatic species, including certain larval stages of fish and invertebrates.

2.5.3 Exploring the Dual Role of Biofilms in Larval Growth, Nutrition, and Disease Prevention

Many aquatic invertebrates often feed on microorganisms present in biofilms. The microorganisms in biofilms can serve as a natural and nutritious food source for the early life stages of aquaculture species. Biofilms can help improve larval nutrition by providing essential fatty acids, vitamins, and proteins. In addition to nutrition, biofilms play a role in disease prevention (Cai & Arias, 2017). Some biofilm-forming bacteria such as *Pseudomonas aeruginosa* produce antimicrobial compounds that can inhibit the growth of pathogenic bacteria (Cary et al., 1989). Biofilms can also serve as a protective barrier, preventing pathogenic bacteria from colonizing the surfaces of tanks and aquatic organisms. Tank biofilms are complex microbial communities that form on surfaces within aquaculture systems (Couturier et al., 1995). They are essential for maintaining water quality, providing nutrition to the larval stages of aquatic species, but also contributing to disease in the marine world. Understanding and managing biofilms in aquaculture systems is crucial for the overall success and sustainability of aquaculture operations.

2.6 Scallop Aquaculture and Nursery Development

Successful scallop aquaculture depends on reliable conditioning for their successful growth and survival during the different production stages. Significant variations in growth and survival during the different production steps from larvae to spat are a worldwide experience (Ó Foighil et al., 1990; Bourne & Hodgson, 1991; Heasman et al., 1994b; Couturier et al., 1995; Robert & Gérard, 1999; Heasman et al., 2002; Le Pennec et al., 2003; Rupp et al., 2004b; Torkildsen & Magnesen, 2004) and a bottleneck in the development of viable scallop culture industries. The scallops go through several critical cultural stages, from broodstock conditioning to 15-20 mm, the commercial spat size in Norway. The physical and nutritional environment is manipulated during conditioning, and the broodstock diet affects the fecundity and viability of eggs and embryos (Utting & Millican, 1998; Andersen & Ringvold, 2000).

Food quality and quantity are essential for larvae to go through metamorphosis and survive the post-larval stages successfully (Whyte et al., 1987, 1992; Delaunay et al., 1992; Farías et al., 1998; Lu et al., 1999b; Milke et al., 2004; Rupp et al., 2004b), and substrate type, water circulation, food availability, temperature, and salinity are important factors determining larval settlement (Shumway, 1991). Temperature, salinity, and food availability are critical environmental factors during post-larval and spat growth. In hatcheries, it is possible to enhance the output of scallop larvae and post-larvae, i.e., obtain higher spat yields than in nature (Loosanoff & Davis, 1963; Le Pennec et al., 2003; Torkildsen & Magnesen, 2004). Nevertheless, *P. maximus* spat yields are shown to be variable in culture (Robert & Gérard, 1999; Bergh & Strand, 2001).

A primary requisite in any cultural operation is a reliable, plentiful, and inexpensive seed supply, i.e., spats or juveniles (Bourne, 2000). Booming scallop culture industries exist, as in China and Japan, but they are in short supply in several other countries. Information regarding the environmental requirements of post-larvae and minor spats is scarce. Bourne (2000) emphasizes the need for considerable research to be carried out in all phases of culture to develop large scallop industries.

Descriptions of scallop spat culture procedures in large (commercial) scale systems exist primarily as hatchery manuals and confidential protocols, but also in more general literature as for *Placopecten magellanicus* (Couturier et al., 1995), *P. fumatus* (Heasman et al., 1994b, 2002), *Patinopecten yessoensis* (Ventilla, 1982; Bourne et al., 1989; Bourne & Hodgson, 1991), *Argopecten purpuratus*, *Argopecten ventricosus*, *Euvola ziczac, Nodipecten nodosus* and *N. subnodosus* (Uriarte et al., 2001; Merino et al., 2001), *Argopecten irradians* (Widman et al., 2001), *Argopecten irradians* (Sastry, 1965; Lu & Blake, 1997b), and *P. maximus* (Millican, 1997; Hardy, 1991; Robert & Gérard, 1999). Thus, published research on the biology of the early life stages of scallops is mainly obtained from studies in laboratory or hatchery situations and related to reproduction, larval phase, and the growth of larger juveniles.

Due to the development of scallop aquaculture industries over the last decades, the focus on the hatchery-nursery phase has increased. The post-larval growth stages are crucial in spat production, and adequate rearing conditions are fundamental. A nursery bridges the gap between intensive production in hatcheries and grow-out in the sea (Claus, 1981; Bourne et al., 1989) and covers the rearing of recently metamorphosed larvae (ca. 0.25 mm) until the juvenile size of 20–30mm shell-height (Bourne, 2000). Nursery growth may be divided into a primary and a secondary stage, with the primary nursery growth reaching approximately 2mm, usually in close connection with the hatchery. Due to rapid growth, the increase in food demand during the post-larval stage of bivalves is substantial. It makes it easier to use cost-effective conditions in semi-intensive secondary nurseries that can be located on land or in the sea and use natural or grown algae as food (Claus, 1981; Manzi et al., 1986; Strand, 1996; Hadley et al., 1999). The spat is kept in different enclosures (i.e., nets or trays) suspended from long lines in the sea, raceways, or tanks with horizontal, downwelling, or upwelling water flow. In 1981, attention was paid to using semi-intensive growth systems or nurseries in bivalve production (Claus, 1981).

However, the use of nurseries as extensions to the hatchery has developed along with increased interest in cultivation (Claus, 1981; Rhodes et al., 1981; Rosenthal et al., 1995; Gosling, 2003). Therefore,

most documented research on scallop nursery culture has been conducted during the last 10–15 years. Significant physiological and anatomical development occurs during the post-larval growth stages; thus, small scallops are fragile and vulnerable to changes in the rearing conditions. It is essential to think about how changes in development are affected by the environment because moving and handling animals can cause them to die, which can lower the number of spat that are produced during stressful culture operations (Maguire et al., 1999; Maeda-Martnez et al., 2000).

Limited information exists about nursery-sized scallops in general. The knowledge concerning the most minor spat of ≤2 mm size is restricted to a few species of commercial interest. Little research has been done on this size group of *P. maximus*. Specifically, the impact of food amount, seawater flow, and temperature on post-metamorphic development (Robert & Nicolas, 2000; Nicolas & Robert, 2001) and different diet compositions (Laing & Psimopoulous, 1998) has been investigated.

2.7 Bacteria Associated with Bivalves

Limited information exists on the bacterial communities associated with Atlantic Sea scallops. The study conducted by Crone et al. in 2020 revealed that the bacterial communities found in sea scallop larvae consist of a wide range of different taxa. The detection of *Flavobacterium, Pseudomonas, Psychrobacter,* and *Suttonella* in eggs and larvae obtained from the hatchery indicates that these microorganisms are likely acquired from the broodstock, other larvae, and the surrounding environment. These bacteria are frequently present in maritime environments and associated with marine organisms, suggesting their possible involvement in the microbiome of larvae. Moreover, the specific types of bacteria identified in eggs and larvae obtained from the wild, including *Pelomonas, Polaribacter, Porphyromonas, Oleispira,* and *Sphingobacterium*, emphasize the exceptional microbial communities linked to wild scallop larvae. The variations in the number and variety of bacteria in wild and hatchery environments highlight the impact of environmental microbial communities on marine larvae, emphasizing the random process of acquiring microbial communities in sea scallop larvae (Crone et al., 2020; Nogales et al., 2011).

Other studies provide more detail on bacterial communities associated with Yesso scallops, and point to specific communities associated with different organs. The study by Li et al. (2016) focused on analyzing the culturable bacterial diversity in the larval breeding of Yesso scallops. The findings indicated that the bacterial population in the larvae was primarily composed of *Pseudomonas*, which constituted 38.5% of all bacterial isolates—additional prevailing genera comprised *Vibrio, Bacillus,* and *Staphylococcus*.

Vibriosis, which is caused by *Vibrio* spp., is a highly significant bacterial disease in aquaculture. *Vibrio* is a significant disease for numerous aquatic animals (Reichelt et al., 1976). These organisms have lately been found to cause a significant number of losses in young molluscs that are being farmed in coastal countries. This finding is supported by several studies conducted by Lacoste et al. (2001), Gómez-León et al. (2005), Kesarcodi-Watson et al. (2009), and Jones et al. (2012).

As an illustration, *Vibrio* species are responsible for around 80,000 cases of human diseases in the United States per year, resulting in 100 fatalities (CDC, 2020). Out of them, *Vibrio parahaemolyticus* is the predominant source of infection, although *Vibrio vulnificus* leads to the highest fatality rate, with 1 in 5 cases resulting in death. Given their preference for warm environments, these organisms are particularly worrisome due to the increasing global ocean temperatures (Baker-Austin et al., 2018; Vezzulli et al., 2013). Moreover, due to the natural presence of *Vibrio* spp. in marine and brackish waters, they can withstand depuration techniques that are typically used to eliminate pathogens from oysters before they are consumed. Gaining insight into the determinants of bacterial pathogen accumulation in bivalves is crucial for safeguarding human and ecosystem well-being both presently and in the long term.

The environmental factors influencing the concentrations of human pathogens in coastal waters are well documented. Coastal waters with elevated levels of fecal-borne bacteria are commonly associated with runoff from sources on land and sewage spills. These occurrences often align with high concentrations of nutrients and other pollutants. Marine *Vibrio* species are linked to high water

temperatures and have salinity distributions that are particular to each species (Takemura et al., 2014; Brumfield et al., 2021). This indicates that variables other than the environment are likely to be involved. Individual oysters from the same environment might vary significantly in the number of harmful bacteria they contain. This occurrence, known as the "hot oyster" phenomenon, means that some oysters are riskier to eat than others because they acquire more pathogens (Klein and Lovell (2017), Williams et al. (2017)). Further investigation is required to analyze many elements that may elucidate the connections between environmental conditions, the transmission of human pathogens from water to oysters, and the accumulation and persistence of pathogens in oysters.

2.7.1 Identification of scallop tank bacterial isolates using different medium.

2.7.1.1 TCBS media

Thiosulphate citrate bile salt (TCBS) agar is a selective agar used in microbiology laboratories to isolate *Vibrio* species (Aryal, 2022). It is a selective and differential medium for isolating and growing *Vibrio* organisms, including *Vibrio cholera,* from clinical specimens and other materials. Citrate, sodium thiosulfate, sodium chloride, bile salts, sucrose, bromothymol blue, and agar comprise the medium. The TCBS medium evaluates a bacteria's capacity to ferment sucrose and create yellow colonies due to the acid produced during fermentation. TCBS agar is effective for isolating and differentiating *V. cholerae* and *V. parahaemolyticus*, it may prevent the growth of or be less effective for cultivating other *Vibrio* species, such as *V. vulnificus* and some strains of *Vibrio alginolyticus*, which may not grow as well or produce distinctive colonies on this medium. These species are often less bile tolerant compared to *V. cholerae* and *V. parahaemolyticus*, and their growth may be inhibited by the bile salts present in TCBS agar (Aryal, 2022).

Sucrose fermentation is advantageous because *V. cholerae,* the cholera-causing pathogen, exhibits it as a fundamental trait. The capacity to ferment sucrose is connected to other pathogenic

features of *V. cholerae* (Shrestha & Shakya, 2021), such as its capacity to colonize and wreak disease in humans, in addition to the generation of yellow colonies on TCBS agar.

While the FDA has a specific protocol for identification of this pathogen from food, the current gold standard for detection of *V. cholerae* is culturing stool or rectal swabs on the selective media thiosulphate citrate bile salts sucrose (TCBS) or taurocholate-tellurite gelatin agar (TTGA) either directly or after enrichment in alkaline peptone water (APW), followed by serotyping and biotyping with antisera (CDCP, 1999; Ramamurthy, 2020). The culture method requires competent laboratory support and technical skills, which are only sometimes available at health facilities or laboratories where cholera is endemic. In addition, this method is not sensitive and can take up to three days after collecting fecal specimens, which is not ideal for an outbreak.

2.7.1.2 TSI media

Triple Sugar Iron Agar (TSI) is a differential and selective medium used for the cultivation and identification of microorganisms, particularly the Enterobacteriaceae family, in their ability to ferment carbohydrates to form acids, gases, and H_2S (Chusniati et al., 2012; Ma et al., 2021). It also includes peptones, yeast extract, sodium chloride, ferrous sulfate, the pH indicator phenol red, and the three sugars glucose, lactose, and sucrose. Oliver et al.'s (1983) study found that lactose-positive strains of *Vibrio* from different samples were positively related to the pH and turbidity of the water, the amount of *Vibrio* in the sediment and oysters, and the total number of bacteria in oysters. The study also found sucrose-negative *Vibrio* in seawater, sediment, plankton, and animal species. TSI media tests for several different properties of microorganisms. Lysine-decarboxylase and oxidase assays should be included to aid in identifying this type of organism (Sanyal et al., 1973). It is used to differentiate bacteria based on their ability to ferment glucose, lactose, and sucrose and produce gas from these sugars. TSI media is also used to test for the production of sulfur and the metabolism of the amino acid arginine (Sukmawati & Pursetyo, 2021).

A shift in hue occurs in TSI media when bacteria ferment carbohydrates. If the bacteria can ferment the sugar, the medium's pH will become acidic, turning it yellow. The medium remains orange/red if the bacteria are unable to ferment the sugar, and instead are using the proteins and creating alkaline byproducts. The appearance of bubbles or fissures in the medium indicates that gas is being produced. The appearance of a black precipitate indicates that hydrogen sulfide is being produced (Ma et al., 20121). In 1973, Sanyal et al. wrote about a study done in Calcutta that showed *Vibrio cholerae* strains usually do not ferment lactose after being left to sit overnight (18-24 hours). However, some bacteria turn lactose into acid after being left to sit for longer periods of time. The ability of bacteria to metabolize various sugars and amino acids and their capacity to create gas and sulfur can be used to identify them using a TSI medium. It is frequently used in microbiology labs to identify Enterobacteriaceae, including *Salmonella* and *Escherichia coli*. *Vibrio cholerae* exhibit acidic reactions in the aerobic and anaerobic portions, and do not produce gas or hydrogen sulfide (Centers for Disease Control and Prevention, 1999).

2.7.1.3 Arginine media

The capacity of microorganisms to digest carbohydrates, metabolize the amino acid arginine, or utilize iron in the medium is frequently assessed using arginine media, a form of microbiological growth medium. Usually, it consists of a base medium supplemented with particular nutrients and markers to check for the necessary metabolic pathways. The following three elements comprise arginine media: iron is frequently present as ferric citrate, which some microorganisms can use as an electron acceptor during metabolism. Some microorganisms can metabolize the amino acid arginine to produce ammonia and other byproducts. Sugars are a carbon and energy source for microorganisms to grow and reproduce. Based on their capacities for metabolism, distinct species of hatchery bacteria can be distinguished using arginine media. While other bacteria cannot ferment carbohydrates, creating gas and acid as byproducts, some can. Some bacteria can also break down arginine into ammonia, raising the medium's pH. Others

can conduct electron transfer processes using the iron in the media. On arginine media, microorganisms thrive and produce metabolic byproducts that can be observed, allowing scientists to distinguish and categorize various types of bacteria based on their metabolic capacities. *Vibrio cholerae* produces an alkaline reaction in anaerobic conditions and acidic reaction in aerobic conditions (Centers for Disease Control and Prevention, 2019).

2.8 Use of Antibiotics in the United States of America

Several recent reports, press releases, and ongoing investigations have raised legitimate public concerns about the safety of antibiotic drug usage in aquaculture (examples include Alderman & Hastings, 1998; Goldburg et al., 2001). The widespread use of antibiotics in aquaculture poses public health and environmental problems, including the emergence of antibiotic-resistant bacteria that can make human infections harder and more expensive to cure, potentially increasing mortality. Aquaculture pollution can impair aquatic ecosystems, non-target species, and microbial populations, affecting animal and human health. Antibiotic residues in seafood create food safety and customer trust concerns. The lack of worldwide antibiotic standards in aquaculture exacerbates these difficulties, emphasizing the need for tougher controls, increased surveillance, and alternate disease management options including vaccines and probiotics. Aquaculture must overcome these problems to remain a safe and sustainable food source without harming public health or the environment.

Even though US aquaculture of bivalves does not use antibiotics, these can persist in coastal waters from human wastewater sources. Establishing the exact level of drug use and potential dangers is complex due to a lack of data, fragmented laws, regulations, jurisdictions, and interpretations of reporting guidelines (OTA, 1995), and large quantities of aquaculture imports from countries where legal and illegal drug use may escape documentation.

The use of antibiotics in the United States has been a significant aspect of healthcare for several decades. Antibiotics are powerful medications designed to kill or inhibit the growth of bacteria, thereby

treating bacterial infections (Madigan et al., 2008). They have been critical to saving countless lives and improving public health. However, their use also raises concerns about antibiotic resistance and the emergence of resistant strains of bacteria that can render these medications ineffective (Byrne et al., 2019). Except for NAHMS 1997, there are no publicly accessible sources of information on drug use in aquaculture in the United States. There is no reliable basis for regulators and scientists who study fish drug use and the environmental effects of aquaculture to estimate antibiotic drug use, including those from the Joint Subcommittee on Aquaculture (JSA), the Federal and Drug Administration (FDA), the United States Department of Agriculture (USDA), and the Environmental Protection Agency (EPA). There are no licensed antibiotic medications for most aquaculture species, including shrimp and crawfish (Benbrook, 2002). Some estimates of antibiotic usage are based on industry sources and ad hoc information gathered during inspections connected to water quality enforcement activities (Benbrook, 2002). Most of the U.S. aquaculture industry's antibiotic use is reportedly reducing (MacMillan, 2001), although no evidence or a way to confirm this is provided.

The improper use of preventative antibiotics in aquaculture to stop bacterial infections and the fast spread of disease are most likely the main reasons why *V. parahaemolyticus* isolates, or other bacteria like *Aeromonas* sp. isolated from Yesso scallops, have become resistant to many drugs. In addition, the overuse of antibiotics in aquaculture increases the selection of antibiotic-resistant bacteria and the dissemination of antibiotic-resistant genes, and results in antibiotic residues in aquatic organisms such as fish (Miranda et al., 2018).

Antibiotics can be administered orally, topically, or intravenously, depending on the severity and location of the infection. Historically, antibiotics were considered a miracle discovery in the mid-20th century, revolutionizing medicine and significantly reducing mortality rates caused by bacterial infections. The introduction of antibiotics such as penicillin, streptomycin, and tetracycline marked a turning point in healthcare, leading to many more antibiotics over time. However, the overuse and misuse

of antibiotics have contributed to the rise of antibiotic-resistant bacteria. This occurs when bacteria mutate or acquire resistance genes, allowing them to survive the effects of antibiotics.

The United States has implemented various initiatives and policies to address antibiotic resistance. These include promoting appropriate antibiotic use through educational campaigns, guidelines for healthcare providers and surveillance systems to monitor antibiotic resistance patterns. The Centers for Disease Control and Prevention (CDC) and other organizations have launched efforts to improve antibiotic stewardship, which involves optimizing the use of antibiotics to ensure effective treatment while minimizing the development of resistance activities (Benbrook, 2002).

Furthermore, regulatory measures have been taken to control the use of antibiotics in food animals. In 2013, the U.S. Food and Drug Administration (FDA) restricted certain livestock and poultry antibiotics for growth promotion. The aim is to reduce the overall use of antibiotics in animals and limit the potential transmission of antibiotic-resistant bacteria to humans through the food chain.

In recent years, there has been increased awareness about the importance of antibiotic stewardship and responsible antibiotic use among healthcare providers, patients, and the general public (Johnston, 2002). Efforts are being made to improve diagnostics, develop new antibiotics, and explore alternative treatment options to combat antibiotic resistance effectively.

2.8.1 Antibiotics media

Antibiotics are chemical compounds from microorganisms that inhibit or destroy other microbes (Hopwood, 2007; Davies & Davies, 2010). Microbiologists use antibiotic media, a growth medium, to assess bacterial cultures' susceptibility to antibiotics. The presence or absence of bacterial growth on the antibiotic medium can reveal whether or not the bacteria are susceptible to the antibiotic(s) under test. The emergence of multidrug-resistant bacterial species has increased the challenge of treating bacterial infections, posing a huge global public health issue. Testing bacterial cultures for antibiotic resistance is essential to guide appropriate antimicrobial treatment and prevent the dissemination of resistant

microorganisms. Antibiotic-resistant bacteria have been found in a diverse range of ecosystems such as human clinics, animal husbandry, orchards, aquaculture, food, sewage, and chlorinated and unchlorinated water supplies (Chopra & Roberts, 2001). Microbiologists frequently employ Mueller-Hinton, MacConkey, and blood agar as antibiotic media. With the help of these media, it is possible to determine which antibiotics, such as aminoglycosides, beta-lactams, macrolides, tetracyclines, and fluoroquinolones, bacteria are most susceptible to. As evidenced by the rise in fatality rates from 1% to 5.3% following the emergence of drug resistance strains in Guinea-Bissau during the cholera epidemic of 1996–1997, the emergence of microbial resistance to multiple drugs is a serious clinical problem in the treatment and containment of cholera-like diarrhea (Dalsgaard et al., 2000).

2.8.2 What each antibiotics target

2.8.2.1 Streptomycin

Streptomycin is an antibiotic from the class of drugs known as aminoglycosides. It was one of the first antibiotics discovered and was isolated from the bacterium *Streptomyces griseus* in 1943 (Yu et al., 2023). Streptomycin has been widely used to treat various bacterial infections, particularly those caused by *Mycobacterium tuberculosis*, the bacterium responsible for tuberculosis. Streptomycin works by inhibiting protein synthesis in bacteria. It specifically targets the bacterial ribosomes, the cellular structures responsible for assembling proteins. By binding to the bacterial ribosomes, streptomycin interferes with the accurate reading of the genetic code and prevents the synthesis of essential proteins needed for bacterial growth and survival (Daniels & Shafaie, 2000). Generally, *Vibrio* species are recognized to be very susceptible to most antimicrobials (Elmahdi et al., 2016). According to Yu et al. (2023), in a study on shrimp breeding in south China, many *Vibrio* strains showed intermediate antibiotic sensitivity to streptomycin (42.3 %).

In addition, antibiotics have the potential to cause adverse effects in individuals, notably in the auditory and vestibular systems, which can lead to hearing loss and issues with balance (Yu et al. 2023).

Because of this, the use of streptomycin necessitates careful assessment of both the hazards and the benefits, and it should only be prescribed by qualified medical specialists.

2.8.2.2 Penicillin

Penicillin is a group of antibiotics widely used to treat bacterial infections. It was the first antibiotic discovered and is considered one of history's most important medical advancements. The discovery of penicillin by Sir Alexander Fleming in 1928 revolutionized the field of medicine and paved the way for the development of many other antibiotics. Penicillin targets bacteria by interfering with cell wall synthesis (Hopwood, 2007; Fisher & Mobashery, 2020). Bacterial cells have a rigid outer layer called the cell wall, which provides structural support and protection. Ciprofloxacin has more potent antibacterial activity than antibiotics of other groups, such as amino acids, tetracyclines, and penicillin (King et al., 2000; Azargun et al., 2020). Penicillin works by inhibiting the formation of the cell wall, leading to the weakening and eventual rupture of the bacterial cells (Gordon et al., 2000).

Penicillin targets many bacteria, including Gram-positive bacteria like *Streptococcus* and *Staphylococcus* species (Fisher & Mobashery, 2020). These bacteria have a relatively thick cell wall, making them particularly susceptible to the effects of penicillin. However, it is essential to note that penicillin is not effective against all types of bacteria. Some bacteria, such as Gram-negative bacteria, have an outer membrane that protects them from the action of penicillin. Over the years, different forms of penicillin have been developed to enhance their effectiveness and expand their spectrum of activity. These include amoxicillin, ampicillin, and methicillin, among others. Each variant has its own specific characteristics and targets different types of bacteria. It is worth mentioning that the widespread use of penicillin has led to the emergence of antibiotic-resistant bacteria. Some strains of bacteria have developed mechanisms to counteract the effects of penicillin, rendering the antibiotic less effective (King et al., 2000)

2.8.2.3 Ampicillin

Ampicillin is a broad-spectrum antibiotic that belongs to the class of drugs known as penicillin (Yu et al. 2023). It is a derivative of penicillin and is commonly used to treat various bacterial infections. Ampicillin is effective against both Gram-positive and Gram-negative bacteria. Ampicillin works by targeting bacterial cell wall synthesis. It interferes with the enzymes forming peptidoglycan, a crucial bacterial cell wall component. The A class of enzymes known as penicillin-binding proteins (PBPs) are what ampicillin targets. They are what cross-link peptidoglycan strands in the cell walls of bacteria. Penicillin binds to PBPs and inhibits their activity, disrupting the proper assembly of the cell wall structure (Sudha et al., 2014). This makes the bacterial cells more susceptible to osmotic pressure, ultimately destroying them. Due to its broad-spectrum activity, ampicillin is used to treat various bacterial infections, including respiratory tract infections, urinary tract infections, gastrointestinal infections, and skin and soft tissue infections. Like other antibiotics, ampicillin can have side effects in people, including allergic reactions and gastrointestinal disturbances. It is also worth mentioning that the widespread use of ampicillin has led to the emergence of resistant bacteria (Yu et al. 2023).

Aeromonas, which are marine bacteria that can also affiliate with scallops and grow on TCBS plates, were previously shown to be resistant to ampicillin. First-generation antibiotics, such as ampicillin, play a significant role in developing resistance, which lowers the susceptibility profile and makes this medicine less effective at killing *Vibrio* (Sudha et al., 2014).

2.8.2.4 Oxytetracycline

Oxytetracycline is a broad-spectrum antibiotic that belongs to the tetracycline class of antibiotics. It is commonly used to treat various bacterial infections in humans and animals. Oxytetracycline was first discovered in the 1940s and has become essential for combating bacterial diseases. Oxytetracycline targets bacteria by inhibiting protein synthesis within their cells. Bacterial cells rely on protein synthesis for various essential processes, such as growth, reproduction, and the production of enzymes. The

tetracycline antibiotic class has long been the most effective for treating cholera, caused by *Vibrio cholerae* (Shutter & Akhondi, 2022).

Nonetheless, earlier research revealed a global increase in *V. cholera* strains resistant to tetracycline (Dengo-Baloi et al., 2017). Oxytetracycline interferes with the ribosomes, the cellular structures responsible for protein synthesis, thereby preventing the bacteria from producing vital proteins. The broad-spectrum nature of oxytetracycline means it can effectively target a wide range of bacteria. It is particularly effective against both Gram-positive and Gram-negative bacteria, including species such as *E. coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Chlamydia trachomatis*, among others. In addition to its antimicrobial properties, oxytetracycline exhibits anti-inflammatory effects, which can further contribute to its therapeutic benefits in certain conditions (Shutter & Akhondi, 2022). Responsible use of oxytetracycline is essential to preserving its effectiveness and preventing the emergence of antibiotic resistance.

2.9 Implications for Hatchery Management and Future Research

Implementing research findings can lead to more efficient and productive sea scallop hatcheries, contributing to an increased seafood supply. Understanding optimal conditions for scallop larvae can help develop sustainable practices, reducing environmental impact and resource use. Also, enhanced hatchery management can boost the profitability of scallop farming, benefiting both producers and the broader seafood industry.

The research findings have ramifications beyond the general area of improving sea scallop hatchery efficiency. They also provide specific suggestions for better hatchery management. By studying the bacterial communities that are connected to sea scallop larvae, specifically looking at the presence of *Flavobacterium, Pseudomonas, Psychrobacter,* and *Suttonella taxa* (Nicolas et al., 1996), hatchery managers can customize their cleaning methods to stop the growth of specific bacterial strains. Gaining a comprehensive understanding of the resistance profiles exhibited by these bacteria is of utmost

importance (Torkildsen & Magnesen, 2004). This knowledge enables the formulation of precise sanitization protocols that specifically target these bacteria, reducing the risk of contamination and fostering a more conducive environment for the growth and development of scallop larvae.

The practical implementation of these research findings has the potential to enhance the effectiveness of sea scallop hatcheries greatly. Implementing this focused cleaning methodology reduces the likelihood of bacterial-related issues and creates ideal circumstances for the advancement and proliferation of scallop larvae. As a result, this helps improve the long-term viability of hatchery operations by decreasing the adverse effects on the environment caused by antimicrobial drugs and promoting responsible use of resources (Nicolas et al., 1996).

Moreover, incorporating this knowledge into hatchery management procedures is consistent with the overarching objectives of promoting sustainable seafood production. By cultivating scallop larvae in meticulously controlled surroundings that prioritize their welfare, the business can progress towards adopting more conscientious and environmentally sustainable practices. This has a dual advantage since it enhances the profitability of individual hatcheries and strengthens the overall seafood business by guaranteeing a steady and resilient seafood provision (Couturier et al., 1995).

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Introduction

The chapter detailed the culturing procedure followed during this research. The procedure followed was described by Ishaq and Perry labs protocols for identifying scallop tank bacterial isolates.

3.2 Hatchery tank setup

We partnered with three coastal sea scallop hatcheries to obtain biofilm samples for microbiological and genomic analysis. The hatcheries from which samples were sourced are Downeast Institute (DEI), situated directly on the northern Gulf of Maine in Machias, Maine, US (44.4806°N, 67.5986°W); Mook Sea Farms in Walpole, Maine, US (43.5831°N, 69.3317°W); and the Darling Marine Center (DMC) in South Bristol, Maine, US (43.9364°N, 69.5802°W). The latter two are located a few hundred meters upstream on the tidal Damariscotta River, in the mid-coast section of the Gulf of Maine.

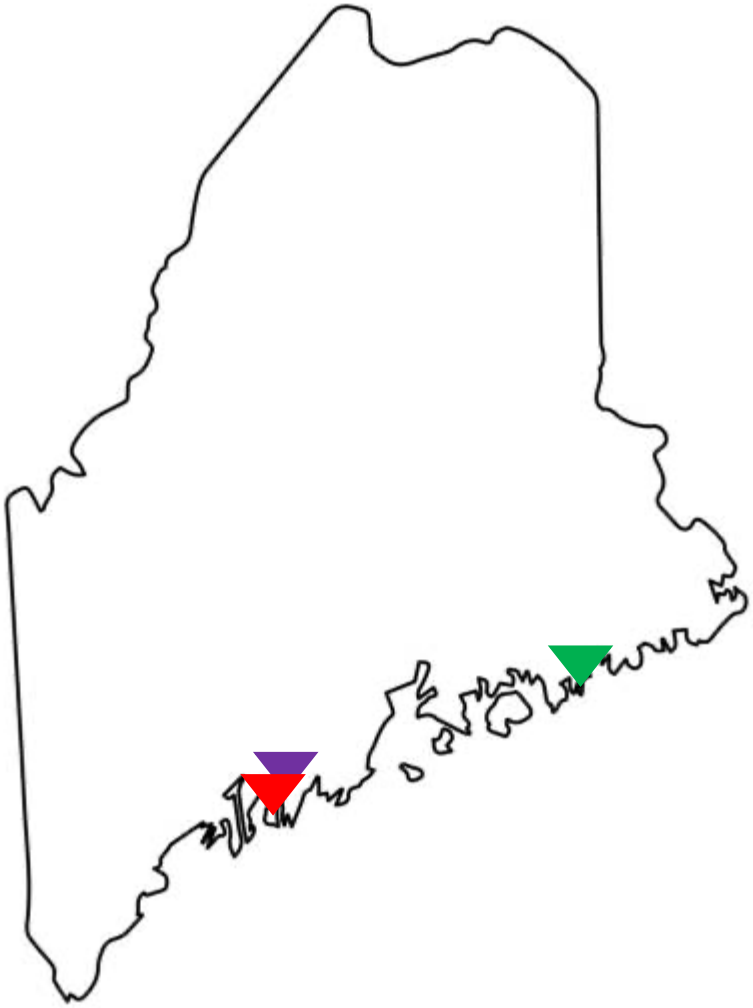

Figure 3.1: Hatchery tank locations in Maine

Down East Institue

Mook Sea Farm

Darling Marine Center

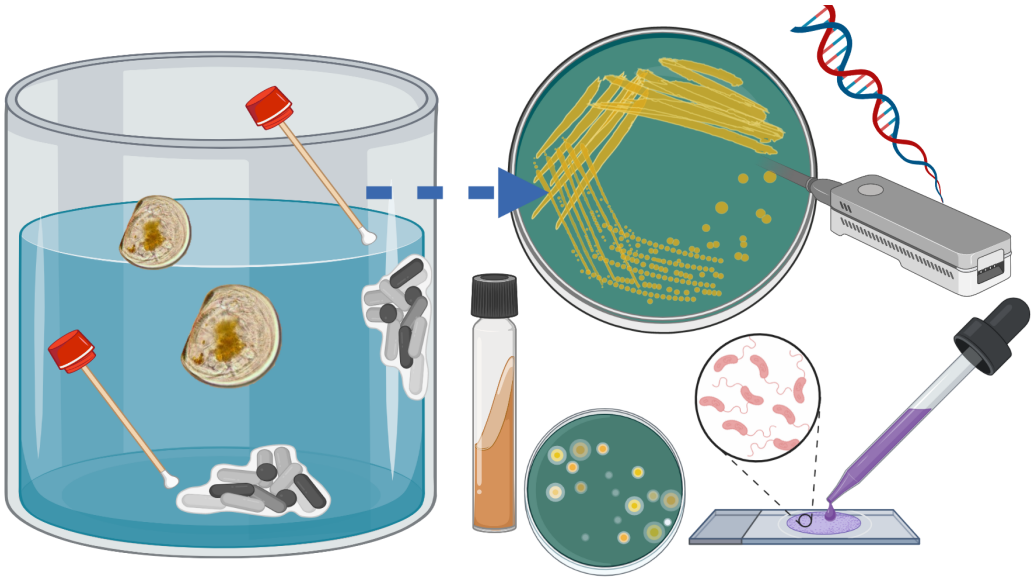

Figure 3.2: Illustrates the experimental design.

Atlantic sea scallop larvae (*Placopecten magellanicus*) hatchery tank biofilms were collected and applied on *Vibrio*-selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar culture plates using streaking technique. The bacterial isolates were analyzed utilizing microbiological and genomic methodologies. Illustration generated using biorender.

Adhering to standard production protocols that imitate industry norms, the water tanks used for larval development remain stationary (i.e., static tanks) for the initial ten days of life of scallop larvae. At this stage, the larvae reach a size of approximately 150 microns (measured along the shortest linear distance during the early stages). They are sufficiently large to be captured by 75-micron water filters. Following ten days, the larvae tanks were either kept in a static state or converted into tanks with a continuous flow of water (i.e., flow-through tanks) for the subsequent stages of development. The tanks were then populated with initial densities of either 5 or 15 larvae per milliliter. Prior research has demonstrated that the settlement of scallop larvae is most abundant and widely distributed when water turbulence is minimal to nonexistent (Pearce et al., 1998). The flow-through tanks employed a 10-micron filter to purify the circulating water further. However, ciliates were detected in the filtered water. With the exception of water circulation in the tanks, all other environmental and managerial characteristics were identical among the groups.

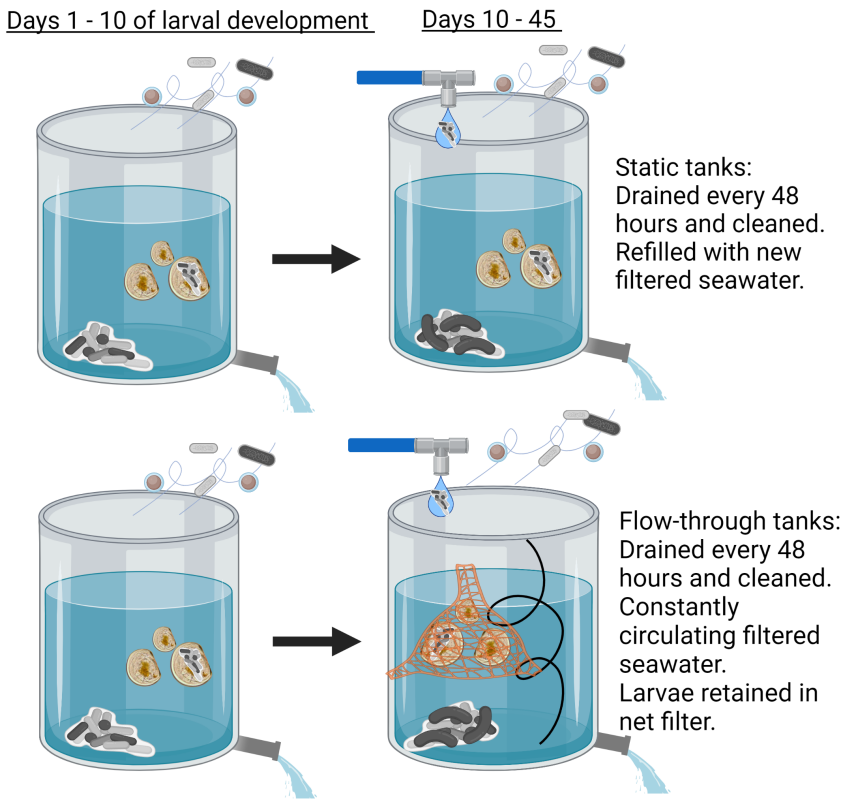

Figure 3.3: Scallop Tank design.

There were two larval tanks, both static-water tanks until day 10 of larval development and then one was switched to a flow-through tank that constantly filters and recirculates. All tanks were drained and cleaned every 48 hours.

Every 48 hours, scallop tanks are refilled with new water. First, scallop larvae are scooped out with a fine mesh net and moved to a temporary bucket. Next, water from the tanks is drained fully through a valve in the bottom. This is referred to as “drain down”, and any samples labeled “draindown” mean they are collected from drained and dirty tanks. Some tanks have a slope from the edge of the bottom to the drain, and some have a flat bottom. The tanks are cleaned with a bleach solution consisting of 1 oz of powdered Tide (Proctor & Gamble, purchased locally) and 100 ml bleach (Clorox, purchased locally) in two gallons of water. A common recommendation is to use a bleach solution at a concentration of approximately 200 ppm (parts per million) of active chlorine. To prepare this, you can dilute standard

household bleach (which typically contains 5-6% sodium hypochlorite) at a rate of approximately 1 milliliter (ml) of bleach per liter of water to achieve a 200-ppm solution. It's crucial to ensure the bleach is thoroughly mixed with the water. The solution is applied with brush bristles, allowing it to settle on the tank surface for two minutes, after which it is thoroughly rinsed. Any samples labeled “clean” have been collected after new seawater has been added.

To collect tank samples, sterile cotton swabs are scraped along the tank surface, covering an area approximately the size of a hand span (10 cm by 10 cm). This is usually done along the bottom of the tank for draindown/dirty tank samples, and on the side of the tank below the water line for cleaned tank samples. This method ensures a representative sample of the microbial population present on the surfaces of interest within the hatchery environment. After collection, swabs of tank surface biofilms from the Downeast Institute (DEI; Beals, Maine), Mook Sea Farms (Walpole, Maine), and Darling Marine Center (South Bristol, Maine) were immediately used at the hatchery site to culture selected bacteria. Swabs were rolled onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (VWR, Bridgeport NJ) as part of their routine pathogen screening because TCBS is selective for some *Vibrio* bacteria, although some other types of bacteria can grow on them, such as *Aeromonas*, *Pseudomonas*, *Proteus,* according to some TCBS manufacturer notes and online forums for microbiology. Plates were left at room temperature (18-22 ℃) for 24 - 48 hours to grow and saved at 4℃ for up to one week before transfer to UMaine.

3.3 Propagating from the initial culture to isolation plate

After the initial TCBS plates were transferred to UMaine, each individual colony was aseptically subcultured onto its own TCBS plate using the streak plate method, shown below.

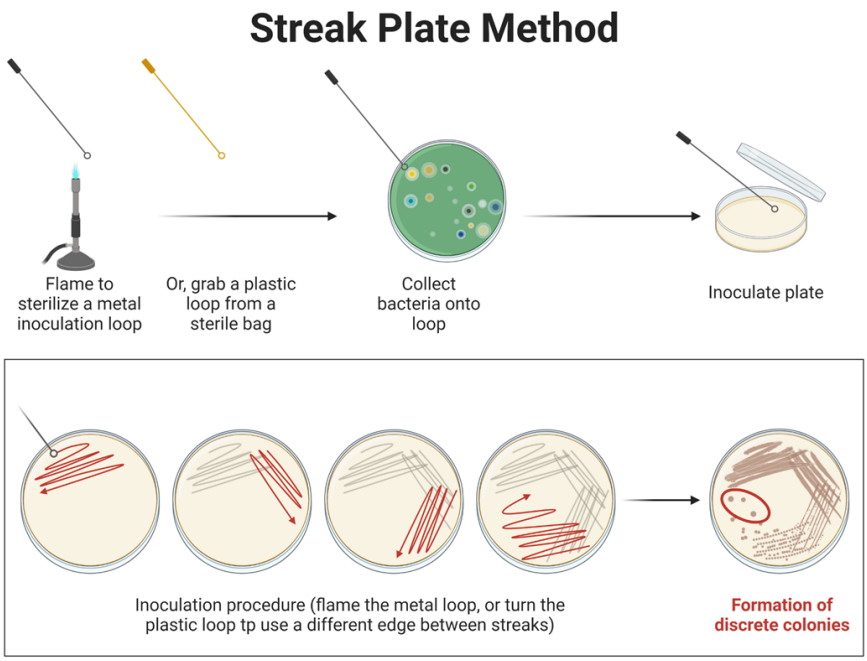

Figure 3.4: Streak Plate Method

The streak plate method is done to spread the cells out over a larger space so that individual bacterial cells will grow into new colonies. Spacing the colonies out helps us make sure that only one strain/isolate regrows on the streak plate. If two strains grow on the new plate, the colonies are picked again and streaked onto new plates until the colonies are isolated.

Isolate culturing

Once the plates were streaked with bacteria from the swabs, plates were incubated at room temperature (18-22 ℃) and examined at 24 and 48 hours, after which they were sealed with parafilm (VWR, Bridgeport NJ) stored at 4℃. Once each colony had developed, they were moved to 1.5mL centrifuge tubes containing 1mL of alkaline peptone water (APW) media (VWR, Bridgeport NJ). After observing growth in the APW media (assessed as turbidity), the multiple aliquots of the samples were moved to a new 1.5 mL cryotube in a 1:1 proportion with sterile 80% glycerol. These samples were then archived at -80°C for future research purposes.

The isolates that grew on TCBS plates were subjected to visual identification and analysis of bacterial morphology. This entailed a thorough scrutiny of the colony on the solid substrate. The evaluated attributes encompassed the bacterial colonies' surface texture, transparency, margin, elevation, and colour or pigmentation. The texture evaluation encompassed descriptors such as smooth, glistening, mucoid, slimy, dry, powdery, and flaky. The assessment of transparency was determined by categorizing colonies as either transparent (permitting visibility), translucent (permitting light transmission), or opaque (possessing a solid appearance). In addition, the colonies were examined for their colour or pigmentation, which exhibited variations including yellow, pink, purple, red, white, or grey. The thorough examination yielded significant observations regarding the structural traits of the bacterial isolates, enhancing our complete comprehension of their attributes within the scope of the research study. This was done because bacteria can exhibit variations in appearance when cultivated on plates among other competing bacteria. Relevant internet resources for describing bacteria and identifying potential *Vibrio* species include the supplier's product information, as well as online sources because of their more detailed images: microbiologyinfo.com, microbiologysociety.org, and microbeonline.com. On TCBS agar, sucrose-fermenting strains grow yellow, due to a pH-indicating dye in the medium which reads to acid, and most other strains grow green, however slow-growing strains may appear green or very faintly yellow after 24 hours. *Pseudomonas* spp.*, Aeromonas* spp. and other bacteria may appear as blue/green colonies (non-sucrose fermenting). Due to the inability to standardize the surface area of swabbing tanks, we are unable to calculate the number of colonies forming units per square area in the tanks. Due to the absence of reported data on no-growth plates from the hatcheries, we are unable to calculate the frequency/proportion of finding any isolate across all plates made.

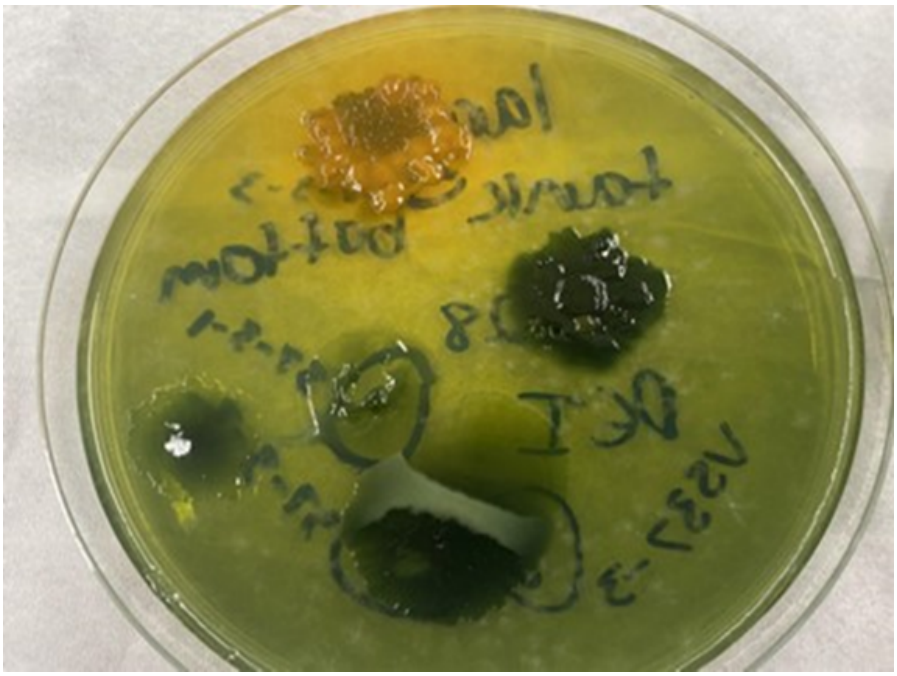

Figure 3.5: Image of suspected Vibrio colonies growing on a TCBS plate that was streaked using a swab of the bottom of a dirty scallop larvae tank

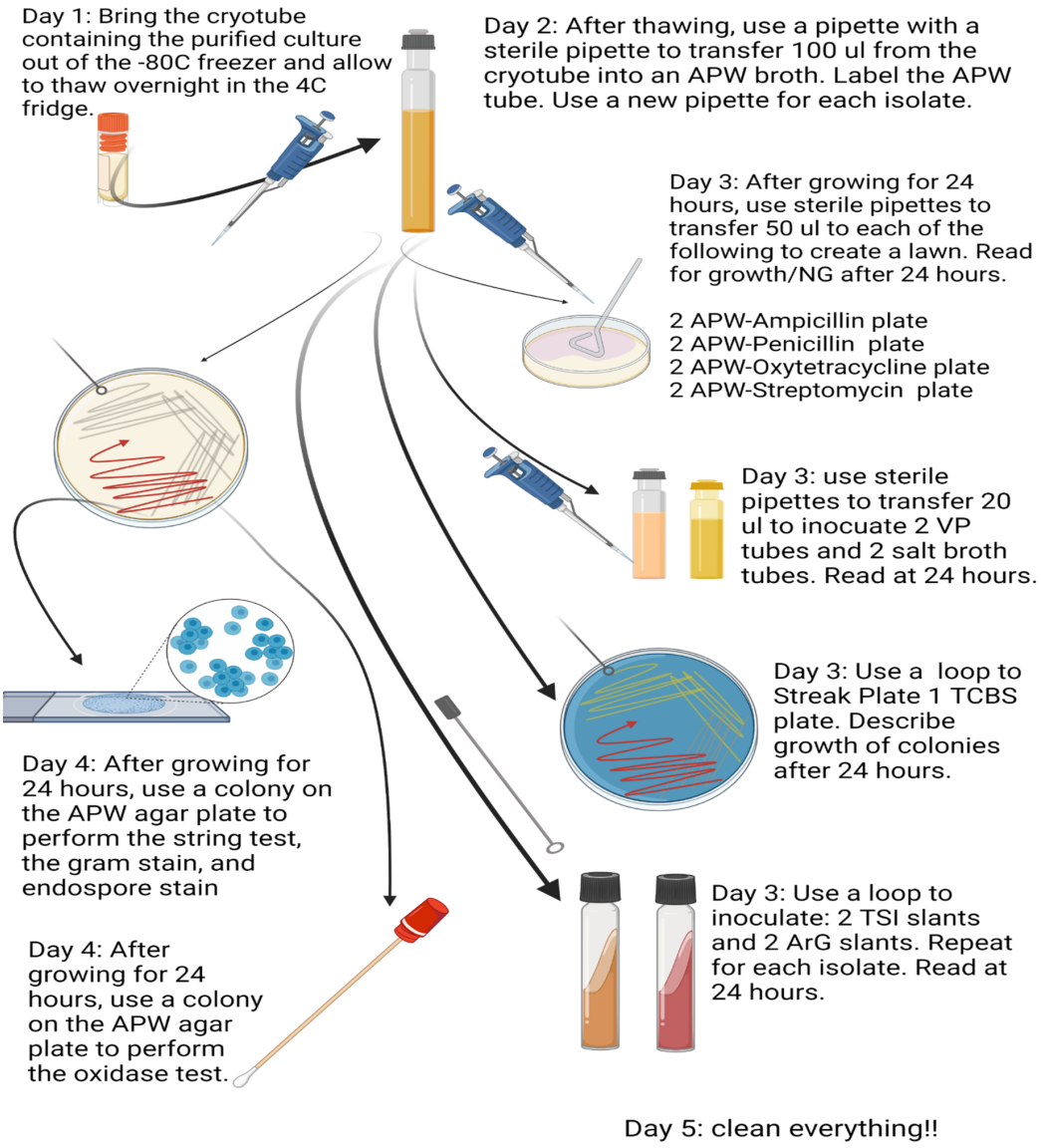

Figure 3.6: Experimental Design showing a sequential process for culturing and testing bacterial isolates obtained from scallop larvae, spanning five days.

The figure describes the procedure for reviving frozen cultures, introducing them into different selective and differential mediums, and conducting tests to determine the identity and characteristics of bacterial isolates.

This procedure is commonly employed in microbiological studies in scallop larvae research to identify harmful bacteria that could impact the health of the larvae. This design aims to separate and

detect any disease-causing microorganisms, analyze their ability to resist antibiotics, and gather information about their metabolic functions. These factors are crucial for effectively maintaining the health of larvae and preventing disease outbreaks in aquaculture environments.

The following is a detailed explanation of the workflow depicted in the figure above, and all tests were completed in duplicate or triplicate:

Day 1: We initiated the thawing process of one aliquot in a cryotube carrying a pure culture from a -80°C freezer by placing it in a 4°C refrigerator and letting it defrost overnight. The purpose of this phase is to delicately reanimate the bacterial culture without causing any abrupt disturbance to the cells, which could potentially originate from scallop larvae.

Day 2: A pipette was used to transfer 100 µl from the thawed cryotube into a labelled APW broth tube. It was ensured that a new pipette was utilized for each isolate to prevent any cross-contamination. APW, also known as Alkaline Peptone Water, is a growth medium that promotes the development of *Vibrio* species. These bacteria are frequently found in marine habitats and may be linked to the presence of scallop larvae.

Day 3: Following 24 hours of growth in APW at 16- ^{0}C in Biosafety Level 2 hood, a sequence of subculturing procedures was performed. Some isolates were also cultured at 4^{0}C in a refrigerated incubator, or 20, 25, and 30^{0}C in a heated incubator, to improve their initial growth. Procedures are as follows:

Using sterile pipettes, 50 µl of the isolate sample was transferred onto agar plates containing 0.1g/l of various antibiotics (Ampicillin, Penicillin, Oxytetracycline, or Streptomycin; VWR). This procedure was conducted to confirm the activities of the antibiotics against the isolates by looking for bacterial growth. The presence or absence of growth on these antibiotic-containing plates would indicate the isolates' susceptibility or resistance to each antibiotic, respectively, thereby providing valuable information on their antimicrobial resistance profiles. This was done to assess the bacterial resistance.

Additionally, 20 µl of the sample was transferred to inoculate tubes for VP (Voges-Proskauer) and high-salt nutrient broth (Beef extract, 3.0 g/l; Peptone 5.0 g/l; Sodium chloride, 10.0 g/l; VWR). Subsequently, we monitored the tubes for 24 at 25-30 ^{0}C hours to evaluate any signs of development and probable metabolic activities.

Fifty microliters of the isolate sample were applied onto a TCBS (Thiosulfate Citrate Bile Salts Sucrose) agar plate, a specialized medium to cultivate *Vibrio* bacteria selectively. The purpose was to examine the formation of bacterial colonies on the plate after 24 hours at 16 ^{0}C to validate the previous colony descriptions. In addition, we introduced the bacteria using the stab and streak procedure to TSI (Triple Sugar Iron) slants and ArG (Arginine) slants for 24 hours at 25-30 ^{0}C to evaluate their capacity for sugar fermentation and arginine utilization.

Day 4: On the fourth day, a colony was cultured on Alkaline Peptone Water (APW) agar to conduct a series of tests. A Gram stain was performed to ascertain the Gram reaction of the microorganisms. A "string test" was performed to visualize viscosity or slime generation, as most *Vibrio* spp. are positive, while *Aeromonas* spp. are negative. The test can provide information about the ability to develop biofilms (Shaik et al., 2015). There was suspicion that the bacteria could form endospores to survive harsh conditions, so an endospore stain was performed. The oxidase test was used to assess the ability to detoxify reactive oxygen species by detecting the presence of cytochrome c oxidase, an enzyme characteristic of specific bacteria, such as certain *Vibrio* species.

Day 5: To maintain the cleanliness and sterility of the laboratory, it was imperative to clean all materials used thoroughly as *Vibrio* are biosafety level II organisms. The task was completed on the fifth day of the isolate culturing test.

3.4 Bacterial Community Sequencing

Bulk DNA was extracted from veligers, swabs from tank surfaces, or no-template (water or the ethanol used to preserve wild veligers) control samples (one from each extraction batch) using

commercially available kits optimized for water and tissue-based microbial communities (Qiagen Powersoil kit for veligers and Zymo Genomic DNA & Concentrator kit for biofilm swabs), and some aliquots were archived by returning the tubes in the -80^{0}C fridge-freezer. DNA extract was roughly quantified and purity-checked with a Nanodrop spectrophotometer (Thermo Scientific, USA). Samples underwent DNA amplicon sequencing of the 16 S rRNA gene V3-V4 region, using primers 341 F (Fadrosh et al., 2014) and 806 R (Caporaso et al., 2011) and protocols consistent with The Earth Microbiome Project (The Earth Microbiome Project WWW Document, 2011), and sequenced on an Illumina MiSeq platform using the 2 × 300-nt V3 kit (Molecular Research Labs, Clearwater, TX).

To examine the bacterial communities in hatchery D-stage veligers, amplicon sequence data was processed using previously curated workflows (Supplemental Material, Ishaq et al., 2023), which used the DADA2 pipeline ver. 1.24 (Callahan, 2022) in the R software environment ver. 4.2 (RcoreTeam, 2022). Plots were made using the ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2022), and phyloseq packages. A subset of the bacterial community data was used in this analysis to compare sequencing results to culturing results.

CHAPTER FOUR

4 RESULTS

4.1 Isolate culturing

In order to assess sucrose fermentation, we categorized the colony morphology of bacterial isolates based on color (Fig. 4.1), as it may vary depending on the culturing circumstances. Yellow colonies indicated sucrose fermentation, whereas green colonies indicated the absence of sucrose fermentation. The predominant bacterial cultures consisted mainly of yellow or green strains, with a few exceptions such as white strains, which could potentially be either enterobacteria or slow-growing *Vibrio* species (Oliver et al., 1983). The colonies exhibiting a green color are most probable to be *V. parahaemolyticus, Pseudomonas,* or *Aeromonas*. On the other hand, the colonies displaying a yellow color might potentially be *V. cholera, V. alginolyticus, V. vulnificus, Proteus* spp*., Bacillus* spp., or *Enterococcus faecalis* (BD, 2003; Aryal, 2022).

Upon receiving plates straight from the hatcheries, it was seen that yellow and green colonies often coexisted on the same plate. In addition, it was frequently seen that the yellow and green colonies would overlap with each other. On most days, colonies of both green and yellow colors were isolated from TCBS plates. However, on certain days, only yellow colonies were obtained (Yellow colonies indicated sucrose fermentation, whereas green colonies indicated the absence of sucrose fermentation with a few exceptions such as white strains and No Account (N/A) which here indicates that the colonies were not described initially and could not be revived after initial archiving.

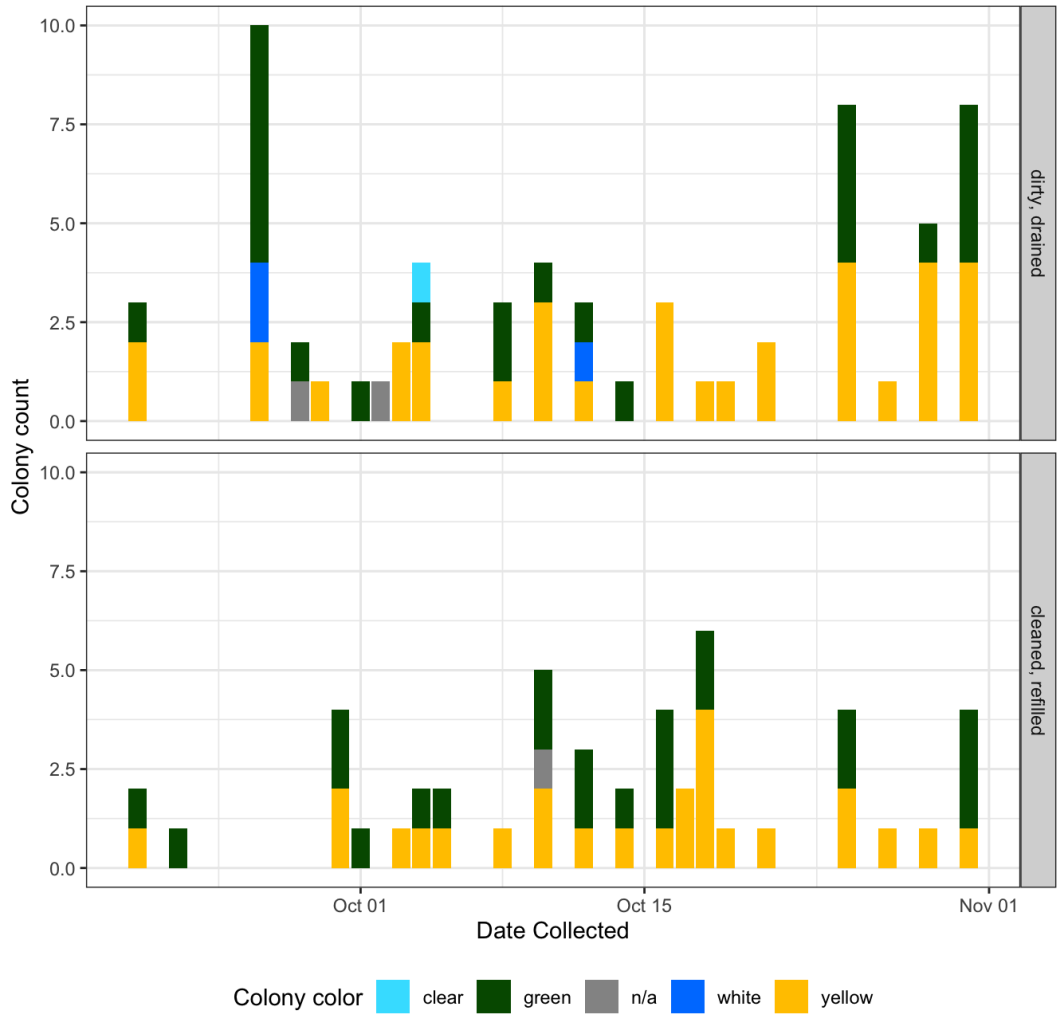

Figure 4.1: Colony morphology of bacterial isolate in tanks based on color and date collection.

Yellow colonies indicated sucrose fermentation, whereas green colonies indicated the absence of sucrose fermentation with a few exceptions such as white strains and No Account (N/A) which here indicates that the colonies were not described initially and could not be revived after initial archiving.

The number of isolates collected from each location varied, with 18 retrieved at Mook Farm, 113 from DEI, and 6 from DMC. The increased number of plates acquired from DEI over an extended duration primarily indicates limitations in sampling across various locations rather than variations in the number of plates showing growth. Based on personal observations, we have seen that the same morphotypes consistently appear in the same tanks when sampled on different days and between

cleanings. Static tanks exhibit greater colony growth and a higher proportion of "yellow" isolates compared to flow-through tanks. However, due to the absence of tank replication (n = 2 tanks per static or flow-through, and flow-through only used for 10 days of the experiment), statistical analysis could not be conducted (Yellow colonies can ferment sucrose in the media, while green colonies cannot. Static and flow-through (constant recirculation) are two main tank conditions for seawater treatment in larvae tanks. No Account (n/a) shows no record obtained as not all tank conditions were swabbed at all hatcheries.

In the DEI larvae tanks across all sampling points and tank setups, there were yellow (59), green (46) colonies, and 7 isolates would not grow. The static environment resulted in the formation of 44 yellow colonies (55%) and 30 green colonies (38%), suggesting a greater prevalence of sucrose fermenting microorganisms than in flow-through tanks. In the flow-through experiment, the number of colonies was reduced compared to the static tank because these tanks were used for a shorter period, and there was a more even distribution of yellow (47%) and green (50%) colonies. The presence of clean conditions led to a slightly higher percentage of yellow colonies in comparison to dirty conditions, suggesting that cleanliness can influence the growth of different *Vibrio* species.

Table 4.1

4.2 TCBS results

Yellow colonies can ferment sucrose in the media, while green colonies cannot. Static and flow-through (constant recirculation) are two main tank conditions for seawater treatment in larvae tanks. No Account (n/a) shows no record obtained as not all tank conditions were swabbed at all hatcheries.

In the DEI larvae tanks across all sampling points and tank setups, there were yellow (59), green (46) colonies, and 7 isolates would not grow. The static environment resulted in the formation of 44 yellow colonies (55%) and 30 green colonies (38%), suggesting a greater prevalence of sucrose fermenting microorganisms than in flow-through tanks. In the flow-through experiment, the number of

colonies was reduced compared to the static tank because these tanks were used for a shorter period, and there was a more even distribution of yellow (47%) and green (50%) colonies. The presence of clean conditions led to a slightly higher percentage of yellow colonies in comparison to dirty conditions, suggesting that cleanliness can influence the growth of different *Vibrio* species.

Table 4.1: Isolate counts by hatchery, sucrose fermentation, and tank setup.

	DownEast Institute			**Darling Marine Center**		**Mook Sea Farms**	
Tank Condition	**Sucrose fermenting isolates**	**Non-sucrose-fermenting isolates**	**No Growth**	**Yellow colonies**	**Green colonies**	**Yellow colonies**	**Green colonies**
Static	44, (55%)	30, (38%)	66, (7%)	2, (29%)	3, (43%)	6, (50%)	6, (50%)
Flow-Through	15, (47%)	16, (50%)	1, (3%)	n/a	n/a	3, (50%)	3, (50%)
Clean	25, (52%)	22, (46%)	1, (2%)	n/a	n/a	n/a	n/a
Dirty	34, (53%)	24, (38%)	6, (9%)	3, (43%)	3, (43%)	9, (50%)	9, (50%)
Static and clean	18, (55%)	14, (42%)	1, (3%)	n/a	n/a	n/a	n/a
Static and dirty	26, (55%)	16, (34%)	5, (11%)	2, (29%)	3, (43%)	6, (50%)	6 (50%)
Flow-through and clean	7, (47%)	8, (53%)	0	n/a	n/a	n/a	n/a
Flow-through and dirty	8, (47%)	8, (47%)	1, (6%)	n/a	n/a	3, (50%)	3, (50%)
Total isolates from Larvae Tanks	59, (53%)	46, (41%)	7, (6%)	3, (43%)	3, (43%)	9, (50%)	9, (50%)

Based on TCBS agar's properties, yellow colonies (Fig. 4.2) indicate the probable presence of *Vibrio* species capable of sucrose fermentation, potentially including *V. cholerae, V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. mimicus* etc. The green colonies are most probably different *Vibrio species* or other bacterial strains such as *Aeromonas, Pseudomonas*, or *Proteus*. These strains can occasionally thrive on TCBS agar but generally do not ferment sucrose (Aryal, 2022)

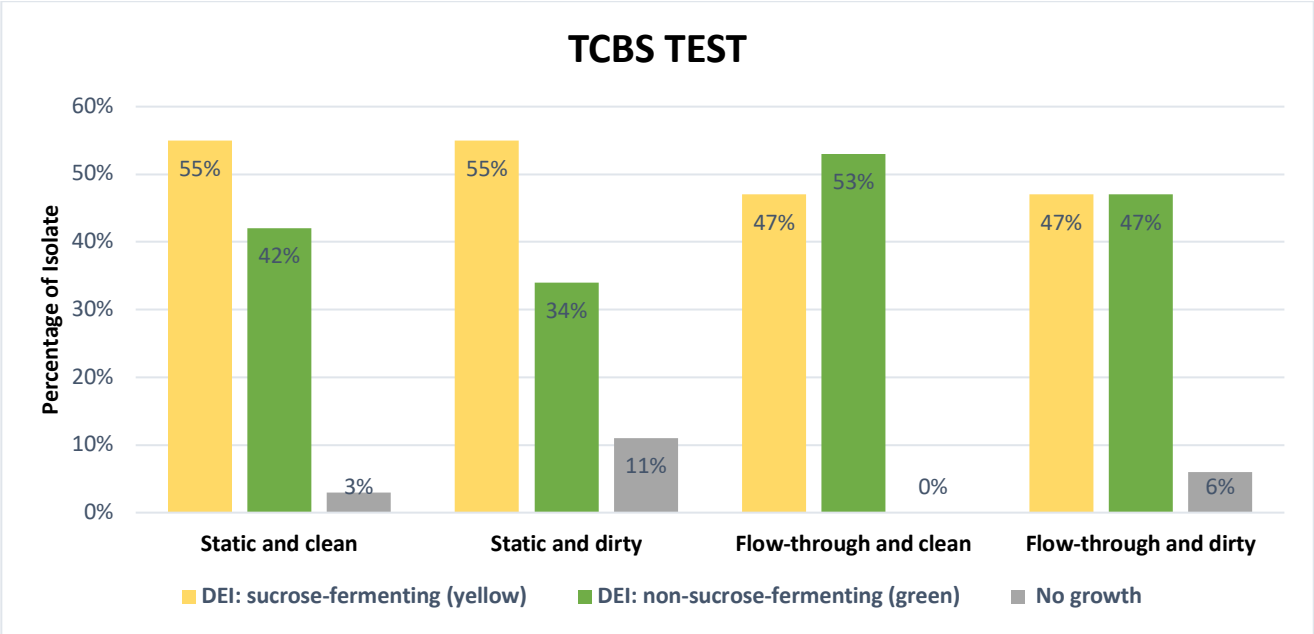

Figure 4.2: TCBS Agar Plate Colony Counts and coloration from DEI Hatchery Tanks.

The bar chart provides a quantitative representation of bacterial isolates cultivated on TCBS agar plates. It indicates the presence of a presumptive *Vibrio* isolate based on the number of yellow and green colonies and the isolate that didn't grow. The colonies are separated based on the cleanliness of the tank conditions (clean or dirty) and the water flow (static or flow-through).

The percentages may indicate that two individual isolates are unlikely to belong to the same species. For instance, in these tanks, bacterial species that can ferment sucrose tend to be more prevalent in static and clean environments. In contrast, they do not exhibit a significant preference for growth in flow-through and dirty conditions.

However, these initial observations from TCBS agar testing are not definitive for species identification. Therefore, additional tests were necessary to complement these findings. Biochemical assays like the oxidase test can differentiate *Vibrio* species from other Gram-negative bacteria, while the

arginine dihydrolase test further distinguishes among *Vibrio* species. Triple Sugar Iron (TSI) agar is also employed to provide a more comprehensive picture of the carbohydrate fermentation profile and gas production, enhancing the specificity of the microbial identification process.

4.3 Triple Sugar Iron (TSI) Results

The table shows the full breakdown of the results from the TSI test, which tells the difference between bacterial isolates based on their ability to ferment sugars (lactose, glucose, and sucrose) in both anaerobic (a yellow butt) and aerobic (a yellow slant) conditions. There are both counts and percentages of positive reactions in the data, which show how metabolically diverse the bacterial communities were in the tank setup. Not all isolates would grow on TSI, thus the total isolates row is specific to this test.

Tank Condition	DEI: yellow butt (sugar fermentation in anaerobic media)	DEI: yellow slant (sugar fermentation in aerobic media)	No Growth
Static	15, (19%)	16, (20%)	49, (61%)
Flow-Through	11, (34%)	11, (34%)	10, (32%)
Clean	12, (25%)	13, (27%)	23, (48%)
Dirty	14, (22%)	14, (22%)	36, (56%)
Static and clean	7, (21%)	8, (24%)	18, (55%)
Static and dirty	8, (17%)	8, (17%)	31, (66%)
Flow-through and clean	5, (33%)	5, (33%)	5, (34%)
Flow-through and dirty	6, (35%)	6, (35%)	5, (30%)
Total isolates from all larvae tanks	26, (23%)	27, (24%)	59, (53%)

Table 4.2: TSI Test Results for Bacterial Isolates from Different Tank Conditions.

The table shows the full breakdown of the results from the TSI test, which tells the difference between bacterial isolates based on their ability to ferment sugars (lactose, glucose, and sucrose) in both anaerobic (a yellow butt) and aerobic (a yellow slant) conditions. There are both counts and percentages of positive reactions in the data, which show how metabolically diverse the bacterial communities were

in the tank setup. Not all isolates would grow on TSI, thus the total isolates row is specific to this test.

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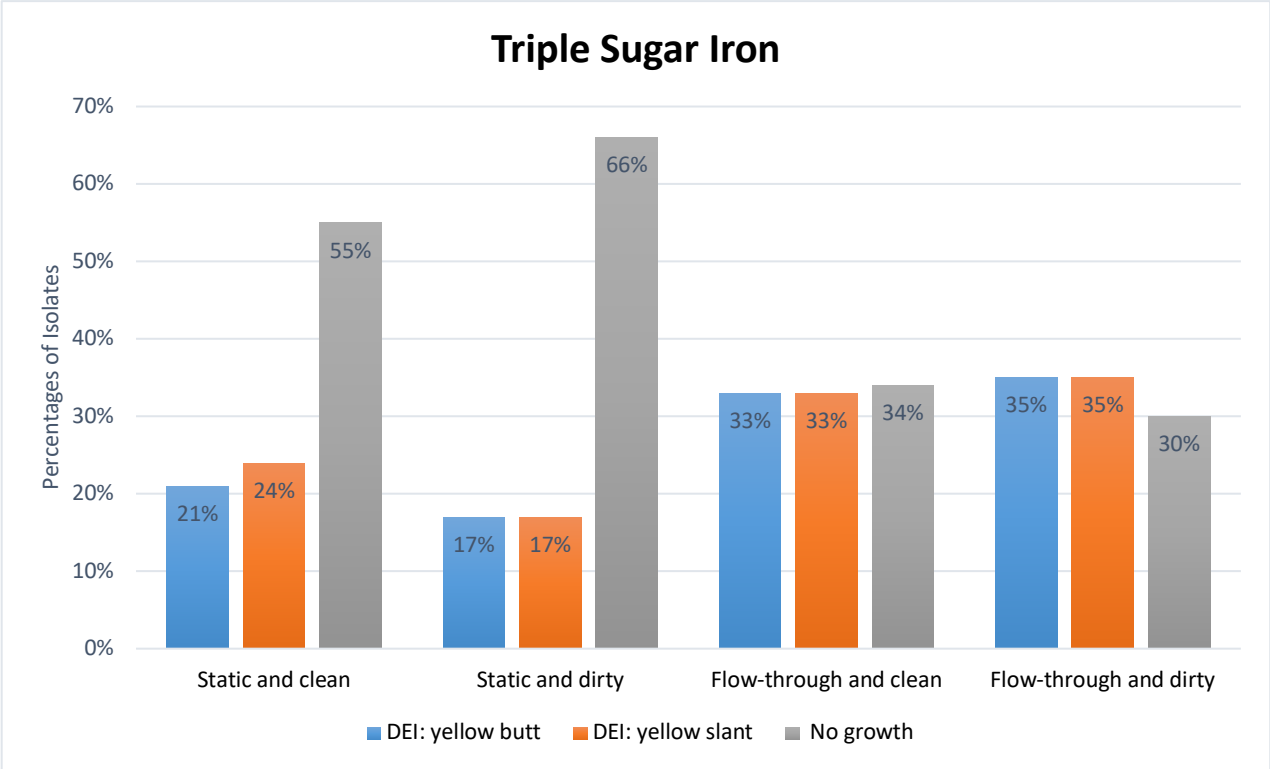

Figure 4.3: TSI Agar Slant Reaction Patterns in Bacterial Isolates

The graph depicts the number of bacterial isolates obtained from scallop hatchery tanks that exhibit a yellow butt or slant reaction on TSI agar. This reaction indicates sugar fermentation under anaerobic and aerobic conditions, respectively. The data is categorized based on the tank's cleanliness and flow conditions.

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presents a concise overview of how environmental conditions can impact hatchery tank bacteria's metabolic activities, specifically sugar fermentation and arginine usage. These results are characteristic of *Vibrio* species' metabolic activities in a TSI slant test, providing useful information for their preliminary identification. It's important to note that the TSI slant test is part of a broader suite of biochemical tests used for the identification of Enterobacteriaceae and other gram-negative rods, and while *Vibrio* species are not members of the Enterobacteriaceae family, their behavior in this test can still offer valuable diagnostic clues.

4.4 Arginine Test

The presented table displays the outcomes of the arginine dihydrolase test, which demonstrates the anaerobic (yellow butt) and aerobic (yellow slant) fermentation of glucose by bacterial isolates from different tank conditions. If the media remains purple, it indicates an alkaline reaction from using arginine instead of the glucose. The numbers represent the overall count of isolates exhibiting a positive response, while the percentages indicate the ratio of positive responses among all isolates tested for each tank condition. Not all isolates would grow on arginine; thus, the total isolates row is specific to this test.

Table 4.3: Arginine dihydrolase test results for bacterial isolates from different tank conditions

Tank Condition	DEI: yellow butt (sugar fermentation in anaerobic portion)	DEI: yellow slant (sugar fermentation in aerobic portion)	No Growth
Static	37, (46%)	29, (36%)	14, (18%)
Flow-Through	18, (56%)	18, (56%)	0
Clean	29, (60%)	25, (52%)	2, (4%)
Dirty	26, (41%)	22, (34%)	16, (25%)
Static and clean	21, (64%)	17, (52%)	0
Static and dirty	16, (34%)	12, (26%)	19, (40%)
Flow-through and clean	8, (53%)	8, (53%)	1, (6%)
Flow-through and dirty	10, (59%)	10, (59%)	1, (6%)
Total isolates from all larvae tanks	55, (49%)	47, (42%)	21, (9%)

The presented table displays the outcomes of the arginine dihydrolase test, which demonstrates the anaerobic (yellow butt) and aerobic (yellow slant) fermentation of glucose by bacterial isolates from different tank conditions. If the media remains purple, it indicates an alkaline reaction from using arginine instead of the glucose. The numbers represent the overall count of isolates exhibiting a positive response, while the percentages indicate the ratio of positive responses among all isolates tested for each tank condition. Not all isolates would grow on arginine; thus, the total isolates row is specific to this test.

Table 4.3: Arginine dihydrolase test results for bacterial isolates from different tank conditions

The presented table displays the outcomes of the arginine dihydrolase test, which demonstrates the anaerobic (yellow butt) and aerobic (yellow slant) fermentation of glucose by bacterial isolates from different tank conditions. If the media remains purple, it indicates an alkaline reaction from using arginine instead of the glucose. The numbers represent the overall count of isolates exhibiting a positive response, while the percentages indicate the ratio of positive responses among all isolates tested for each tank

condition. Not all isolates would grow on arginine; thus, the total isolates row is specific to this test shows a significant percentage of bacteria in all tank conditions can ferment carbohydrates and utilize arginine, as evidenced by the yellow coloration observed in both the butt and slant sections. The "Static and Clean" condition has the highest proportions of yellow butt and slant, indicating that a static water environment may promote bacterial sugar fermentation. The "Static and Dirty" condition exhibits the lowest proportions of both yellow butt and slant, suggesting that this state may be less conducive to these bacterial activities or that distinct bacterial populations are present. The presence of larvae suggests moderate amounts of sugar fermentation and arginine usage, as evidenced by the percentages approaching 50%. The "Flow-Through" condition consistently influences bacterial activity, irrespective of cleanliness, resulting in a more significant proportion of samples exhibiting yellow butt and slant compared to other conditions.

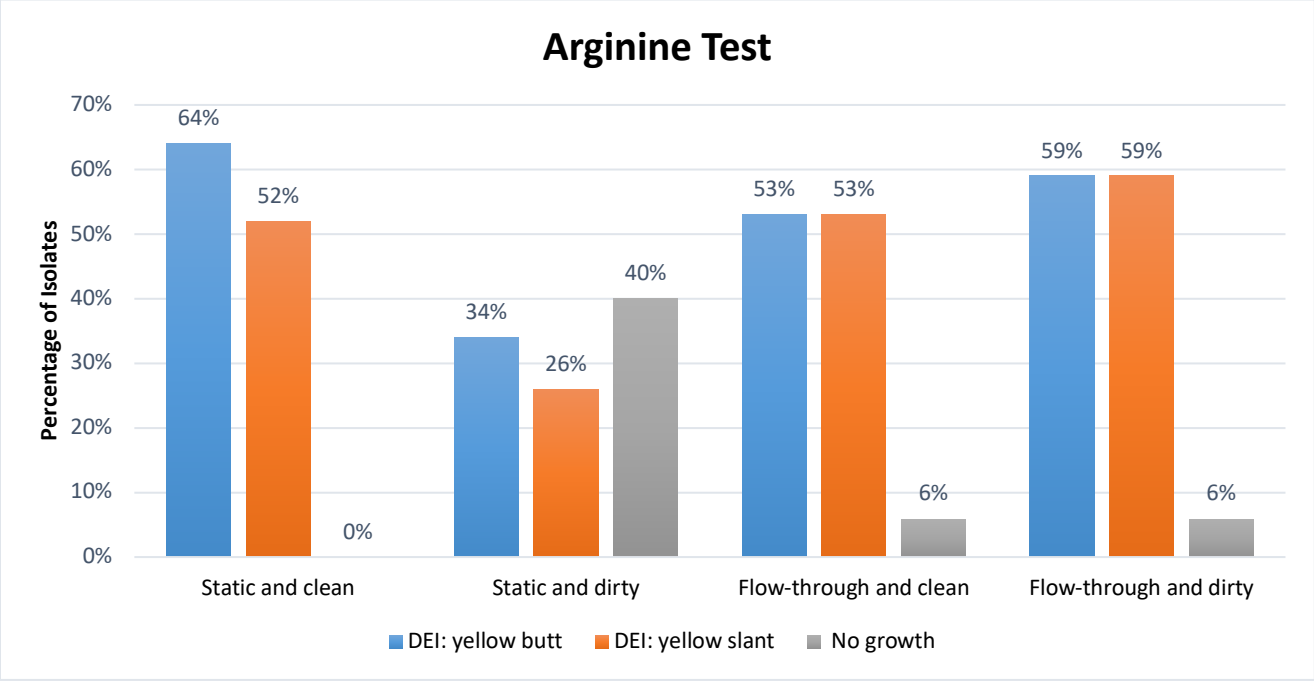


Figure 4.4: Arginine utilization in bacterial isolates from various tank conditions.

This figure displays the isolate count that demonstrates the growth of arginine in different tank conditions at a DEI hatchery. The growth on arginine is categorized by the presence of yellow butt

(anaerobic growth) and yellow slant (aerobic growth), with a comparison to the total isolate count for each condition.

The specific percentages and counts of yellow responses (This figure displays the isolate count that demonstrates the growth of arginine in different tank conditions at a DEI hatchery. The growth on arginine is categorized by the presence of yellow butt (anaerobic growth) and yellow slant (aerobic growth), with a comparison to the total isolate count for each condition.

The butt and slant can be utilized to infer the metabolic traits of the bacterial populations under varying environmental circumstances. This information is crucial for comprehending the ecology of hatchery tank bacteria and can have ramifications for aquaculture practices and disease control in marine environments.

4.5 Antibiotics Result

The resistance levels vary based on the specific antibiotic and the tested bacterial species. Typically, a concentration of 100 mg/l is regarded as high for most antibiotics and can frequently be used to target resistant bacteria specifically. Nevertheless, defining resistance breakpoints for individual antibiotics is crucial by referring to recognized standards or past studies. A concentration of 100 mg/l of ampicillin would typically select for resistant bacteria in various bacterial species. The level of streptomycin resistance might vary, but a concentration of 100 mg/l is generally regarded as high and has the potential to favor the growth of resistant strains. The degrees of resistance to penicillin can vary, but a concentration of 100 mg/l would probably result in the selection of resistant bacteria. Resistance to oxytetracycline can emerge at doses of approximately 100 mg/l, especially when this antibiotic is often employed.

This table shows the number of isolates that grew on APW media with one of four antibiotics (ampicillin, streptomycin, penicillin, or oxytetracycline) added at 100 mg/l, approximately 5 mg in a 50 ml agar plate. The hatchery's static, flow-through, clean, and dirty environments show different numbers of isolates that could grow on this concentration of these antibiotics.

Table 4.4: Antibiotic resistance profile of bacterial isolates

Tank condition	DEI: growth on ampicillin	DEI: growth on streptomycin	DEI: growth on penicillin	DEI: growth on oxytetracycline
Static	29, (36%)	4, (5%)	28, (35%)	8, (10%)
Flow-Through	22, (69%)	4, (13%)	21, (66%)	5, (16%)
Clean	26, (54%)	3, (6%)	24, (50%)	6, (13%)
Dirty	25, (39%)	5, (8%)	25, (39%)	7, (11%)
Static and clean	15, (45%)	2, (6%)	14, (42%)	4, (12%)
Static and dirty	14, (30%)	2, (4%)	14, (30%)	4, (9%)
Flow-through and clean	11, (73%)	1, (7%)	10, (67%)	2, (13%)
Flow-through and dirty	11, (65%)	3, (18%)	11, (65%)	3, (18%)
Total isolates from all larvae tanks	51, (46%)	8, (7%)	49, (44%)	13, (12%)

Ampicillin (A) media demonstrates variability in bacterial resistance across different environments, with the Flow-Through condition revealing the highest proportion of resistant isolates, indicated by a 69% growth rate. These findings indicate that the constant flow of water in tanks may select for bacteria which are slow-growing and do not constantly need cell wall synthesis (the target of ampicillin) or are better at sticking to tanks through the formation of exopolysaccharides or biofilm proteins, which would inhibit the diffusion of antibiotics into the biofilm.

Streptomycin (B) showed negligible bacterial growth under all settings, indicating its broad-spectrum efficacy against many bacterial species. The significant rise in resistance in both Flow-Through

and Dirty tank settings, reaching 18%, suggests the development of bacteria that are either naturally resistant to Streptomycin's effects on protein synthesis or have acquired mutations that provide resistance. The bacteria may be adjusting to environmental challenges by altering their ribosome targets to resist the effects of Streptomycin.

Penicillin (C) and Ampicillin show comparable resistance patterns, indicating a general trend of resistance to β-lactam antibiotics. Similar high resistance rates were recorded in both clean and dirty conditions. Flow-through circumstances at 66% and 65% suggest that environmental factors in these settings may support bacteria with β-lactamase enzymes or other resistance mechanisms that render the antibiotic ineffective in inhibiting cell wall formation.

The reduced resistance levels to Oxytetracycline (D) compared to other antibiotics, as shown by limited growth percentages in all tank settings, indicate an effective mechanism of action against bacterial protein synthesis. Flow-Through tanks show somewhat increased resistance, suggesting that bacteria may be adapting by either reducing Oxytetracycline absorption or increasing efflux to survive and proliferate in the presence of this antibiotic.

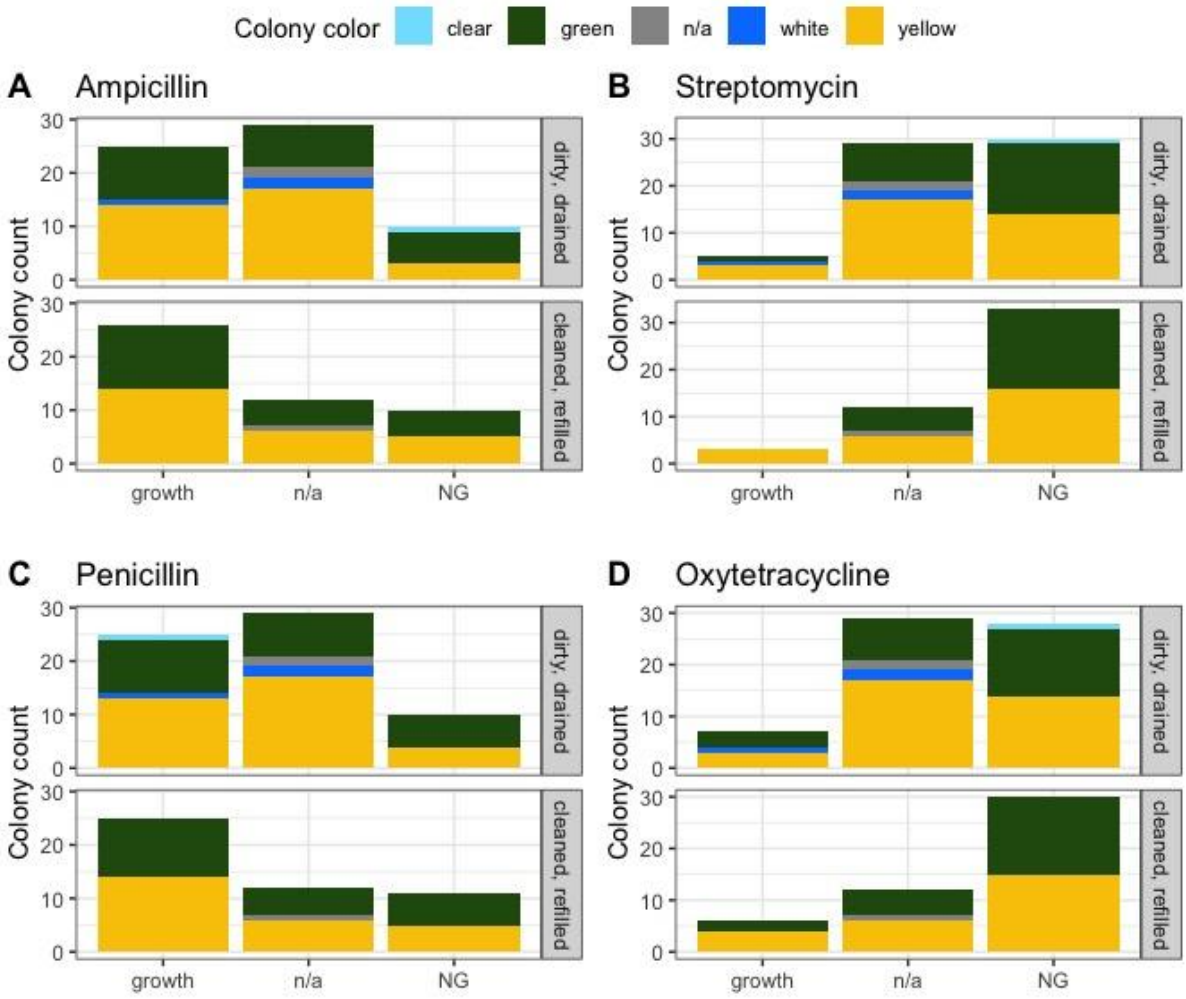


Figure 4.5: Antibiotic susceptibility profiles for bacterial colonies from isolate samples.

Bacteria exposed to both clean and dirty flow-through conditions exhibit increased resistance to ampicillin and penicillin. In certain instances, bacteria found in clean environments may exhibit a relatively high resistance to penicillin and ampicillin, suggesting that physical abrasion and bleach cleaning is not manually removing these biofilms.

Our research suggests that streptomycin and oxytetracycline were effective against these isolates, implying that we are unsure if we have *Vibrio* spp., which is susceptible to both.

4.6 Oxidase result

The oxidase test is used to ascertain the presence of cytochrome c oxidase. This enzyme is a constituent of the electron transport chain in certain bacteria, notably several *Vibrio* species. A positive outcome (shown by changes in color) implies they can process reactive oxygen species and survive being anaerobic conditions.

Table 4.5: Oxidase activity in bacterial isolates from different tank conditions.

Tank condition	**DEI: oxidase positive**	**DEI: oxidase negative**	**No Growth**
Static	17, (21%)	19, (24%)	44, (55%)
Flow-Through	9, (28%)	14, (44%)	9, (28%)
Clean	17, (35%)	16, (33%)	15, (32%)
Dirty	9, (14%)	17, (27%)	38, (59%)
Static and clean	11, (33%)	10, (30%)	12, (37%)
Static and dirty	6, (13%)	9, (19%)	32, (68%)
Flow-through and clean	6, (40%)	6, (40%)	3, (20%)
Flow-through and dirty	3, (18%)	8, (47%)	6, (35%)
Total isolates from all larvae tanks	26, (23%)	33, (29%)	53, (48%)

This table presents the number and proportion of bacterial isolates exhibiting oxidase positive and negative reactions in different tank conditions in the experimental setup. The data is classified based on static and flow-through circumstances, as well as the cleanliness of the environment (clean vs. dirty), emphasizing the impact of these factors on the occurrence of oxidase activity in bacterial populations.

Under static conditions, 17 samples (21%) showed positive results for oxidation, while 19 samples (24%) showed negative results for oxidation. Under flow-through conditions, 9 samples (28%) exhibited oxidase positivity under flow-through conditions, indicating a more significant proportion than static conditions. Nevertheless, a greater proportion exhibited a negative oxidase result (44%). Of the samples

linked to larvae, 26 (23%) tested positive for oxidase, while 33 (29%) tested negative. Clean tank conditions led to a more significant proportion of oxidase-positive samples (35%) compared to dirty tanks (14%). Nevertheless, the occurrence of oxidase-negative samples was higher in unclean conditions (27%) compared to clean conditions (33%).

The aggregate conditions exhibit variability in the outcomes. Under conditions characterized as "Static and clean," there was a more significant occurrence of oxidase-positive results, amounting to 33%, in contrast to the "Static and dirty" conditions, which yielded only 13%. The conditions characterized as "flow-through and dirty" had the lowest proportion (18%) of oxidase-positive samples while having the highest proportion (47%) of oxidase-negative samples.

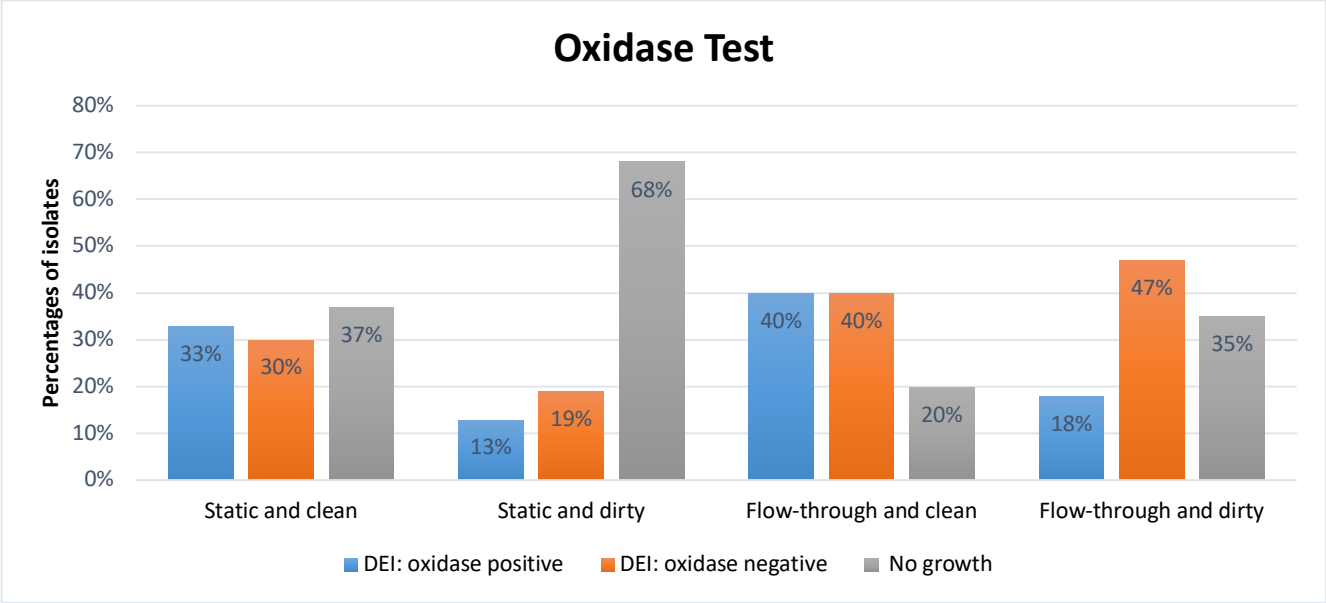


Figure 4.6: Oxidase test results for bacterial isolates from hatchery tanks.

This chart illustrates the number of bacterial isolates obtained from scallop hatchery tanks, categorized as either oxidase positive or oxidase negative. The image also includes a comparison of the overall counts. The isolates are classified according to tank conditions, demonstrating the impact of environmental influences on bacterial enzyme activity.

The composition of bacterial species, particularly the abundance of oxidase-positive bacteria, is influenced by various environmental factors. For instance, flow-through conditions tend to harbor a larger population of oxidase-negative bacteria, suggesting a potential decrease in *Vibrio's* presence or an increase in other bacteria lacking the oxidase enzyme (Figure 4.6). Clean conditions, on the other hand, favor the prevalence of oxidase-positive bacteria compared to dirty tanks. This could suggest that certain *Vibrio* species prefer cleaner environments or that the environmental conditions in clean tanks are more conducive for bacteria with cytochrome c oxidase.

The varying percentages seen in the combined tank conditions demonstrate that the water's status (static or flow-through) and its cleanliness can impact the bacterial population about the presence of the oxidase enzyme. The data can facilitate additional testing and identification of bacteria found in these diverse settings, mainly if the isolation of *Vibrio* species is essential.

4.7 Bacterial communities: Connection of culturing data to amplicon sequence data

The number of distinct color morphotypes observed on TCBS selective media from tank biofilm samples did not show any correlation with the overall bacterial richness in the sequence data. Additionally, it did not align with the abundance of sequences identified as *Vibrio* or other closely related species known to thrive on TCBS media. Nevertheless, *Pseudoalteromonas* believed to act as an antagonist to *Vibrio*, was present in most of the tank and wild veliger samples that included *Vibrio* sequences. *Pseudoalteromonas* exhibited significantly higher abundance and prevalence in wild veligers, while it was completely absent in the hatchery veligers (4.7). *Bacillus* and *Paenibacillus*, usually found together, were present in a small number of tanks grouped together. *Bacillus* and *Paenibacillus* are bacterial taxa that thrive in various habitats, such as marine ecosystems and aquaculture systems, including scallop-hatching tanks. The impact of these bacteria on scallop hatcheries might vary, ranging from advantageous to harmful, depending on the specific species and general conditions of the hatchery. Several sequences classified as *Vibrio* were found in a small number of tanks and wild veliger bacterial

community samples (Fig. 4.7B). *Pseudomonas*, a bacterium that thrives due to human activity and has a hostile relationship with *Vibrio*, was commonly discovered in tanks and hatchery veligers, but was rarely seen in wild veligers (Fig. 4.7B). *Pseudomonas* was the most abundant and commonly shared species variant (SV) between tank biofilms and hatchery veligers, as seen in fugure 4.7

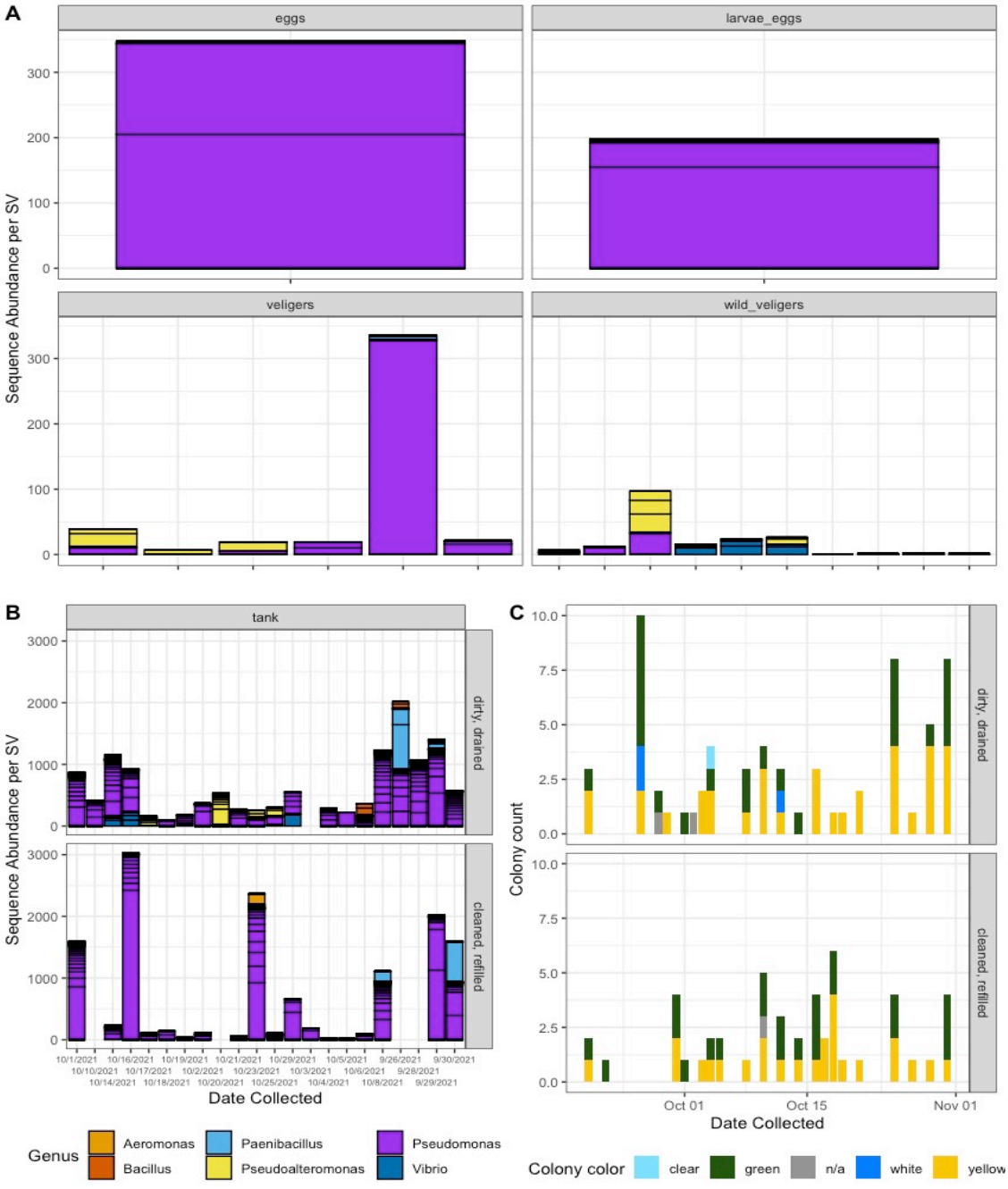


Figure 4.7: Abundance of bacterial genera of interest in sequencing data from scallop veligers (A) and tanks (B), and tank biofilm isolates (C).

Bacteria of interest in panels A and B include *Aeromonas* (yellow-orange), *Pseudoalteromonas* (Yellow), *Bacillus* (Dark Orange), *Paenibacillus* (light Blue), *Pseudomonas* (Purple), and *Vibrio* (Blue), hatchery veligers, and wild veligers. Panel C is colored by bacterial isolate color on TCBS media.

The findings suggest that the bacterial population in the tank biofilms consist of multiple genera, especially among the D-stage veligers. The bacterial load exhibits variability, as seen by the number of sequences, fluctuations in community composition over time, and potential relationships between environmental parameters such as pH and bacterial colony features. The precise ramifications of these discoveries would be contingent upon the broader framework of the experiment, including the veligers' health state, hatchery conditions, and the study's specific goals.

4.8 Other Tests

It is interesting to note that the majority of the isolates thrived in salt broth, which suggests that they favor this medium. We faced an unforeseen obstacle during the fourth day of our bacterial culturing process. Due to a low number of colonies which could be revived from the initial frozen stocks, the proliferation on the APW agar plates needed to be increased to proceed with the intended string test, gram staining, and endospore staining. Interestingly, while conducting the endospore stain, we observed that only a few isolates presented with spores.

The decrease in colony growth can be attributed to various reasons, including the initial quality of the sample and the unique growth requirements of the bacteria under investigation. The string test, which evaluates the generation of extracellular polymeric substances that indicate biofilm formation in conjunction with gram staining and endospore staining, is essential for assessing bacterial species' features and resistance profiles. The failure to perform these tests due to insufficient colony numbers requires an evaluation of our culture techniques and potentially the need to modify our sample collection or incubation methods to achieve more vigorous bacterial growth in future experiments. This setback, however informative, highlights the delicate aspect of working with microbial cultures and the significance of maintaining ideal growth conditions to attain reliable outcomes.

CHAPTER 5

DISCUSSION & CONCLUSION

When scallops are raised in artificial environments instead of their natural habitat, suboptimal conditions may result, making the scallops more sensitive to changes in the environment (Maguire 1998, Lodeiros et al. 2001, Lafrance et al. 2003). One of the important parts of the aquaculture sector is the hatchery system culture of sea scallops (*Placopecten magellanicus*), which aims to guarantee a sustainable and effective production of this commercially valuable species. The microbial community found in the tank biofilms is one important element affecting the hatchery's performance. Comprehending the functions of the bacteria extracted from these biofilms is crucial for enhancing the well-being, development, and overall productivity of larvae. Tank biofilms are complex ecosystems made up of organic debris, extracellular polymeric materials, and bacteria. These biofilms contain bacteria that are essential to the maintenance of water quality, the cycling of nutrients, and the provision of an environment that supports larval growth. The overall health of sea scallop larvae can be greatly impacted by the makeup and activity of bacterial populations.

In undertaking this research, three coastal sea scallop hatcheries were partnered with to obtain biofilm samples for microbiological and genomic analysis. The objectives of the study were: to identify microbes in scallop tank biofilms and compare static vs. flow-through tanks, to determine if static vs. flow-through tanks recruit different species of *Vibrio* bacteria and is a long-term goal, standardize management practices to improve scallop health and aquaculture success. Many of the biofilms that were cultured onto plates contained multiple morphotypes of *Vibrio*, as well as occasional isolates which are possibly Pseudomonas and enterobacterial types. Isolates were cultured from dirty tanks which had housed scallop larvae for 48 hours, as well as clean tanks which had been refilled with filtered sea water.

Based on this study, dirty static tanks contained more strains that could ferment sucrose, presenting as yellow colonies on TCBS agar, but flow-through tanks contained equal numbers of sucrose-fermenting (yellow) and non-sucrose-fermenting (green) isolates. This could be because site selection is of major importance when culturing scallops, and the growth and survival of scallop spat is very much dependent on the grower's choice of culture method, system, equipment and nursery location (Grecian et al. 2000, Frenette et al. 2001, Rupp & Parsons 2004). In a hatchery situation, scallops are held in a modified environment where the temperature and food availability are elevated compared with natural conditions. The incoming seawater is filtered and the scallops are usually fed a mixture of cultured algae rather than a single species diet to meet the required nutritional quality for good growth (Laing & Psimopoulous 1998).

The only significant association identified in the study was between arginine utilization and flow-through tanks, based on Chi-square analysis. This suggests that flow-through systems may influence the recruitment of bacterial strains capable of utilizing arginine. For the purposes of this thesis, it is important to highlight that this was the only significant association found. Other potential associations, such as those relating to different nutrients or environmental factors in the tanks, did not show statistical significance. This information is crucial for understanding the specific conditions that favor the proliferation of certain bacterial species over others in scallop aquaculture environments.

4.9 Trends in Scallop Hatchery Tanks Related to Antibiotic Resistance

Much of the research conducted in the developing field of environmental antimicrobial resistance has concentrated chiefly on wastewater, a prominent origin of antimicrobial-resistant bacteria and antimicrobial-resistant genes transmitted to water sources. Untreated wastewater influent, also known as raw sewage, includes high concentrations of both pathogenic and nonpathogenic microorganisms (Eftim et al., 2017; Soller et al., 2018) and antibiotics (Jury et al., 2011).

Antimicrobials are employed in agriculture, encompassing animal husbandry, plant commodities, and aquaculture, for the purpose of treating and preventing infections, as well as enhancing growth and feed efficiency (USEPA 2013). According to estimates, some 196,000 kg (433,000 pounds) of antibiotics are used each year in the United States' aquaculture industry alone (Done et al., 2015). Despite being a major consumer of fish and fishery products, the United States only produces 5% to 7% of these items domestically. The remaining 90% of seafood consumed in the U.S. is imported (Done et al., 2015).

Hence, the worldwide utilization of antimicrobials in aquaculture is highly probable to be significantly greater. In the United States, the quantity of antibiotics applied to plants is estimated to be less than 0.5% of the total annual production of around 22,680,000 kg of antibiotics (Levy, 1998). Moreover, the quantity of antibiotics utilized in plant agriculture accounts for roughly 0.12% of the total antibiotics employed in animal agriculture, as reported by Stockwell et al. in 2012.

When considering all *Vibrio* species, streptomycin is linked to reduced mortality. However, penicillin alone is not notably effective—based on our data, ampicillin, and penicillin had the lowest efficacy as antibiotics against bacterial infections. Based on our research findings, streptomycin, and oxytetracycline have shown greater efficacy in treating bacterial infections due to the lower resistance exhibited by bacteria towards these antibiotics in all circumstances. Therefore, environmental factors substantially impact bacterial resistance to medications, and a combination of multiple treatments may be necessary to achieve effective treatment.

The infection mechanism of *Vibrio* species is a complex interplay of invasive and toxic mechanisms. When the concentration of *Vibrio* sp. in the gut exceeds a certain threshold, the digestive enzymes can cause the bacteria to break down, releasing a significant quantity of toxins such as ciliostatic toxin, proteases, and endotoxins. These toxins can disrupt the normal digestion process and initiate tissue breakdown. The subsequent proliferation of bacteria intensifies this assault until all tissues have been infiltrated. This intricate mode of infection aligns with the type III pathogenesis described by Elston and Leibovitz (1980), adding another layer of complexity to our understanding of these infections.

4.10 Interrelationship Between Wild Scallop Larvae and Their Surroundings at A Microbial Level

The composition of microbial communities in ocean waters can change fast due to the influence of currents and phages that specifically target microorganisms, causing populations to turn out quickly (Breitbart et al., 2018). Nevertheless, extensive investigations of northwestern Atlantic Ocean waters indicate a consistent state of equilibrium regarding seasonal patterns and a persistent presence of comparable microbial communities (Zorz et al., 2019). Microorganisms in ocean waters can introduce and establish themselves in the microorganism communities associated with marine species (Sousa et al., 2021). Alterations in these water communities can potentially impact the populations of microorganisms associated with the hosts (Ishaq et al., 2022). The study conducted by Boscaro et al. (2022) revealed that wild larvae of different marine species harbor a significant abundance of marine-associated bacteria they acquire from their surroundings. However, it remains uncertain whether this phenomenon is also observed in scallops or if environmental bacteria predominantly shape the microbial communities in marine bivalves.

Furthermore, it remains to be seen if the bacterial communities found at various depths in the water column (Cui et al., 2019) will lead to larvae adopting distinct bacterial communities depending on their depth during their pelagic phases. The warming of ocean waters could enable a more favorable habitat for *Vibrio* spp., posing significant risks to both marine life and public health.

4.11 Limitations and New Hypotheses

While biofilms are ubiquitous in aquaculture facilities, and debris, planktonic cells, and other chemical outputs may be released from biofilms into tank water, biofilms do not appear to negatively affect the health of the adult scallops in these facilities (personal communications). However, scallop larvae might have a different response to these biofilms as juvenile animals typically have a different microbial community and immune system capabilities than adults. Several strategies in hatcheries reduce

the incidence of disease, including the use of mechanical- or bio-filtration of water, continuous-flow instead of static-water tank systems, as well as broad-spectrum or low-concentration antibiotics to reduce infections and boost production (Andersen et al., 2011; Nicolas et al., 1995; Torkildsen and Magnesen, 2004). In terrestrial agriculture, this practice is being restricted and replaced as it contributes to antimicrobial resistance and threats to public health (Executive Order 13676 - Combating Antibiotic-Resistant Bacteria, 2014). While antibiotics are still used in aquaculture facilities, this practice is unsustainable, both ecologically and financially, and has mixed benefits. Antibiotics can increase mortality in some marine species, partly by encouraging antibiotic resistance and partly by removing beneficial or symbiotic bacteria (Prado et al., 2010; Schmidt et al., 2017).

It has been speculated that the diverse community associated with adult shellfish may offer some protective effects toward larvae. One idea to ensure the formation of a healthy microbial community in larvae is to present them with "conditioned" adult tank water as an *in situ* probiotic. For example, adult oyster tanks contained a higher abundance of the bacterial genus *Alteromonas*, shellfish commensals (Song 2022). Unfortunately, adult tank water becomes too foul with adults' feces and pseudofeces to be used as a direct source for larvae tanks (Beal, communications). Even filtering adult tank water to recover only microorganisms for use as a probiotic in oyster larvae tanks has mixed results depending on the season (Song 2022). Alternatively, exogenously sourced probiotics that encourage the growth of symbiotic bacteria have been shown to improve aquaculture animal survival, but a further understanding of *P. magellanicus* microbial communities is still needed to develop effective therapeutics and management practices (Prado et al., 2010; Schmidt et al., 2017).

4.12 Potential for Impact

Scallop has long been a source of food and economic opportunity (The Centre for Indigenous Peoples' Nutrition and Environment, 2017; Tremblay et al., 2020). Wild populations of scallops are currently at an increased risk of decline because of climate change (Marushka et al., 2019; Rheuban et

al., 2018), and on top of natural variation in scallop populations, this makes the industry financially vulnerable (Coleman et al., 2021; rbouvier Consulting, 2019). Shellfish farms are of significant ecological and economic value, including the use of shellfish farms to remove nitrogen runoff and clean coastal waters (Bayer 2022), or to create artificial reefs and habitats to improve other biodiversity (Mercaldo-Allen 2022). Yet, much is unknown about scallops, particularly their interactions with microorganisms in wild and hatchery settings.

CONCLUSION

To ensure the sustainability and viability of sea scallop aquaculture, it is imperative to investigate the activity of bacteria isolated from tank biofilms in sea scallop hatchery systems. In addition to expanding our knowledge of the dynamics of microbiological communities in hatchery settings, this research creates new opportunities for raising the general production efficiency and health of sea scallop larvae.

REFERENCES

Alderman, D.J. and T.S. Hastings. 1998. "Antibiotic Use in Aquaculture: Development of Resistance – Potential for Consumer Health Risks." *International Journal of Food Science and Technology*. 33(2): 139-155.

Andersen, S., & Ringvold, H. (2000). Seasonal differences in effect of broodstock diet on spawning success in the great scallop. *Aquaculture International: Journal of the European Aquaculture Society*, *8*(2), 259-265.

Andersen, S., Christophersen, G., & Magnesen, T. (2011). Spat production of the great scallop (Pecten maximus): a roller coaster1This review is part of a virtual symposium on current topics in aquaculture of marine fish and shellfish. *Canadian Journal of Zoology*, *89*(7), 579-598.

Aryal, S. (August 10, 2022). *Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar - Composition, Principle, Uses, Preparation, and Colony Morphology. MicrobiologyInfo.com]*. Retrieved April 17, 2024, from https://microbiologyinfo.com/thiosulfate-citrate-bile-salts-sucrose-tcbs-agar-composition-principle-uses-preparation-and-colony-morphology/

Assefa, A., & Abunna, F. (2018). Maintenance of Fish Health in Aquaculture: Review of Epidemiological Approaches for Prevention and Control of Infectious Disease of Fish. *Veterinary Medicine International*, *2018*, 5432497.

Azargun, R., Gholizadeh, P., Sadeghi, V., Hosainzadegan, H., Tarhriz, V., Memar, M. Y., Pormohammad, A., & Eyvazi, S. (2020). Molecular mechanisms associated with quinolone resistance in Enterobacteriaceae: review and update. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *114*(10), 770-781.

Baker-Austin, C., Oliver, J. D., Alam, M., Ali, A., Waldor, M. K., Qadri, F., & Martinez-Urtaza, J. (2018). Vibrio spp. infections. *Nature Reviews. Disease Primers*, *4*(1), 8.

Beal, B.F., 2014. Final Report [NA10NMF4270214]: Enhancing Sea Scallop Stocks in Eastern Maine Through Applied Aquaculture Research and Technology Transfer (No. NA10NMF4270214). Downeast Institute.

Benbrook, C.M. (2002). Antibiotic drug use in U.S. Aquaculture. The Northwest Science and Environmental Policy Centre, Sandpoint, Idaho, 1-18

Beninger, P., & Pennec, M. (2006). Chapter 3 Structure and function in scallops. *Developments in Aquaculture and Fisheries Science*, *35*, 123-227.

Bergh, Ø. & Strand, Ø. (2001). Great scallop, Pecten maximus, research and culture strategies in Norway: a review. Aquaculture International 9:305-318.

Betancourt, R. J., Pérez, J. E., Vélez, A., Freites, L., & Segnini, M. I. (1995). Efectos de la consanguinidad en la vieira Euvola ziczac (L.). Inst. Oceanogr. Venez. Univ …, 34, 69–75.

Bopp, C., Ries, A., & Wells, J. (1999). *Laboratory methods for the diagnosis of epidemic dysentery and cholera*. Centers for Disease Control and Prevention. Available at: https://www.cdc.gov/cholera/pdf/Laboratory-Methods-for-the-Diagnosis-of-Epidemic-Dysentery-and-Cholera.pdf

Boscaro, V., Holt, C. C., Van Steenkiste, N. W. L., Herranz, M., Irwin, N. A. T., Ã€lvarez-Campos, P., Grzelak, K., Holovachov, O., Kerbl, A., Mathur, V., Okamoto, N., Piercey, R. S., Worsaae, K., Leander, B. S., & Keeling, P. J. (2022). Microbiomes of microscopic marine invertebrates do not reveal signatures of phylosymbiosis. *Nature Microbiology*, *7*(6), 810-819.

Bourne, N. & Hodgson, C.A. (1991). Development of a viable nursery system for scallop culture. In: S.E. Shumway and P.A. Sandifer, (Eds.). Scallop Biology and Culture. World Aquaculture Workshops, No. 1. World Aquaculture Society, Baton Rouge, LA. 273 - 286.

Bourne, N. F. (2000). The potential for scallop culture - the next millenium. *Aquaculture International: Journal of the European Aquaculture Society*, *8*(2), 113-122.

Bourne, N., Hodgson, C.A. and J.N.C. Whyte. 1989. A manual for scallop culture in Brittish Columbia. Can. Tech. Rep. Fish. and Aquat. Sci. 1694: 215pp

Branch, G.M., C.L. Griffiths, Branch M.L. & L.E. Beckley. 2010. Two Oceans. A Guide to the Marine Life of southern Africa. Random House Struik Publishers. pp. 456.

Brand, A. R. (1991). *Scallop ecology: distributions and behavior. in SE Shumway, editor. Scallops: biology, ecology and aquaculture*. Elsevier, Amsterdam-Oxford-New York-Tokyo, *517-584*.

Breitbart, M., Bonnain, C., Malki, K., & Sawaya, N. A. (2018). Phage puppet masters of the marine microbial realm. *Nature Microbiology*, *3*(7), 754-766.

Brumfield, K. D., Usmani, M., Chen, K. M., Gangwar, M., Jutla, A. S., Huq, A., & Colwell, R. R. (2021). Environmental parameters associated with incidence and transmission of pathogenic Vibrio spp. *Environmental Microbiology*, *23*(12), 7314-7340.

Byrne, M. K., Miellet, S., McGlinn, A., Fish, J., Meedya, S., Reynolds, N., & van Oijen, A. M. (2019). The drivers of antibiotic use and misuse: the development and investigation of a theory driven community measure. *BMC Public Health*, *19*(1), 1425.

Cai, W., & Arias, C. R. (2017). Biofilm Formation on Aquaculture Substrates by Selected Bacterial Fish Pathogens. *Journal of Aquatic Animal Health*, *29*(2), 95-104.

Callahan, B. (2022). *DADA2: Fast and accurate sample inference from amplicon data with single-nucleotide resolution (1.16)*.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, *108 Suppl 1*(Suppl 1), 4516-4522.

Cary, S. C., Leighton, D. L., & Phleger, C. F. (2009). Food and feeding strategies in culturing larval and early juvenile purple-hinge rock scallops, *Hinnites multirugosus* (Gale). *Journal of the World Aquaculture Society*, *12*(1), 156-169.

CDC. 2020. Vibrio species causing vibriosis. Centers for Disease Control and Prevention. https://www.cdc.gov/vibrio/faq.html. Accessed 20 June 2022.

Centers for Disease Control and Prevention (1999). Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera. CDC. Available from: https://stacks.cdc.gov/view/cdc/6669

Center for Disease Control, C., Prevention, & Others. (2019). Vibrio species causing vibriosis. In *2019-03-05)[2020-08-28]. https://www. cdc. gov/vibrio/index. html*.

World Health Organization *Cholera*. (n.d.). Retrieved April 17, 2024, from https://www.who.int/health-topics/cholera

Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews: MMBR*, *65*(2), 232-260 ;.

Chusniati, S., Handijatno, D., Sudarno, & Kusdarwarti, R. (2012). Petunjuk Praktikum Mikrobiologi (Surabaya: Fakultas Perikanan dan Kelautan Universitas Airlangga) (in Indonesia). 17-20

Coleman, S., Cleaver, C., Morse, D., Brady, D. C., & Kiffney, T. (2021). The coupled effects of stocking density and temperature on Sea Scallop (Placopecten magellanicus) growth in suspended culture. *Aquaculture Reports*, *20*, 100684.

Concha, C., Figueroa, E., & Winkler, F. M. (2011). AsociaciÃ³n entre la tasa de autofecundaciÃ³n y la frecuencia de larvas malformadas en poblaciones cultivadas del ostiÃ³n del norte Argopecten purpuratus (Lamarck, 1819). *Latin American Journal of Aquatic Research*, *39*(2), 327-337.

Couturier, C.Y., Dabinett, P. and Lanteigne, M. 1995. Scallop culture in Atlantic Canada. In: A. Boghen (Editor). ColdWater Aquaculture in Atlantic Canada. Canadian Institute for Research on Regional Development, Université de Moncton, Moncton, NB, pp.

297–340,

Cragg, S. M. (2006). Chapter 2 Development, physiology, behaviour and ecology of scallop larvae. In S. E. Shumway & G. J. Parsons (Eds.), *Developments in Aquaculture and Fisheries Science* (Vol. 35, pp. 45-122). Elsevier.

Crone, S., Vives-Florez, M., Kvich, L., Saunders, A. M., Malone, M., Nicolaisen, M. H., MartÃ-nez-GarcÃ-a, E., Rojas-Acosta, C., Catalina Gomez-Puerto, M., Calum, H., Whiteley, M., Kolter, R., & Bjarnsholt, T. (2020). The environmental occurrence of Pseudomonas aeruginosa. *APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, *128*(3), 220-231.

Cui, Y., Chun, S.-J., Baek, S. H., Lee, M., Kim, Y., Lee, H.-G., Ko, S.-R., Hwang, S., Ahn, C.-Y., & Oh, H.-M. (2019). The water depth-dependent co-occurrence patterns of marine bacteria in shallow and dynamic Southern Coast, Korea. *Scientific Reports*, *9*(1), 9176.

Culliney, J. L. (1974). Larval development of the giant scallop *Placopecten magellanicus* (Gmelin). *The Biological Bulletin*, *147*(2), 321-332.

Dalsgaard, A., Forslund, A., Serichantalergs, O., & Sandvang, D. (2000). Distribution and content of class 1 integrons in different Vibrio cholerae O-serotype strains isolated in Thailand. *Antimicrobial Agents and Chemotherapy*, *44*(5), 1315-1321.

Daniels, N. A., & Shafaie, A. (2000). A Review of Pathogenic Vibrio Infections for Clinicians. *Infections in Medicine*, *17*(10). http://dx.doi.org/ 25-28

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews: MMBR*, *74*(3), 417-433.

Dengo-Baloi, L. C., SemÃ¡-Baltazar, C. A., Manhique, L. V., Chitio, J. E., Inguane, D. L., & Langa, J. P. (2017). Antibiotics resistance in El Tor Vibrio cholerae 01 isolated during cholera outbreaks in Mozambique from 2012 to 2015. *PloS One*, *12*(8), e0181496.

Done, H. Y., Venkatesan, A. K., & Halden, R. U. (2015). Does the Recent Growth of Aquaculture Create Antibiotic Resistance Threats Different from those Associated with Land Animal Production in Agriculture? *The AAPS Journal*, *17*(3), 513-524.

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging Infectious Diseases*, *8*(9), 881-890.

Drew, G. A. (1906). The Habits, Anatomy, and Embryology of the Giant Scallop*, (Pecten Tenuicostatus, Mighels)*. University of Maine.

Du, M., Chen, J.X., Zhang, X.H., Li, A.J., Li, Y., 2007. Characterization and resuscitation of viable but nonculturable Vibrio alginolyticus VIB283. Arch. Microbiol. 188, 283–288.

earthmicrobiome. (n.d.). Retrieved December 29, 2023, from https://earthmicrobiome.org/

Eftim, S. E., Hong, T., Soller, J., Boehm, A., Warren, I., Ichida, A., & Nappier, S. P. (2017). Occurrence of norovirus in raw sewage - A systematic literature review and meta-analysis. *Water Research*, *111*, 366-374.

Elmahdi, S., DaSilva, L. V., & Parveen, S. (2016). Antibiotic resistance of Vibrio parahaemolyticus and Vibrio vulnificus in various countries: A review. *Food Microbiology*, *57*, 128-134.

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., & Ravel, J. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome*, *2*(1), 6.

FAO 2014. Food and Agriculture Organization of the United Nations Statistics. www.fao.org/fishery/statistics/en.

FAO Yearbook. Fishery and Aquaculture Statistics 2014. Retrieved April 17, 2024, from https://www.fao.org/documents/card/en/c/570fc84b-5d02-4345-a6b3-5876279e4a8d

FAO, (2004). Hatchery culture of bivalves: A practical manual. FAO fisheries technical paper 1-203.

FAO, (2022). The state of world fisheries and aquaculture: Towards a blue transformation. Rome, 1-266.

Fisher, J. F., & Mobashery, S. (2020). Constructing and deconstructing the bacterial cell wall. *Protein Science: A Publication of the Protein Society*, *29*(3), 629-646.

Fletcher, G. L., Shears, M. A., Yaskowiak, E. S., King, M. J., & Goddard, S. V. (2004). Gene transfer: potential to enhance the genome of Atlantic salmon for aquaculture. *Australian Journal of Experimental Agriculture*, *44*(11), 1095-1100.

Food and Agriculture Organization of the United Nations. (2018). *2016 THE STATE OF WORLD FISHERIES AND AQUACULTURE: CONTRIBUTING TO FOOD SECURITY AND NUTRITION FOR ALL*. Food & Agriculture Org.

Center for Disease Control, C., Prevention, & Others. (2019). Vibrio species causing vibriosis. In *2019-03-05)[2020-08-28]. https://www. cdc. gov/vibrio/index. html*.

Global Task Force on Cholera Control. (n.d.). Retrieved April 17, 2024, from https://www.gtfcc.org/

Goldburg, Rebecca J., Matthew S. Elliot and Rosamond L. Naylor. 2001. “Marine Aquaculture in the United States: Environmental Impacts and Policy Options.” Report prepared for the Pew Oceans Commission. Arlington, Virginia. 16-19

Gomez-Leon, J., Villamil, L., Lemos, M. L., Novoa, B., & Figueras, A. (2005). Isolation of Vibrio alginolyticus and Vibrio splendidus from aquacultured carpet shell clam (Ruditapes decussatus) larvae associated with mass mortalities. *Applied and Environmental Microbiology*, *71*(1), 98-104.

Gordon, E., Mouz, N, Duee, E. & Dideberg, O. (2000). The crystal structure of the penicillin-binding protein 2x from streptococcus pneumoniae and its acyl-enzyme form: implication in drug resistance. J. Mol Biol 299(2): 477-85.

Gosling, E. (2003) Bivalve Molluscs Biology, Ecology and Culture. Fishing New Books, Blackwell Publishing,. - References - Scientific Research Publishing. (p 443). Retrieved April 17, 2024, from https://www.scirp.org/reference/referencespapers?referenceid=1249539

Gosner, K.L. 1978. A field guide to the Atlantic seashore. Houghton Mifflin Co. Boston

Grecian, L. A., Parsons, G. J., Dabinett, P., & Couturier, C. (2000). Influence of season, initial size, depth, gear type and stocking density on the growth rates and recovery of sea scallop, *Placopecten magellancius*, on a farm-based nursery. *Aquaculture International: Journal of the European Aquaculture Society*, *8*(2), 183-206.

Guo, X., Ford, S. E., & Zhang, F. (1999). Molluscan aquaculture in China. *Journal of Shellfish Research*, *18*(1), 19-31.

Hasegawa, H., Lind, E. J., Boin, M. A., & HÃ¤se, C. C. (2008). The extracellular metalloprotease of Vibrio tubiashii is a major virulence factor for pacific oyster (Crassostrea gigas) larvae. *Applied and Environmental Microbiology*, *74*(13), 4101-4110.

Heasman, M.P., O’Connor, W.A. and A.W.J. Frazer. 1994. Ontogenic changes in optimal rearing temperatures for the commercial scallop, Pecten fumatus Reeve. J. Shellfish Res. 15: 627 - 634.

Helm, M. M., Bourne, N., & Lovatelli, A. (2004). Hatchery culture of bivalves, a practical manual. FAO Fisheries Technical Paper 471. Food and Agriculture Organization of the in the laboratory. *Marine Biology*, *15*, 350-355.

Hodgson, C. A., N. Boume, and D. Mottershead. 1988. A selected bibliography of scallop literature. Can. MS Rep. Fish. Aquat. Sci. 1965: 133 p.

Holbach, M., Robert, R., Boudry, P., Petton, B., Archambault, P., & Tremblay, R. (2015). Scallop larval survival from erythromycin treated broodstock after conditioning without sediment. *Aquaculture* , *437*, 312-317.

Hopwood, D. A. (2007). *Streptomyces in Nature and Medicine: The Antibiotic Makers*. Oxford University Press, USA. 312-316

Goldburg, R., Elliott, M. S., & Naylor, R. L. (2001). *Marine aquaculture in the United States: Environmental impacts and policy options*. Retrieved from https://www.iatp.org/sites/default/files/Marine_Aquaculture_in_the_United_States_Envir o.pdf

Ishaq, S. L., Hosler, S., Dankwa, A., Jekielek, P., Brady, D. C., Grey, E., Haskell, H., Lasley-Rasher, R., Pepperman, K., Perry, J., Beal, B., & Bowden, T. J. (2023). Bacterial community trends associated with sea scallop, *Placopecten magellanicus*, larvae in a hatchery system. *Aquaculture Reports*, *32*, 101693.

Ishaq, S. L., Turner, S. M., Tudor, M. S., MacRae, J. D., Hamlin, H., Kilchenmann, J., Lee, G., & Bouchard, D. (2022). Many Questions Remain Unanswered About the Role of Microbial Transmission in Epizootic Shell Disease in American Lobsters (Homarus americanus). *Frontiers in Microbiology*, *13*, 824950.

Jay Parsons, G., & Robinson, S. M. C. (2006). Chapter 16 Sea scallop aquaculture in the northwest atlantic. In *Scallops: Biology, Ecology and Aquaculture* (pp. 907-944). Elsevier.

Johnston, N. J., Mukhtar, T. A., & Wright, G. D. (2002). Streptogramin antibiotics: mode of action and resistance. *Current Drug Targets*, *3*(4), 335-344.

Jones, J. L., LÃ¼deke, C. H. M., Bowers, J. C., Garrett, N., Fischer, M., Parsons, M. B., Bopp, C. A., & DePaola, A. (2012). Biochemical, serological, and virulence characterization of clinical and oyster Vibrio parahaemolyticus isolates. *Journal of Clinical Microbiology*, *50*(7), 2343-2352.

Jury, K. L., Khan, S. J., Vancov, T., Stuetz, R. M., & Ashbolt, N. J. (2011). Are Sewage Treatment Plants Promoting Antibiotic Resistance? *Critical Reviews in Environmental Science and Technology*, *41*(3), 243-270.

Kassambara, A. (2022). Ggpubr: 'Ggplot2' Based Publication Ready Plots. *Scientific Research Publishing*, volume(issue), pages. Retrieved from https://www.scirp.org/reference/referencespapers?referenceid=3546076

Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J., & Gibson, L. F. (2009). Challenge of New Zealand Greenshell mussel Perna canaliculus larvae using two Vibrio pathogens: a hatchery study. *Diseases of Aquatic Organisms*, *86*(1), 15-20.

Kilburn, R., & Rippey, E. (1982). *Sea shells of Southern Africa*. Macmillan South Africa; Distributed by ISBS.

King, D. E., Malone, R., & Lilley, S. H. (2000). New classification and update on the quinolone antibiotics. *American Family Physician*, *61*(9), 2741-2748.

Klein, S. L., & Lovell, C. R. (2017). The hot oyster: levels of virulent Vibrio parahaemolyticus strains in individual oysters. *FEMS Microbiology Ecology*, 91-93 (2). https://doi.org/10.1093/femsec/fiw232

Krassoi, R., Anderson, I. and D. Everett. 1997. Larval abnormalities in doughboy scallops *Chlamus (Mimachlamus) asperrima L*. in response to test conditions and six reference toxicants. Aust. J. Ecotox. 3: 65 - 74.

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., GÃ©lÃ©bart, F., Cordevant, C., Lange, M., & Poulet, S. A. (2001). A Vibrio splendidus strain is associated with summer mortality of juvenile oysters Crassostrea gigas in the Bay of Morlaix (North Brittany, France). *Diseases of Aquatic Organisms*, *46*(2), 139-145.

Laing, I., & Psimopoulous, A. (1998). Hatchery cultivation of king scallop (Pecten maximus) spat with cultured and bloomed algal diets. *Aquaculture* , *169*(1), 55-68.

Le Pennec, M., Paugam, A., & Le Pennec, G. (2003). The pelagic life of the pectinid *Pecten maximus* a review. *ICES Journal of Marine Science: Journal Du Conseil*, *60*(2), 211-233.

Levy, L., Parsons, G.J. & Dabinett, P. (1998). Effect of deployment date and environmental conditions at a farm-based nursery on growth and recovery of hatchery-reared sea scallops (*Placopecten magellanicus*). Bulletin of the Aquaculture Association Canada 98-2:73-75.

Levy, L., Parsons, G.J. & Dabinett, P. 1998. Effect of deployment date and environmental conditions at a farm-based nursery on growth and recovery of hatchery-reared sea scallops (Placopecten magellanicus). Bulletin of the Aquaculture Association Canada 98-2:73-75.

Li, M., Liu, J. C., Sun, X. Y., Zhao, X. W., Liang, J., Sun P. H., and Ma, Y. X., 2016. Culturable bacterial diversity in larval breeding in Yesso scallop *Patinopecten yessoensis*. Journal of Dalian Ocean University, 31: 374-3379

Liu, B., Dong, B., Tang, B., Zhang, T., & Xiang, J. (2006). Effect of stocking density on growth, settlement and survival of clam larvae, Meretrix meretrix. *Aquaculture* , *258*(1), 344-349.

Liu, J., Liu, Z., & Sun, X. (2011). The Effects of Inbreeding on Production Traits of the Southern Bay ScallopArgopecten irradians concentricus. *Journal of Shellfish Research*, *30*(1), 109-113.

Lodeiros, C., Maeda-Martinez, A. N., Freites, L., & Sicard, M. T. (2001). Ecofisiología de pectínidos Iberoamericanos. In *Los Moluscos Pectínidos de Iberoamérica: Ciencia y Acuicultura* (Vol. 4, pp. 77–88).

Lu, Y. T., & Blake, N. J. (1997). Clearance and ingestion rates of Isochrysis galbana by larval and juvenile bay scallops, argopecten irradians concentricus (Say). *Oceanographic Literature Review*, *12*(44), 1540.

Lu, Y.T. & Blake, N.J. 1996. Optimum concentrations of Isochrysis galbana for growth of larval and juvenile bay scallops, Argopecten irradians concentricus (Say). Journal of Shellfish Research 15:635-643.

Ma, Y., Yang, X., Wang, H., Qin, Z., Yi, C., Shi, C., Luo, M., Chen, G., Yan, J., Liu, X., & Liu, Z. (2021). CBS-derived H2S facilitates host colonization of Vibrio cholerae by promoting the iron-dependent catalase activity of KatB. *PLoS Pathogens*, *17*(7), e1009763.

MacMillan, J.R. (2001). "Aquaculture and Antibiotic Resistance: A Negligible Public Health Risk?" World Aquaculture. June. Pp. 49-51:68.

Madigan, M.T., Martinko, J.M., Dunlap, P.V., Clark, D.P. (2008). Brock biology of micro-organisms 12th edition. International Microbiology, 11: 65-73.

Maeda-MartÃ-nez, A. N., Reynoso-Granados, T., Monsalvo-Spencer, P., Sicard, M. T., MazÃ³n-SuÃ¡stegui, J. M., HernÃ¡ndez, O., Segovia, E., & Morales, R. (1997). Suspension culture of catarina scallop Argopecten ventricosus (=circularis) (Sowerby II, 1842), in Bahia Magdalena, Mexico, at different densities. *Aquaculture* , *158*(3), 235-246.

Magnesen, T., Bergh, Ã˜., & Christophersen, G. (2006). Yields of great scallop, Pecten maximus, larvae in a commercial flow-through rearing system in Norway. *Aquaculture International: Journal of the European Aquaculture Society*, *14*(4), 377-394.

Maguire, J. A., Cashmore, D., & Burnell, G. M. (1999). The effect of transportation on the juvenile scallop*Pecten maximus*(L.). *Aquaculture Research*, *30*(5), 325-333.

Maguire, J. A., Oâ€™Connor, D. A., & Burnell, G. M. (1999). An Investigation into Behavioural Indicators of Stress in Juvenile Scallops. *Aquaculture International: Journal of the European Aquaculture Society*, *7*(3), 169-177.

Marine, H.,Rene, R., Pierre, B., Bruno, P., Philippe, A. and R. Tremblay. 2015. Scallop larval survival from erythromycin treated broodstock after conditioning without sediment. Aquacul. 437: 132 - 317.

Coastal Enterprises, Inc. (2019). *Market Analysis of Maine Farm-Raised Sea Scallops*. Brunswick, ME: Author. Available at: https://www.ceimaine.org/wp-content/uploads/2019/02/Market-Analysis-of-Maine-Farm-Raised-Sea-Scallops.pdf

Martin, S. J., Mather, C., Knott, C., & Bavington, D. (2021). â€œLandingâ€ salmon aquaculture: Ecologies, infrastructures and the promise of sustainability. *Geoforum; Journal of Physical, Human, and Regional Geosciences*, *123*, 47-55.

Martinko, Clark, D. P., Martinko, J. M., & Dunlap, P. V. (2009). *Brock Biology of Microorganisms 12th International Edition* (12th ed.). Pearson Education. 11-15

Marushka, L., Kenny, T.-A., Batal, M., Cheung, W. W. L., Fediuk, K., Golden, C. D., Salomon, A. K., Sadik, T., Weatherdon, L. V., & Chan, H. M. (2019). Potential impacts of climate-related decline of seafood harvest on nutritional status of coastal First Nations in British Columbia, Canada. *PloS One*, *14*(2), e0211473.

Mercaldo-Allen, R., Clark, P., Liu, Y., Phillips, G., Redman, D., Auster, P. J., Estela, E., Milke, L., Verkade, A., & Rose, J. M. (2021). Exploring video and eDNA metabarcoding methods to assess oyster aquaculture cages as fish habitat. *Aquaculture Environment Interactions*, *13*, 277-294.

Merino, G., Uribe, E., Soria, G., & von Brand, E. (2009). A comparison of larval production of the northern scallop, Argopecten purpuratus, in closed and recirculating culture systems. *Aquacultural Engineering*, *40*(2), 95-103.

Milke, L. M., Bricelj, V. M., & Parrish, C. C. (2006). Comparison of early life history stages of the bay scallop, Argopecten irradians: Effects of microalgal diets on growth and biochemical composition. *Aquaculture* , *260*, 272-289.

Millican, P. F. (1997). *The hatchery rearing of king scallop (Pecten maximus)* (Great Britain. Centre for Environment, Fisheries and Aquaculture Science (Ed.)). 11-15

Minchin, D. (2003). Introductions: some biological and ecological characteristics of scallops. *Aquatic Living Resources*, *16*(6), 521-532.

Minchin, D. 1992. Biological observations on young scallops, Pecten maximus. J. Mar. Biol. Assoc. U.K. 72: 807 - 819.

Miranda, C. D., Godoy, F. A., & Lee, M. R. (2018). Current Status of the Use of Antibiotics and the Antimicrobial Resistance in the Chilean Salmon Farms. *Frontiers in Microbiology*, *9*, 1284.

N.A.H.M.S. (1997). National Animal Health Monitoring System. "Catfish `97 Study." APHIS/USDA. Washington, D.C. 6-8

Naidu, K.S., Fournier, R., Marsot, P. and Worms, J. 1989. Culture of sea scallop *Placopecten magellanicus*. In: A.D. Boghen (Editor). Cold-Water Aquaculture in Atlantic Canada. The Canadian Institute for Research on Regional Development, Université de Moncton, Moncton, NB, Canada, pp. 211–239.

Narvarte, M. A., & Pascual, M. S. (2003). Fertilization, larval rearing and post-larval growth of the Tehuelche scallop *Aequipecten tehuelchus* D'Orb., 1846. *Aquaculture* , *217*(1), 259–274.

Nicolas, J. L., Corre, S., Gauthier, G., Robert, R., & Ansquer, D. (1996). Bacterial problems associated with scallop Pecten maximus larval culture. *Diseases of Aquatic Organisms*, *27*, 67-76.

Niles, M., Barrell, J., Sameoto, J., Keith, D. and Sonier, R. 2021. Scallop Fishery Assessment of the Southern Gulf of St. Lawrence in 2018: Commercial Fishery and Survey Data. DFO Can. Sci. Advis. Sec. Res. Doc. 2021/038. xii + 178 p

Nogales, B., Lanfranconi, M. P., Piña-Villalonga, J. M., & Bosch, R. (2011). Anthropogenic perturbations in marine microbial communities. *FEMS Microbiology Reviews*, *35*(2), 275–298.

Oliver, J. D., Warner, R. A., & Cleland, D. R. (1983). Distribution of Vibrio vulnificus and other lactose-fermenting vibrios in the marine environment. *Applied and Environmental Microbiology*, *45*(3), 985-998.

OTA. 1995. Office of Technology Assessment. U.S. Congress. "Selected Technology Issues in US Aquaculture.

Parsons, G.J. and S.M.C. Robinson. 2006. Sea scallop aquaculture in the Northwest Atlantic. In: S. E. Shumway and G. J. Parsons, (Eds.). Scallops: Biology, Ecology and Aquaculture. Amsterdam: Elsevier. pp. 907 - 944.

Paulet, Y. M., Lucas, A., & Gerard, A. (1988). Reproduction and larval development in two Pecten maximus (L.) populations from Brittany. *Journal of Experimental Marine Biology and Ecology*, *119*(2), 145-156.

Pearce, C. M., Gallager, S. M., Manuel, J. L., Manning, D. A., Oâ€™Dor, R. K., & Bourget, E. (1998a). Effect of thermoclines and turbulence on depth of larval settlement and spat recruitment of the giant scallop Placopecten magellanicus in 9.5 m deep laboratory mesocosms. *Marine Ecology Progress Series*, *165*, 195-215.

Pearce, C. M., Gallager, S. M., Manuel, J. L., Manning, D. A., Oâ€™Dor, R. K., & Bourget, E. (1998b). Effect of thermoclines and turbulence on depth of larval settlement and spat recruitment of the giant scallop Placopecten magellanicus in 9.5 m deep laboratory mesocosms. *Marine Ecology Progress Series*, *165*, 195-215.

Peña, J.B. (2001). Taxonomía, morfología, distribución y hábitat de los pectínidos Iberoamericanos. In: A.N. Maeda-Martínez (ed.). Los moluscos pectínidos de Iberoamérica: ciencia y acuicultura, Editorial Limusa, México,1-25.

Prado, S., Romalde, J. L., & Barja, J. L. (2010). Review of probiotics for use in bivalve hatcheries. *Veterinary Microbiology*, *145*(3-4), 187-197.

Ramamurthy, T., Das, B., Chakraborty, S., Mukhopadhyay, A. K., & Sack, D. A. (2020). Diagnostic techniques for rapid detection of Vibrio cholerae O1/O139. *Vaccine*, *38 Suppl 1*, A73-A82.

Reichelt, J. L., Baumann, P., & Baumann, L. (1976). Study of genetic relationships among marine species of the genera Beneckea and Photobacterium by means of in vitro DNA/DNA hybridization. *Archives of Microbiology*, *110*(1), 101-120.

Rhamtulla, S., Ramana, C. V., & Byragi Reddy, T. (2015). Isolation of Vibrio parahaemolyticus from the gut of Octopus (Octopus spp.) and mussel (Perna indica). *International Journal of Current Microbiology and Applied Sciences*, 4(1), 543-551. Available at: https://www.ijcmas.com/vol-4-1/Shaik%20Rhamtulla,%20et%20al.pdf

Rheuban, J. E., Doney, S. C., Cooley, S. R., & Hart, D. R. (2018). Projected impacts of future climate change, ocean acidification, and management on the US Atlantic sea scallop (Placopecten magellanicus) fishery. *PloS One*, *13*(9), e0203536.

Robert, R., & Nicholas, L. (2000). The effect of seawater flow and temperature on metamorphosis and postlarval development in great scallop. *Aquaculture International: Journal of the European Aquaculture Society*, *8*(6), 513-530.

Rojas, R., Miranda, C. D., Opazo, R., & Romero, J. (2015). Characterization and pathogenicity of Vibrio splendidus strains associated with massive mortalities of commercial hatchery-reared larvae of scallop Argopecten purpuratus (Lamarck, 1819). *Journal of Invertebrate Pathology*, *124*, 61-69.

Rosenthal, H., Allen, J.H., Helm, M.M. & McInerney-Northcott, M. 1995. Aquaculture technology: Its application, development and transfer. In: Boghen, A.D. (ed.). ColdWater Aquaculture in Atlantic Canada. The Canadian Institute for Research on Regional Development, Canada, pp. 393-450.

Rudders, D. B., Benoît, H. P., Knotek, R. J., Mandelman, J. A., Roman, S. A., & Sulikowski, J. A. (2022). Discard mortality of sea scallops *Placopecten magellanicus* following capture and handling in the U.S. dredge fishery. *Marine and Coastal Fisheries: Dynamics, Management , and Ecosystem Science*, *14*(2). https://doi.org/10.1002/mcf2.10197

Rupp, G. S. (2020). Cultivo de vieiras em Santa Catarina: tecnologias utilizadas e influência de fatores ambientais. *Agropecuária Catarinense*, *33*(3), 14–17.

Rupp, G. S., Thompson, R. J., & Parsons, G. J. (2004). Influence of food supply on postmetamorphic growth and survival of hatchery-produced lion's paw scallop, *nodipecten nodosus*. *Journal of Shellfish Research*, *23*, 5+.

S. A. U., &. M. A. K. (2006). The use of antibiotics in shrimp hatcheries in Bangladesh. *Journal of Fisheries and Aquatic Science*, *1*(1), 64-67.

Sanyal, S. C., Sakazaki, R., Prescott, L. M., & Sinha, R. (1973). Isolation of rapidly lactose-fermenting Vibrio cholerae strains. *Journal of Medical Microbiology*, *6*(1), 119-120.

Sarkis, S. (2007). *Installation and Operation of a Modular Bivalve Hatchery*. Food & Agriculture Org. 17-20

Schmidt, V., Gomez-Chiarri, M., Roy, C., Smith, K., & Amaral-Zettler, L. (2017). Subtle Microbiome Manipulation Using Probiotics Reduces Antibiotic-Associated Mortality in Fish. *mSystems*, *2*(6).14-17. https://doi.org/10.1128/mSystems.00133-17

Davidson, & Dadswell, Michael & GJ, Parsons,. (2018). *Sea Scallop (Placopecten magellanicus) Culture in Northeastern North America Background.*

Shrestha, B. K., & Shakya, J. (2021). *Simple method devised for rapid isolation and identification of Vibrio cholerae from water resources of Sunsari District, Nepal*. 33-38.

Shumway, S. E. (Ed.). (1991). *Scallops: Biology, Ecology and Aquaculture (DEVELOPMENTS IN AQUACULTURE AND FISHERIES SCIENCE)*. Elsevier Science Ltd. 600-604

Shumway, S. E., & Jay Parsons, G. (2011). *Scallops: Biology, Ecology and Aquaculture*. Elsevier.

Shutter MC, Akhondi H. Tetracycline. [Updated 2023 Jun 5]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK549905/

Soller, J. A., Eftim, S. E., & Nappier, S. P. (2018). Direct potable reuse microbial risk assessment methodology: Sensitivity analysis and application to State log credit allocations. *Water Research*, *128*, 286-292.

Song, L., Wang, L., Zhang, H., & Wang, M. (2015). The immune system and its modulation mechanism in scallop. *Fish & Shellfish Immunology*, *46*(1), 65-78.

Sousa, R., Vasconcelos, J., Vera-Escalona, I., Delgado, J., Freitas, M., Gonzalez, J. A., & Riera, R. (2021). Major ocean currents may shape the microbiome of the topshell *Phorcus sauciatus* in the NE Atlantic Ocean. *Scientific Reports*, *11*(1), 12480.

Squires, H.J., 1962. Giant scallops in Newfoundland coastal waters. Bulletin of the Fisheries Research Board Canada 135.

Stockwell, V. O., & Duffy, B. (2012). Use of antibiotics in plant agriculture. *Revue Scientifique et Technique* , *31*(1), 199-210.

Stokesbury, K. D. E. (2012). Stock Definition and Recruitment: Implications for the U.S. Sea Scallop (Placopecten magellanicus) Fishery from 2003 to 2011. *Reviews in Fisheries Science*, *20*(3), 154-164.

Stotz, W. (2000). When aquaculture restores and replaces an overfished stock:Is the conservation of the Species assured? The case of the scallop *Argopecten purpuratus* in Northern Chile. *Aquaculture International: Journal of the European Aquaculture Society*, *8*(2), 237-247.

Sudha, S., Mridula, C., Silvester, R., & Hatha, A. (2014). *Prevalence and antibiotic resistance of pathogenic Vibrios in shellfishes from Cochin market*. https://www.semanticscholar.org/paper/64c3f206090ea2f196d03786b16ab152d3e69345

Sukmawati, A. R., & Pursetyo, K. T. (2021). The study of live and frozen marine fish in commodities imports using conventional and the Polymerace Chain Reaction (PCR). *IOP Conference Series: Earth and Environmental Science*, *718*(1), 012023.

Takemura, A. F., Chien, D. M., & Polz, M. F. (2014). Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Frontiers in Microbiology*, *5*, 38.

The hatchery culture of bivalves: a practical manual. (*FAO Fisheries Technical Papers, No. 471*) .Retrieved April 17, 2024, from https://www.fao.org/in-action/globefish/publications/details-publication/en/c/338709/

Torkildsen, L., & Magnesen, T. (2004). Hatchery Production of Scallop Larvae (Pecten maximus) - Survival in Different Rearing Systems. *Aquaculture International: Journal of the European Aquaculture Society*, *12*(4), 489-507.

Tremblay, R., Landry-Cuerrier, M., & Humphries, M. (2020). Culture and the social-ecology of local food use by Indigenous communities in northern North America. *Ecology and Society*, p. 25. https://doi.org/10.5751/ES-11542-250208

Us Epa, O. (2016). *Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2015*. https://www.epa.gov/ghgemissions/inventory-us-greenhouse-gas-emissions-and-sinks-1990-2015

Utting, S., & Millican, P. (1998). The Role of Diet in Hatchery Conditioning of *Pecten Maximus* L.: A Review. *Aquaculture*, 167(3-4), 215-227. https://doi.org/10.1016/S0044-8486(98)00268-3

Ventilla, R.F. 1982. The scallop industry in Japan. Advan. Mar. Biol. 20: 310 - 382.

Vezzulli, L., Colwell, R. R., & Pruzzo, C. (2013). Ocean warming and spread of pathogenic vibrios in the aquatic environment. *Microbial Ecology*, *65*(4), 817-825.

Wang, S., Duncan, P.F., Knibb, W. & Degnan, B.M. 2002. Byssal attachment of Amusium balloti (Bernardi, 1861) (Bivalvia:Pectinidae) spat. Journal of Shellfish Research 21:563-569.

Wang, S., Zhang, J., Jiao, W., Li, J., Xun, X., Sun, Y., Guo, X., Huan, P., Dong, B., Zhang, L., Hu, X., Sun, X., Wang, J., Zhao, C., Wang, Y., Wang, D., Huang, X., Wang, R., Lv, J., â€¦ Bao, Z. (2017). Scallop genome provides insights into evolution of bilaterian karyotype and development. *Nature Ecology & Evolution*, *1*(5), 120.

Wickham, H., Francois, R., Henry, L., Müller, K., 2015. dplyr: A Grammar of Data Manipulation. R package version 0.4. 3. R Found. Stat. Comput, Vienna. ⟨https://CR AN.R-project.org/package=dplyr..

Williams, T. C., Froelich, B. A., Phippen, B., Fowler, P., Noble, R. T., & Oliver, J. D. (2017). Different abundance and correlational patterns exist between total and presumed pathogenic Vibrio vulnificus and V. parahaemolyticus in shellfish and waters along the North Carolina coast. *FEMS Microbiology Ecology*, *93*(6). https://doi.org/10.1093/femsec/fix071

Winkler, F., & Estevez, B. F. (2003). Effects of self-fertilization on growth and survival of larvae and juveniles of the scallop Argopecten purpuratus L. *Journal of Experimental Marine Biology and Ecology*, *292*, 93-102.

Yu, Y., Tang, M., Wang, Y., Liao, M., Wang, C., Rong, X., Li, B., Ge, J., Gao, Y., Dong, X., & Zhang, Z. (2023). Virulence and antimicrobial resistance characteristics assessment of Vibrio isolated from shrimp (Penaeus vannamei) breeding system in south China. *Ecotoxicology and Environmental Safety*, *252*, 114615.

Zorz, J., Willis, C., Comeau, A. M., Langille, M. G. I., Johnson, C. L., Li, W. K. W., & LaRoche, J. (2019). Drivers of Regional Bacterial Community Structure and Diversity in the Northwest Atlantic Ocean. *Frontiers in Microbiology*, *10*, 281.

APPENDICES

APPENDIX A: Sampling Protocol for MFAC Scallop Vibrio Project

Design: Following DEI production protocols which mimic Industry standards, the larval development tanks are a static system, in which every 48 hours, larvae are removed with fine filtering, water is completely changed out, tanks cleaned with soap, water, and a low concentration bleach solution, and larvae are replaced into the water.

Sample collection targets:

- Eggs/sperm in water samples, at time of breeding
    - Used for culturing?
    - Save at least 3 2-ml tubes worth in freezer (or in ethanol at room temperature)
- D-veliger larvae in water samples, in the first few days
    - Used for community sequencing.
    - Save at least 3 2-ml tubes (> 20 larvae per tube if possible) worth in ethanol at room temperature for transport to Grey Lab at UMaine
- Adult scallop tanks, every 2 days when tanks emptied (if they are placed in 500-L tanks) OR collect some from the bulk tank they are in
    - Swab sides of tank, using three different swabs in three different locations
    - Use each of the three swabs to create a TCBS culture plate
        - Grow these at room temperature to look for Vibrio growth. If positive, save in fridge for transport back to Perry lab at UMaine
    - Save each of the three swab heads (after they have been used for the culture plates) by snapping them off into their own individual 2-ml tubes filled with PBS
        - Save these in the freezer for transport back to Ishaq Lab at UMaine
- Larvae scallop tanks, every 2 days when tanks emptied until after population crash
    - Swab sides of tank, using three different swabs in three different locations
    - Use each of the three swabs to create a TCBS culture plate
        - Grow these at room temperature to look for Vibrio growth. If positive, save in fridge for transport back to Perry lab at UMaine
    - Save each of the three swab heads by snapping them off into their own individual 2-ml tubes filled with PBS
        - Save these in the freezer for transport back to Ishaq Lab at UMaine
- A control tank/water sample that has no animals in it, every 2 days
    - Swab sides of tank, using three different swabs in three different locations
    - Use each of the three swabs to create a TCBS culture plate
        - Grow these at room temperature to look for Vibrio growth. If positive, save in fridge for transport back to Perry lab at UMaine
    - Save each of the three swab heads by snapping them off into their own individual 2-ml tubes filled with PBS
        - Save these in the freezer for transport back to Ishaq Lab at UMaine

APPENDIX B: Thiosulfate-citrate-bile salts-sucrose (TCBS) Agar

TCBS Agar is used to selectively isolate Vibrio cholerae and other enteropathogenic vibrios. This is used as a starting point for other microbiological tests.

This media is purchased as a pre-mixed powder. Add it to boiling water but do not autoclave it.

- Boil 1000 ml of deionized (MilliQ) water in an electric tea kettle which is set inside a chem hood.
- While that is heating, weigh 89 grams of dehydrated TCBS agar powder, preferably in another chem hood away from the moisture. This powder creates a lot of fine dust, it would help to wear a facemask if this being weighed outside of a chem hood.
- Make sure it says “agar” on the bottle, so that it will solidify later.
- Once the water has boiled, the kettle has automatically turned itself off, and the water is no longer bubbling (but still hot), pour in the 89 grams of powder. Use a large metal spoon to mix it in enough to disrupt the large clumps.
- Turn the kettle back on for about 10 seconds, then turn it back off again. Do not leave it alone when you turn it back on.
- You want it to roil/bubble just enough to mix it in. If you leave it for more than 30 seconds it will boil over!
- Allow the media to cool in the kettle for about 5 - 10 minutes. It will be around 45-50°C when cool.
- During this time, you can set up plates in the Media Prep Station.
- Mix the kettle by gentle shaking, and pour into sterile Petri plates, so that the media fills about 40% of dish. Place the lid half-on the the top of the plate to allow evaporation.
- Allow the plates to cool for at least 15 minutes, or until the media remains solid when you jiggle the plate. Put the lid fully on and flip the plate over.
- Store upside down in a refrigerator until use, ideally within 3 weeks. Wrap with parafilm to extend their shelf life.

APPENDIX C: Alkaline Peptone Water (APW) agar

This is used as a starting point for other microbiological tests.

1. In an autoclaved flask add the following ingredients, with low heat and stirring:

Ingredients	Amount (gm/L)	What it is used for
dH20	1 L	
NaCl	20 g	
BactoPeptone	20 gm	Peptone, a protein decomposition product, is made by incomplete hydrolysis process of the protein originated from beef, casein, milk powder, gelatin, soy protein, silk protein, fibrin, etc.
Agar	15 g	

2. Use HCL or OH to adjust the final pH to 8.4
3. Autoclave at 121°C for 20 minutes, with the "slow" setting selected so it will increase/decrease the pressure gradually and prevent over-boiling/exploding the media bottles.

4. Allow the media to cool in the bottle for about 5 - 10 minutes. It will be around 45-50°C when cool.

a. During this time, you can set up plates in the Media Prep Station.

5. Mix the bottle by gentle shaking, and pour into sterile Petri plates, so that the media fills about 40% of dish. Place the lid half-on the the top of the plate to allow evaporation.
6. Allow the plates to cool for at least 15 minutes, or until the media remains solid when you jiggle the plate. Put the lid fully on and flip the plate over.

Store upside down in a refrigerator until use, ideally within 3 weeks. Wrap with parafilm to extend their shelf life.

APPENDIX D: Oxidase test

This tests the presence of a cytochrome oxidase system that will catalyze the transport of electrons between electron donors in the bacteria and a redox dye- tetramethyl-p-phenylene-diamine. The dye is reduced to a deep purple color. This test is used to assist in the identification of Pseudomonas, Neisseria, Alcaligens, Aeromonas, Campylobacter, Vibrio, Brucella and Pasteurella, all of which produce the enzyme cytochrome oxidase.

Note: this test can be unreliable as a differential tool, and should be combined with other methods.

Reagents and Equipment

- Bacterial isolates which have been grown on a TCBS plate for 24 hours at room temperature
- 1% dimethyl-*p*-phenylenediamine dihydrochloride (diluted in ultrapure water)
- Sterile cotton swabs, 1 for each isolate
- Bunsen burner and metal inoculating loop
    - Or, you can just use sterile plastic inoculating loops, 1 for each isolate
- Nutrient Agar plates, 1 for each isolate. Recipe is provided above.

Direct Plate Method

1. You must use bacterial isolates grown on TCBS agar for 24 hours, so they are all in the same phase of growth. If these plates are not already made, follow steps a-d. Otherwise, proceed to Step 2.
    a. Use the Streak Plate Method to inoculate each bacterial isolate onto their own TCBS agar plate.
    b. Label the bottom of the plate with the isolate ID number, and the date.
    c. Let the TCBS plates sit at room temperature for 24 hours, with the plates stacked upside-down.
    d. Plates may be left on the lab counter, inside a plastic container to prevent contamination.
2. For each isolate, take the TCBS plate and use a sterile inoculating loop to pick a colony. Use the Streak Plate Method (zones 1,2,3) to spread the bacteria on the inoculating loop onto a Nutrient agar plate.
    a. Label the bottom of the plate with the isolate ID number, and the date.
3. Grow the Nutrient or Muller-Hinton plates at room temperature for 24 hours.
    a. Plates may be left on the lab counter, inside a plastic container to prevent contamination.

Perform these steps at least twice for each isolate:

4. Swab a single colony from the plate and hold over an empty petri dish lid or other dish to collect liquid drops

5. Add 2 -3 drops of 1% dimethyl-*p*-phenylenediamine dihydrochloride (diluted in ultrapure water) reagent directly to the swab. Do not flood the plate with the reagent.
6. Then observe a color change within 10 seconds.
a. Results can be recorded on the “Isolates” tab of the Vibrio tracking sheet.
b. **Positive**: Dark purple or black color appears
c. **Negative**: No color change

APPENDIX E: TSI, Triple Sugar Iron agar media

The triple sugar iron agar determines whether bacteria can use glucose, lactose, and/or sucrose, and whether they can produce hydrogen sulfate (H_2S) from sodium thiosulfate. This agar is used in test tubes which are slanted when the agar is poured in and cooled.

Reagents and Equipment

- Bacterial isolates which have been grown on a TCBS plate for 24 hours at room temperature
- Bunsen burner and metal inoculating loop
    - Or, you can just use sterile plastic inoculating loops, 1 for each isolate
- TSI agar in test tubes, 3 tubes for each isolate, plus 3 tubes for negative control

Ingredient	**Amount per liter**	**What it is used for**
dH2O	1 L	
Agar	15 - 16 g	
Pancreatic digest of casein	10 g	Carbon source, amino acids
Peptone	10 g	Carbon source, amino acids
Lactose	10 g	Carbon source
Sucrose	10 g	Carbon source
Glucose	1 g	Carbon source
Sodium chloride (NaCl)	5 g	
Ferrous sulfate, ferrous ammonium sulfate, or ferrous ammonium citrate	0.2 g	Reacts with H2S gas to form black precipitate
Sodium thiosulfate	0.3 g	Used to produce H2S gas
Phenol red	0.024 g	pH indicator

To make the media:

1. Use a 1000 ml or larger flask, beaker, or other glass bottle to mix in. Add an autoclaved magnetic stir rod and place on a magnetic stir plate.

a. It is recommended that the stir plate is placed in a chemical safety hood, so the dust, fumes, and evaporation from the media are carried out of the room.

2. Mix all ingredients. May need to heat slightly to dissolve.
3. Autoclave at 121°C for 20 minutes, with the "slow" setting selected so it will increase/decrease the pressure gradually and prevent over-boiling/exploding the media bottles.
4. When the media comes out of the autoclave, it will already have started to harden in the bottom of the bottle.

a. Put the bottle onto the magnetic hot plate to spin and reheat it

b. While that liquifies again, get racks of test tubes ready in the BSL2 hood, propped up on a 1.5 ml tube tray so test tubes are at an angle

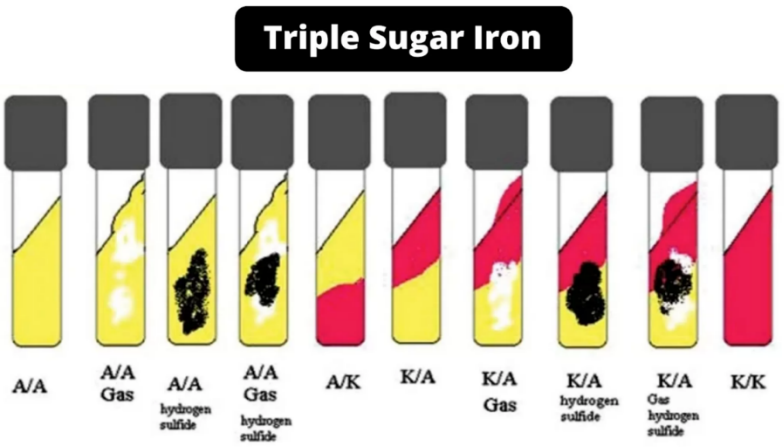
5. Move the media bottle to a magnetic stir plate in the hood, and put about 200 ml in a beaker.

a. Pipette 4 - 5 ml into test tubes in the BSL2 hood, allow to cool at that angle
b. When cool, cap with a sterile rubber stopper or aluminum foil over the tube of the tube caps.
c. It can be saved in the fridge for several weeks.

To run the test:

6. You must use bacterial isolates grown on TCBS agar for 24 hours, so they are all in the same phase of growth.
7. When inoculating the TSI agar in the test tubes, take 1 inoculating loop worth of colony from the TCBS plate, and stab the butt of the test tubes almost to the bottom. Then, pull the loop out and use it to streak the slant in a zig-zag pattern.
a. Make 3 tubes for each isolate, and leave 3 tubes as sterile to be the negative control.
b. Cap the test tubes, and write date and isolate # on the tube.
8. Incubate the tubes at room temperature 66°C for 18 - 24 hours in the incubator with ambient air (no carbon dioxide gas added to the incubator). (Butt will be anaerobic)
9. Then observe a color change at 24 hours.
a. Results can be recorded on the "Isolates" tab of the Vibrio tracking sheet.

Result label	Colors (slant/butt)	Reaction (slant/butt)	Description
K / A	Red / yellow	Alkaline / acid	Uses Glu, acid in butt Slant: b/c glu low peptones used, produces NH3
A / A	Yellow / yellow	Acid / acid	Uses Glu and Lac/Suc, lots of acid produced
A /A	Yellow / black	Acid / acid	Uses Glu and Lac/Suc, lots of acid, H2S produced (only happens in acidic and anaerobic)
K / A	Red / black	Alkaline / acid	Glu used + H2S production (only in acidic and anaerobic)
K / NC	Red / orange	Alkaline / No change	No carbs used, just peptones, orange butt means can't use peptones anaerobically.
K / NC	Black blue / orange	Alkaline / No change	No carbs used, just peptones aerobically, pigment produced



https://microbiologynote.com/triple-sugar-iron/

APPENDIX F: Arginine glucose slant

Reagents and Equipment

- Bacterial isolates which have been grown on a TCBS plate for 24 hours at room temperature
- Bunsen burner and metal inoculating loop
    - Or, you can just use sterile plastic inoculating loops, 1 for each isolate
- Arginine glucose agar in test tubes, 3 tubes for each isolate, plus 3 tubes for negative control

Ingredient	**Amount per liter**	**What it is used for**
dH2O	1 L	Dissolve the ingredients and create volume in the media
Agar	15 - 16 g	Creates a solid structure to the media
BactoPeptone	5 g	Peptone, a protein decomposition product, is made by incomplete hydrolysis of the protein from beef, casein, milk powder, gelatin, soy protein, silk protein, fibrin, etc.
Yeast extract	3 g	Carbon source, amino acids
Tryptone	10 g	Amino acids. Peptides formed by the digestion of casein by the protease trypsin
Glucose	1 g	Carbon source
Sodium chloride (NaCl)	20 g	
Ferrous +/- ammonium) sulfate, or ferrous ammonium citrate	0.5 g	Reacts with H2S gas to form black precipitate
Sodium thiosulfate	0.3 g	Used to produce H2S gas
Bromocresol purple	0.02 g	pH indicator

To make the media:

1. Use a 1000 ml or larger flask, beaker, or other glass bottle to mix in. Add an autoclaved magnetic stir rod and place on a magnetic stir plate.

a. It is recommended that the stir plate is placed in a chemical safety hood, so the dust, fumes, and evaporation from the media are carried out of the room.

2. Mix all ingredients. May need to heat slightly to dissolve.
3. Pipette 5 ml into test tubes in the BSL2 hood, and cap so there is a little opening for air to move through. Put aluminum foil over the tube of the tube caps.
4. Autoclave at 121°C for 20 minutes, with the “slow” setting selected so it will increase/decrease the pressure gradually and prevent over-boiling/exploding the media bottles.
5. When the media comes out of the autoclave, it will already have started to harden in the bottom of the bottle.

a. Put the bottle onto the magnetic hot plate to spin and reheat it

b. While that liquifies again, get racks of test tubes ready in the BSL2 hood, propped up on a 1.5 ml tube tray so test tubes are at an angle

6. Move the media bottle to a magnetic stir plate in the hood, and put about 200 ml in a beaker.

a. Pipette 4 - 5 ml into test tubes in the BSL2 hood, allow to cool at that angle

b. When cool, cap with a sterile rubber stopper or aluminum foil over the tube of the tube caps.

c. It can be saved in the fridge for several weeks.

To run the test:

7. When inoculating the ArG agar in the test tubes, take 1 inoculating loop worth of colony from the TCBS plate, and stab the butt of the test tubes almost to the bottom. Then, pull the loop out and use it to streak the slant in a zig-zag pattern.

a. Make 3 tubes for each isolate, and leave 3 tubes as sterile to be the negative control.

b. Cap the test tubes, and write date and isolate # on the tube.

8. Incubate the tubes at room temperature 66°C for 18 - 24 hours in the incubator with ambient air (no carbon dioxide gas added to the incubator). (Butt will be anaerobic)

Results can be recorded on the "Isolates" tab of the Vibrio tracking sheet.

APPENDIX G: Voges-Proskauer test

Resources for Voges-Proskauer test. This test identifies Enterobacteriacae, which we would not want in a hatchery. We expect that the Vibrio and other marine bacteria will not grow in this media, and if they do grow, they will provide a negative result.

Reagents, make and set aside:

1. 5% α-naphthol (1 g / 20 mL) in 95% ethyl alcohol. The reagent should be stored at 4-8°C in the dark. The shelf life is 2-3 weeks.
2. 40% potassium hydroxide (KOH): Dissolve 40 g of potassium hydroxide pellets in a polyethylene bottle in 100 ml of distilled water. Keep the bottle in a cool water bath during preparation.

To make the media:

1. In an autoclaved flask add the following ingredients, with low heat and stirring:

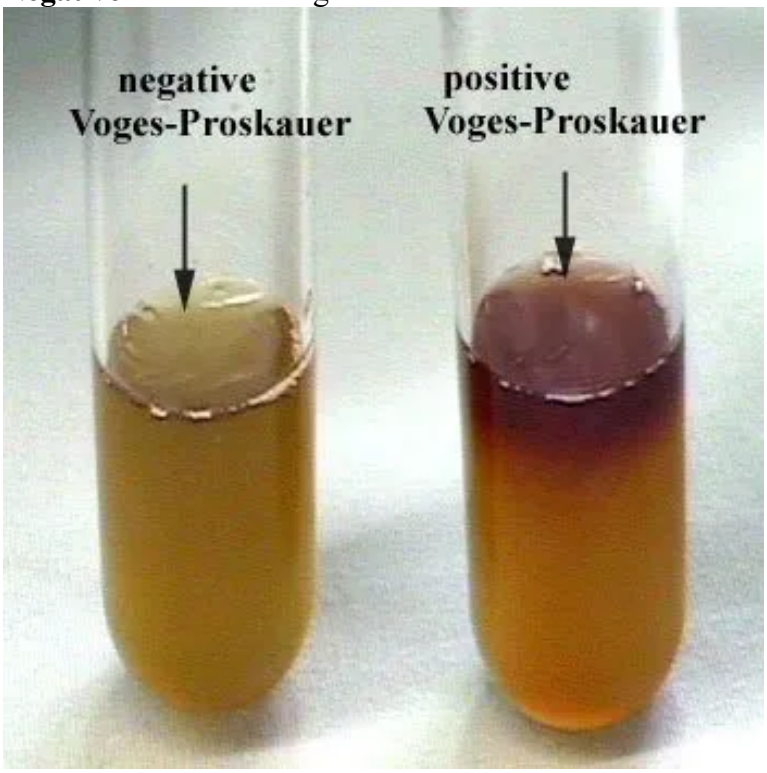
Ingredients for VP broth	**Amount per liter**	**What it is used for**
dH2O	1L	
Polypeptone	7 g	Amino acid source
Glucose	5 g	Carbon source
Dipotassium phosphate	5 g	

2. If needed, use HCL or OH to adjust the final pH to 6.9.
3. Pipette 5 ml into test tubes in the BSL2 hood, and cap so there is a little opening for air to move through. Put aluminum foil over the tube of the tube caps.
4. Autoclave at 121°C for 20 minutes, with the "slow" setting selected so it will increase/decrease the pressure gradually and prevent over-boiling/exploding the media bottles.
5. Allow to cool to room temperature before use. It can be saved in the fridge for several weeks.

To run the test:

6. Add 1 inoculating loopful of colony from the TCBS plate.
a. Make 3 tubes for each isolate, and leave 3 tubes as sterile to be the negative control.
b. Write the date and isolate number on the tube.
7. Incubate for 24 - 48 hours at 35°C.
8. At the end of this time, aliquot 1 mL of broth into a clean test tube.
9. Add 0.6mL of 5% α-naphthol*, followed by 0.2 mL of 40% KOH.

a. (Note: The reagents must be added in this order.)
10. Shake the tube** gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 minutes.
11. Then observe a color change after 15 minutes and before 30 minutes.
a. Results can be recorded on the "Isolates" tab of the Vibrio tracking sheet.
b. **Positive**: development of a pink-red color at the surface within 15 minutes or more after the addition of the reagents.
c. The test should not be read after standing for over 1 hour because negative Voges-Proskauer cultures may produce a copper-like color, potentially resulting in a false-positive interpretation.
d. **Negative**: no color change



APPENDIX H: Susceptibility to Ampicillin

Ingredients for Nutrient Agar	Amount (gm/L)	What it is used for
dH20	1 L	
NaCl	20 gm	
BactoPeptone	20 gm	Peptone, a protein decomposition product, is made by incomplete hydrolysis process of the protein originated from beef, casein, milk powder, gelatin, soy protein, silk protein, fibrin, etc.
Ampicillin	100 mg	
Agar	15 g	

1. Use HCL or OH to adjust the final pH to 8.4 ± 0.2.
2. Pour into multiple bottles before autoclaving, so that each bottle is only half full.
3. Autoclave at 121°C for 20 minutes, with the "slow" setting selected so it will increase/decrease the pressure gradually and prevent over-boiling/exploding the media bottles.
4. Allow the media to cool in the bottle for about 5 - 10 minutes. It will be around 45-50°C when cool.

a. During this time, you can set up plates in the Media Prep Station.

5. Mix the bottle by gentle shaking, and pour into sterile Petri plates, so that the media fills about 40% of dish. Place the lid half-on the the top of the plate to allow evaporation.
6. Allow the plates to cool for at least 15 minutes, or until the media remains solid when you jiggle the plate. Put the lid fully on and flip the plate over.
7. Store upside down in a refrigerator until use, ideally within 3 weeks. Wrap with parafilm to extend their shelf life.
8. To plate, use the lawn or streak plate method to see if the isolate will grow or not.

APPENDIX I: Susceptibility to Oxytetracycline

Ingredients for Nutrient Agar	Amount (gm/L)	What it is used for
dH20	1 L	
NaCl	20 gm	
BactoPeptone	20 gm	Peptone, a protein decomposition product, is made by incomplete hydrolysis process of the protein originated from beef, casein, milk powder, gelatin, soy protein, silk protein, fibrin, etc.
Oxytetracyclyine	100 mg	
Agar	15 g	

1. Use HCL or OH to adjust the final pH to 8.4 ± 0.2.
2. Pour into multiple bottles before autoclaving, so that each bottle is only half full.
3. Autoclave at 121°C for 20 minutes, with the "slow" setting selected so it will increase/decrease the pressure gradually and prevent over-boiling/exploding the media bottles.
4. Allow the media to cool in the bottle for about 5 - 10 minutes. It will be around 45-50°C when cool.

a. During this time, you can set up plates in the Media Prep Station.

5. Mix the bottle by gentle shaking, and pour into sterile Petri plates, so that the media fills about 40% of dish. Place the lid half-on the the top of the plate to allow evaporation.
6. Allow the plates to cool for at least 15 minutes, or until the media remains solid when you jiggle the plate. Put the lid fully on and flip the plate over.
7. Store upside down in a refrigerator until use, ideally within 3 weeks. Wrap with parafilm to extend their shelf life.
8. To plate, use the lawn or streak plate method to see if the isolate will grow or not.

APPENDIX J: Susceptibility to Streptomycin sulfate

Ingredients for Nutrient Agar	Amount (gm/L)	What it is used for
dH20	1 L	
NaCl	20 gm	
BactoPeptone	20 gm	Peptone, a protein decomposition product, is made by incomplete hydrolysis process of the protein originated from beef, casein, milk powder, gelatin, soy protein, silk protein, fibrin, etc.
Streptomycin sulphate	100 mg	
Agar	15 g	

1. Use HCL or OH to adjust the final pH to 8.4 ± 0.2.
2. Pour into multiple bottles before autoclaving, so that each bottle is only half full.
3. Autoclave at 121°C for 20 minutes, with the "slow" setting selected so it will increase/decrease the pressure gradually and prevent over-boiling/exploding the media bottles.
4. Allow the media to cool in the bottle for about 5 - 10 minutes. It will be around 45-50°C when cool.
    a. During this time, you can set up plates in the Media Prep Station.
5. Mix the bottle by gentle shaking, and pour into sterile Petri plates, so that the media fills about 40% of dish. Place the lid half-on the the top of the plate to allow evaporation.
6. Allow the plates to cool for at least 15 minutes, or until the media remains solid when you jiggle the plate. Put the lid fully on and flip the plate over.
7. Store upside down in a refrigerator until use, ideally within 3 weeks. Wrap with parafilm to extend their shelf life.
8. To plate, use the lawn or streak plate method to see if the isolate will grow or not.

APPENDIX K: Susceptibility to Benzyl Penicillin

Ingredients for Nutrient Agar	Amount (gm/L)	What it is used for
dH20	1 L	
NaCl	20 gm	
BactoPeptone	20 gm	Peptone, a protein decomposition product, is made by incomplete hydrolysis process of the protein originated from beef, casein, milk powder, gelatin, soy protein, silk protein, fibrin, etc.
Benzyl Penicillin	100 mg	
Agar	15 g	

1. Use HCL or OH to adjust the final pH to 8.4 ± 0.2.
2. Pour into multiple bottles before autoclaving, so that each bottle is only half full.
3. Autoclave at 121°C for 20 minutes, with the "slow" setting selected so it will increase/decrease the pressure gradually and prevent over-boiling/exploding the media bottles.
4. Allow the media to cool in the bottle for about 5 - 10 minutes. It will be around 45-50°C when cool.
    a. During this time, you can set up plates in the Media Prep Station.
5. Mix the bottle by gentle shaking, and pour into sterile Petri plates, so that the media fills about 40% of dish. Place the lid half-on the the top of the plate to allow evaporation.
6. Allow the plates to cool for at least 15 minutes, or until the media remains solid when you jiggle the plate. Put the lid fully on and flip the plate over.
7. Store upside down in a refrigerator until use, ideally within 3 weeks. Wrap with parafilm to extend their shelf life.
8. To plate, use the lawn or streak plate method to see if the isolate will grow or not.

BIOGRAPHY OF THE AUTHOR

Ayodeji Olaniyi was born in Iwo, Osun state Nigeria on September 30, 1992. He was raised in Iwo, Osun state and graduated from Our Lady of Fatima Academy, Iwo. He attended the Federal University of Agriculture Abeokuta and graduated in 2015 with a bachelor's degree in animal production and health. He gained admission to University Maine and entered the Animal Science graduate program at The University of Maine in the spring of 2022. After receiving his degree, Ayodeji will join Indianapolis University of Purdue Indianapolis, to begin his career in the Hydrocephalus lab. Ayodeji is a candidate for the Master of Science degree in Animal Science from the University of Maine in May 2024.