Microbiome associated with *Ulva lacinulata* and seawater in an integrated abalone (*Haliotis midae*)–*Ulva* system with partial recirculation

MKHNOK047

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Abstract

Several commercial abalone farms in South Africa grow *Ulva* in D-ended raceways to bioremediate farm effluent water, allowing water to be recirculated back to the abalone, while *Ulva* is often used as a supplementary feed source. Despite *Ulva'*s versatility in integrated multi-trophic aquaculture (IMTA) systems, there are biosecurity concerns with the recirculation of seawater and the use of effluent-grown *Ulva* as abalone feed, preventing wider adoption of this technology. To better understand the potential disease risks, as well as the benefits of this practise, this study aimed to characterise the bacterial, fungal, and oomycete communities associated with seawater and *Ulva* in an integrated abalone-*Ulva* farm with 50% water recirculation. The study was conducted on a commercial abalone farm along the South-Western Cape coast of South Africa. Water (N = 40) and *Ulva* (N = 20) samples were collected from two different systems. One system consisted of *Ulva* raceway tanks that received seawater directly from the adjacent coastline, hereafter referred to as the seawater (SW) raceway or non-IMTA system. The other systems comprised raceways receiving abalone effluent water, with 50% recirculation between the abalone and *Ulva* raceways, referred to as abalone effluent water (AEW) or IMTA systems. *Ulva* samples were collected from within each raceway, whereas water samples were collected at the inlet (effluent water) and outlet (bioremediated water) of each raceway. One SW raceway (only one exists in the farm) and 4 AEW raceways were sampled in summer, autumn, winter, and spring.

The first experimental chapter (Chapter 2) of the study investigated the impact of various DNA extraction kits on the quality and quantity of DNA obtained from environmental samples and compared commonly used bioinformatic pipelines for the 16S rDNA (QIIME2 vs. mothur) and ITS2 (PIPITS vs. QIIME2) genes to evaluate how different bioinformatics pipelines influence taxonomic classification and subsequent diversity analyses. In chapter 3, culture-dependent techniques were used to assess changes in the abundance of specific bacteria on *Ulva* and in seawater using three selective media types, namely tryptic soy agar (TSA; a general media routinely used for isolation of marine bacteria), thiosulfate-citrate-bile-sucrose (TCBS) agar (*Vibrio* selective), and Ulvan agar, where the primary carbohydrate of *Ulva* (Ulvan) was utilised as the main carbohydrate source. A non-culture-based next-generation sequencing (NGS) approach was subsequently used to describe the bacterial microbiome

associated with the IMTA and non-IMTA systems, by sequencing the V4 hyper-variable region of the 16S rDNA gene. In Chapter 4, fungi and oomycetes were targeted by sequencing the internal transcriber 2 (ITS2) gene region of nuclear ribosomal DNA. Following NGS using an Illumina MiSeq sequencing platform, sequence data were processed using QIIME2 and reads were mapped against the SILVA 16S rDNA database for the bacterial microbiome and the UNITE database for the mycobiome. The summarised taxonomic abundance was assessed using MicrobiomeAnalyst.

The Qiagen QIAamp DNA Micro kit and QIIME2 bioinformatic pipeline exhibited the best overall performance out of the DNA extraction kits and bioinformatic pipelines tested in chapter 2, combining high sensitivity with excellent specificity, and were both used for subsequent analyses. Chapter 3 results showed that culturable bacterial numbers were significantly higher (ANOVA; $p < 0.05$) in the IMTA raceway systems receiving abalone effluent water than in *Ulva* raceways receiving seawater directly from the adjacent coastline. Bacterial abundance on all three selective media types was also higher on *Ulva* blades sampled from AEW systems. However, in both systems, *Ulva* appeared to have a modulatory effect on the number of culturable bacteria in the water column, as indicated by the general reduction in bacteria recovered from seawater from the inlets to the outlets of both systems ($p < 0.001$). A greater reduction in marine bacteria between the inlet and outlet of water samples was observed in the effluent water system (IMTA) when compared to the seawater system (non-IMTA), which had lower nutrient levels. A total of 2822 individual bacterial amplicon sequence variants (ASVs) were identified, belonging to 203 family-, 305 genus-, and 320 species-level taxonomic groups. Alpha diversity analyses, based on Chao1, Shannon, and Simpson indices, showed statistically significant differences (ANOVA; $p < 0.05$) between the respective cohorts, where the greatest amount of diversity was observed in the water cohorts, whereas the *Ulva* cohorts had the lowest bacterial abundance and diversity relative to the water systems (SW and AEW). The beta diversity analyses (non-metric multidimensional scaling) showed a partial degree of overlap between the water cohorts and *Ulva* samples from different systems. However, a separation of IMTA and non-IMTA systems was observed. Various genera associated with marine environments were identified, predominantly belonging to *Vibrio*, *Pseudoalteromonas*, and *Granulosicoccus*. Differential abundance analysis (DESeq2) revealed that general marine bacteria such as *Roseobacter*, *Granulosicoccus*, and *Algitalea* were

present in high abundance and potentially pathogenic bacteria such as *Vibrio* exhibited reduced abundance in both system types due to the presence of *Ulva*. Chapter 4 assessed the fungal communities associated with effluent water and seawater through next-generation sequencing of the ITS2 region and revealed the presence of 169 individual ASVs belonging to 54 family-, 63 genus-, and 71 species. The alpha diversity analyses based on Chao1, Shannon, and Simpson indices displayed a higher degree of fungal diversity ($p < 0.001$) in the water cohorts (AEW and SW) than in the *Ulva* cohorts (AEW_*Ulva* and SW_*Ulva*), supporting findings from the bacterial microbiome studies. Moreover, the Chao1 richness estimator was significantly ($p < 0.001$) higher in the AEW (AEW_In and AEW_Out) cohorts than in the SW cohorts. Beta diversity analyses, including principal co-ordinate analysis (PCoA), showed a separation between water samples (AEW and SW), collected from the two system types, which was consistent with the nonmetric multidimensional scaling (NMDS) analysis, demonstrating that sample types tended to have different fungal communities ($p < 0.001$). Within each cohort, ASVs commonly associated with marine environments were found, predominantly belonging to Ascomycota and Basidiomycota.

Collectively, the results of this study indicate that the water cohorts exhibited greater relative abundance and diversity of bacteria and fungi than the *Ulva* cohorts. Furthermore, the availability of nutrients had a significant impact on the overall diversity of these microbial communities wherein the level of diversity in the IMTA system was notably higher compared to the non-IMTA system. This is because microorganisms tend to proliferate in environments characterised by high nutrient availability. The current study highlights the capability of 16S and ITS2 metabarcoding techniques for assessing microbial diversity within complex environments and has for the first time provided critical in-depth information on the microbiome of an abalone-*Ulva* IMTA systems and its contribution to system and animal health. Moreover, the observed positive modulatory effect of *Ulva* on the microbiome of the IMTA system contributes towards a growing body of literature on the benefits of including seaweed(s) in aquaculture systems and aquafeeds. The findings from this study provides critical information on biosecurity of IMTA systems, species health and system health that may promote broader uptake of these more sustainable aquaculture production technologies.

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List of Abbreviations

AEW	Abalone effluent water
SW	Seawater
IMTA	Integrated multi-trophic aquaculture
TSA	Tryptic Soy Agar
TCBS	Thiosulfate-Citrate-Bile-Sucrose
UA	Ulvan agar
QIIME	Quantitative Insights Into Microbial Ecology
°C	Degrees Celsius
%	Percentage
<	Less than
>	More than
±	Plus-minus
β	Beta
µL	Microlitres
µg	Micrograms
µg/mL	Micrograms per millilitre
µM	Micromolar
µm	Micrometre
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
bp	Base pair
cm	Centimetres
CV	Coefficient of variation
DAFF	Department of Agriculture, Forestry and Fisheries
DEFF	Department of Forestry, Fisheries, and the Environment
ddH2O	Double distilled water
DNA	Deoxyribonucleic acid
e.g.	Exempli gratia (for example)
et al.	et alii (and others)
EtBr	Ethidium bromide
FDR	False discovery rate
F-value	Statistic representing the ratio of variances in ANOVA
g	grams
LDA	Linear discriminant analysis
LefSe	Linear discriminant analysis effect size
Log2FC	Log fold change

MgCl2	Magnesium chloride
dNTPs	Deoxynucleoside triphosphates
mL	Millilitres
mm	Millimetres
mM	Millimolar
N	Sample number
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NGS	Next-generation sequencing
ng.µL	Nanogram per microlitre
ITS	Internal transcribed spacer
NMDS	Non-metric multidimensional scaling
NRF	National Research Foundation
PCoA	Principal co-ordinates analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
p-value	Probability value (as a statistically significant threshold)
qPCR	Quantitative polymerase chain reaction
r	Pearson correlation coefficient
rDNA	Ribosomal deoxyribonucleic acid
RLE	Relative log expression
RNA	Ribonucleic acid
s	Seconds
SD	Standard deviation
SE	Standard error
spp.	Several species
US$	United States of America dollar
V	Volume
DADA	Divisive Amplicon Denoising Algorithm
Cm	Chloramphenicol
Kan	Kanamycin
CFU	Colony forming units
OD	Optical density
Mt	Million tonne
PYGS	Peptone-yeast-glucose-saline
ASW	Autoclaved seawater
NaCl	Sodium chloride
Rpm	Revolutions per minute

Chapter 1: Literature review

1.1 Status of aquaculture production globally, in Africa, and in South Africa

1.1.1 Trends in global aquaculture

Aquaculture is defined as the cultivation of aquatic organisms including fish, molluscs, crustaceans and aquatic plants (FAO, 2020). It is the fastest-growing food production sector in the world and presently accounts for more than 50% of the world's fish used for food (FAO, 2020). Other food-producing sectors, such as the poultry sector, have a greater growth rate in terms of quantity (Edwards et al., 2019), but these sectors have a slower pace of expansion than the aquaculture sector. Since the late 1980s, wild capture fisheries landings have remained stagnant, whereas marine and freshwater aquaculture production has increased significantly over the same period (FAO, 2020) (Fig. 1.1). This can mainly be attributed to the increased worldwide per capita fish consumption and the demand for fish products linked to a growing global population (Guillen et al., 2019). In 2017, more than 80 Mt of fish and shellfish and 32 Mt of seaweed were produced from aquaculture, representing over 425 farmed species (FAO, 2019).

The growth and maturation of the aquaculture sector can be defined by three major trends of development: 1) a continuous increase in the volume and value chains of aquaculture; 2) breakthroughs in fish nutrition, genetics, and the use of other types of feeds or feed ingredients that reduce the need to include wild fish in aquafeed formulations; and 3) an expanded culture of extractive species (such as mussels and various seaweeds) with the potential to supply a vast array of food, industrial, and environmental services (Naylor et al., 2021). Aquaculture has evolved to be more integrated into the global food system, characterised by rapid growth and significant transformations in feed ingredients, production methods, farm management strategies and value chains (Naylor et al., 2021). By 2020, global aquaculture production reached a new high of 122.6 Mt, with an estimated farm gate value of US$ 281.5 billion. Asia continues to dominate the global aquaculture industry, producing 91.6% of the total world production over this period. Algae accounted for 35.1 Mt of global production in 2020, whereas aquatic animals accounted for 87.5 Mt (FOA, 2022).

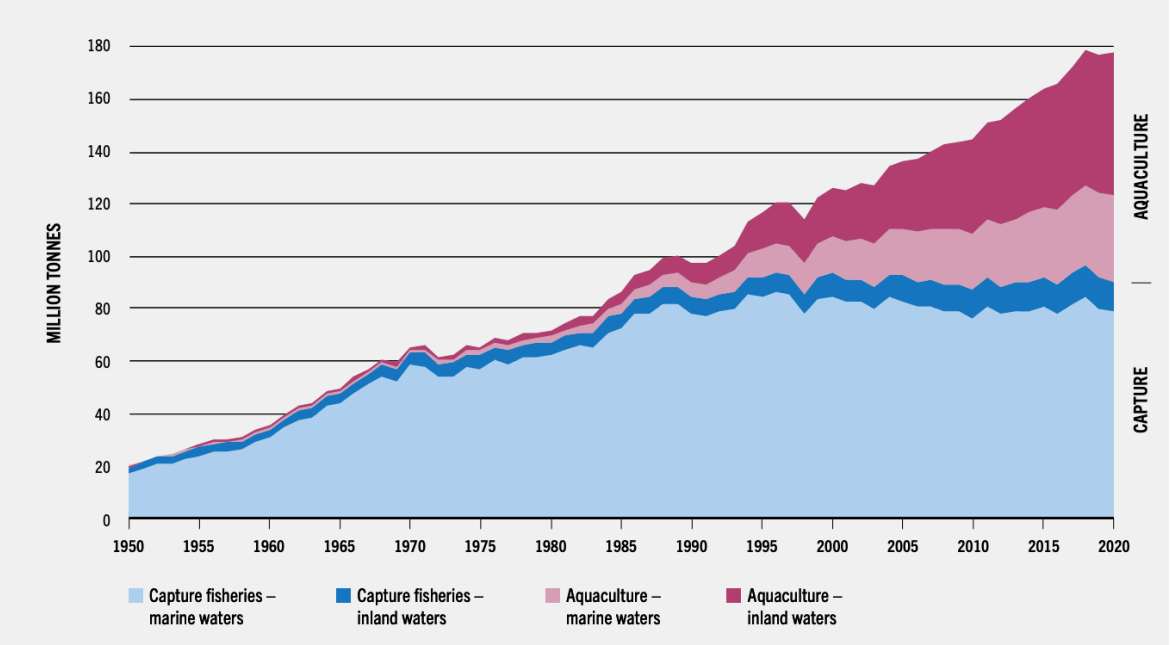

Figure 1.1. World production of capture fisheries and aquaculture from 1950 to 2020 (Source: FAO, 2022).

Since the early 1960s, there has been a notable increase in the worldwide consumption of aquatic foods (excluding algae), with an average annual growth rate of 3%, compared to the 1.6% annual population growth rate. The average per capita consumption of aquatic foods increased steadily from 9.9 kg during the 1960s to 20.5 kg in 2019, followed by a slight decrease to 20.2 kg in 2020 (FAO, 2022). It is anticipated that rising incomes and urbanisation, improvements in post-harvest practices, and changes in dietary trends will lead to a 15% increase in the consumption of aquatic foods, to an average of 21.4 kg per capita in 2030 (FAO, 2022). The primary contributors to aquaculture production in 2017 were seaweeds, carps, bivalves, tilapia, and catfish, collectively accounting for 75% of the total output (Naylor et al., 2021). Although the production of marine and diadromous fish species and crustaceans has also increased rapidly over the past decade, freshwater fish species currently dominate aquaculture fish production (Naylor et al., 2021), with freshwater carps and cyprinids accounting for 53.1% of the total fish production. This is followed by miscellaneous freshwater fish species accounting for 19.5%, tilapia and other cichlids at 11%, and diadromous salmonids at 6.5% (Gunnarsson et al., 2020). The rapid growth of the aquaculture industry on a global scale has unfortunately frequently occurred at the detriment of the environment; therefore, more sustainable aquaculture practices are

required to meet the increasing demand for aquatic foods (FAO, 2022). In a move toward increased diversification and sustainability, the global proportion of plant-based ingredients in aquafeed has increased, as has the production of extractive species (molluscs and seaweed) (Fig. 1.2) that filter nutrients from terrestrial and marine feed sources and those excreted by co-cultured species in integrated systems (Pereira et al., 2008; Chopin & Tacon, 2021).

1.1.2 Molluscan and seaweed aquaculture

Molluscan aquaculture presently comprises ca. 65 documented species, the majority of which are bivalves such as clams, oysters, scallops, and mussels (Tacon, 2020). For example, two-thirds of the total mollusc production is made up of clams, including the Japanese littleneck (carpet shell, *Venerupis philippinarum*) and Pacific cupped oysters, *Magallana gigas* (formally *Crassostrea gigas*). The global production of farmed molluscs increased at an annual rate of 3.5% between 2000 and 2017 (Tacon, 2020). Given that China is the world's top mollusc consumer and producer, bivalve culture in China has expanded to meet consumer demand, with China presently accounting for ca. 84% of the global cultivated mollusc volume. In addition to seafood, molluscan aquaculture produces various industrial products, including pharmaceuticals, fertilizers, poultry grit, construction materials, and nutraceuticals (de Montaudouin, 2014; Smaal et al., 2019). Molluscs and seaweeds are more sustainable species because they are extractive, meaning that they filter particles and extract nutrients from the surrounding water column and consequently do not rely on formulated feeds (Naylor et al., 2021). However, certain high-value farmed molluscs, such as abalone and conchs, generally require formulated feeds to ensure optimal growth rates for aquaculture production, but these species only account for 2.4% of the total output of cultured molluscs (Tacon, 2020). Nonetheless, the sustainability of feed ingredients incorporated into the formulated feeds needs to be carefully considered to ensure long-term sustainability of the farming of these species, as is the case for the abalone (*Haliotis midae* Linnaeus, 1758) farming industry in South Africa, where cultured abalone are frequently fed formulated feeds supplemented with fresh wild collected kelp (*Ecklonia maxima*) and/or farmed *Ulva* spp. (Bolton et al., 2016; Rothman et al., 2020).

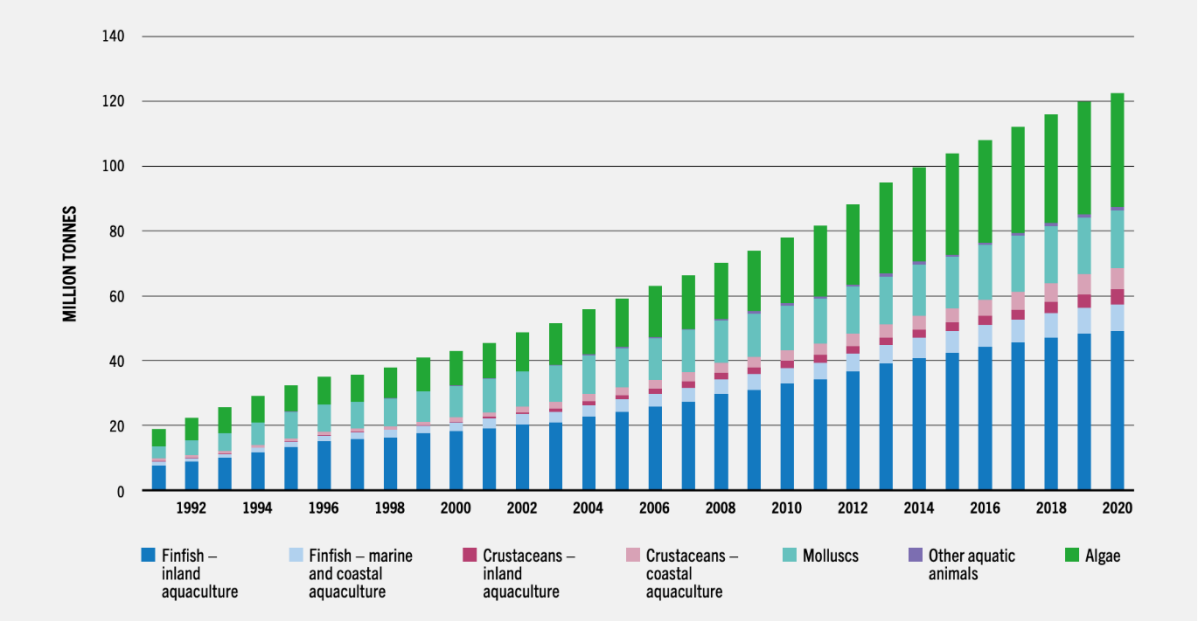

Figure 1.2. World aquaculture production, 1991–2020 (Source: FAO,2022)

Since 2000, there has been an increased demand for macroalgae, with global production tripling from 10 Mt (wet weight) in 2000 to more than 32 Mt in 2017, with aquacultured macroalgae accounting for more than 97% of the production (Barbier et al., 2020; FAO, 2020). More than 99% of cultured macroalgae are produced in Asia, and 31-38% of the 32 Mt of cultivated algae are consumed directly as food. Seaweed culture, like molluscan aquaculture, is widely recognised for its ecosystem service values that extend beyond the production of food and feed, including the bioremediation and carbon sequestration capacity of seaweeds. However, many producers have not been able to capitalize on this value (Chopin & Tacon, 2020). This might be due to a lack of awareness and knowledge about the benefits of seaweeds and a lack of infrastructure and technology to efficiently farm and process seaweeds.

1.2 African aquaculture production

Global aquaculture production increased in all regions by 2020, except for Africa, most notably due to a decline in production in Egypt and Nigeria, which are Africa's two largest aquaculture producing nations (FOA, 2022). Despite large-scale investment in aquaculture, the contribution of African countries such as Ghana, Egypt, Uganda, and Nigeria, which presently produce substantial quantities of fish (Cai et al., 2017; FAO, 2018), to global

aquaculture production remains relatively low (~2.7%) (Halwart, 2020; Hinrichsen et al., 2022). Despite this, from 1995 to 2018, the African region's production increased twenty-fold, from 110,200 to 2,196,000 tonnes, with a compound annual growth rate (CAGR) of 15.55% (FAO, 2016; Halwart, 2020). The remarkable growth and expansion of aquaculture on the African continent over the last decade can be attributed to various factors, such as research and development, credit facility access, capacity building in critical subject areas, good governance, and most importantly, the promotion of aquaculture development led by the private sector (Satia, 2011).

1.2.1 Aquaculture production and the species produced in South Africa

Aquaculture practices began in the mid-1670s in South Africa with the cultivation of indigenous oysters, a practise known as mariculture, which is the farming of marine organisms (Kar, 2021). However, it was not until 1948 that commercial operations became economically feasible (FAO, 2010–2020b). Rainbow trout (*Oncorhynchus mykiss*) farming in South Africa is considered to be the oldest subsector of the freshwater aquaculture sector (DAFF, 2018a). In 1896, the country received its first batch of rainbow trout eggs, and dry pelleted feeds were introduced in 1956 (Hecht & Britz, 1990). South Africa has favourable environmental conditions for the growth of the aquaculture industry, in addition to vast commercial production prospects for numerous cultivated species. Nevertheless, the aquaculture industry has not lived up to its potential and contributes minimally to the country's fisheries products and gross domestic product (GDP) (FAO, 2010–2020b). Compared to Nigeria, Egypt, Uganda, and other countries globally, the South African aquaculture sector remains rather small, contributing less than 1% of Africa's aquaculture production. Based on aquaculture output and monetary value in 2018, South Africa ranked as the tenth highest aquaculture producing country in Africa, contributing 0.28% of the continent's total edible fish aquaculture production (FAO, 2010 – 2020b; IDC/Urban-Econ, 2015). In 2015, the total production of aquaculture in South Africa, comprising both marine and freshwater production, was 5,418 tonnes (excluding seaweed) worth R696 million (US$ 48.2 million), with the mariculture subsector accounting for 3,592 tonnes (72%) and the freshwater aquaculture subsector accounting for 1,826 tonnes (28%) of the total production (DAFF, 2015; Britz & Venter, 2016; DAFF, 2017).

Compared to 2000, aquaculture production increased significantly from 2,819 tonnes to 9,753 tonnes in 2020 (Fig. 1.3). A yearly growth rate of 6.4% was better than the global average but below regional and sub-regional norms. In 2020, the country ranked as Africa's 12th largest producer of aquacultured products. According to the Food and Agriculture Organization's (FAO) report of 2022, the production of mussels in 2020 reached the highest quantity of 2,276 tonnes, followed by abalone with 1,977 tonnes and trout with 1,006 tonnes. On the other hand, the production of tilapia, catfish, oyster, and marron is still in its early stages. Currently, the South African mariculture sector comprises a total of 200 active marine and freshwater farms and accounts for more than 80% of the overall output value in the country, mostly due to the contribution of the well-established, high-value abalone subsector (Britz & Venter, 2016; DAFF, 2017; FAO, 2022).

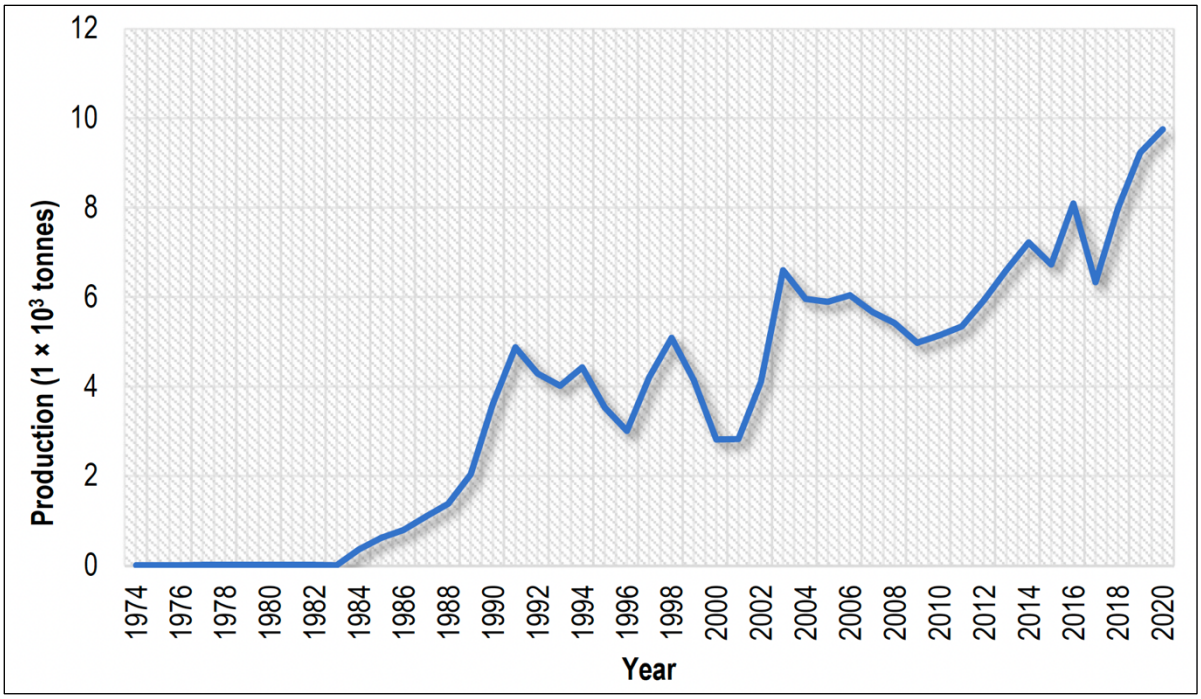

Figure 1.3. Aquaculture production in South Africa (1974-2020) (Source: FAO, 2021).

1.2.2 Challenges confronting the South African aquaculture industry

In South Africa, several challenges impede the growth and development of the aquaculture industry. According to Britz and Venter (2016), while South Africa is equipped with good facilities, commercial institutions, and supply chains, its aquaculture production potential is limited by factors such as its high-energy coastline, limited number of sheltered bays, and scarcity of freshwater inland. Other challenges facing the local aquaculture industry that are impeding expansion include suboptimal climatic conditions, including a wide temperature range and aridity, along with macroeconomic constraints such as a lack of skilled human resources, fish pricing, a poorly developed value chain, and complicated authorisation procedures (FAO, 2010–2020b; Britz & Venter, 2016). The aforementioned challenges have resulted in the emergence of predominantly land-based aquaculture and the cultivation of high-value species, such as abalone, *H. midae,* oysters, and trout, as a means of counterbalancing the elevated production expenses associated with land-based aquaculture.

1.2.3 Abalone aquaculture in South Africa

Abalone (family Haliotidae) are marine gastropods inhabiting temperate and tropical coastal waters near rocky shorelines and reefs (Degnan et al., 2006; Raemaekers & Britz, 2009). There are 56 documented species in the family Haliotidae, all of which belong to the genus *Haliotis* (Geiger, 1999; Geiger & Poppe, 2000). Numerous members of this genus, including *Haliotis rufescens, Haliotis cracherodii, Haliotis iris, and Haliotis midae*, have attained commercial status as fisheries and/or aquaculture species and are of great economic importance. The South African Abalone *H. midae* (Fig. 1.4), also known locally as "perlemoen", occurs along the South African coast from St. Helena Bay on the west coast to Port St. Johns on the east coast (Fig. 1.5) (Geiger, 2000; Evans et al., 2004; Reddy-Lopata et al., 2006). It is the only indigenous species of commercial significance among the five *Haliotis* species: *H. parva* Linnaeus (1758); *H. spadicea* Donovan (1808); *H. queketti* Smith (1910); and *Haliotis alfredensis* Bartsch (1951), formerly *H. speciosa* Reeve (1846) (see Owen 2006).

Figure 1.4. *Haliotis midae* in South Africa (Two Oceans Aquarium, 2018).

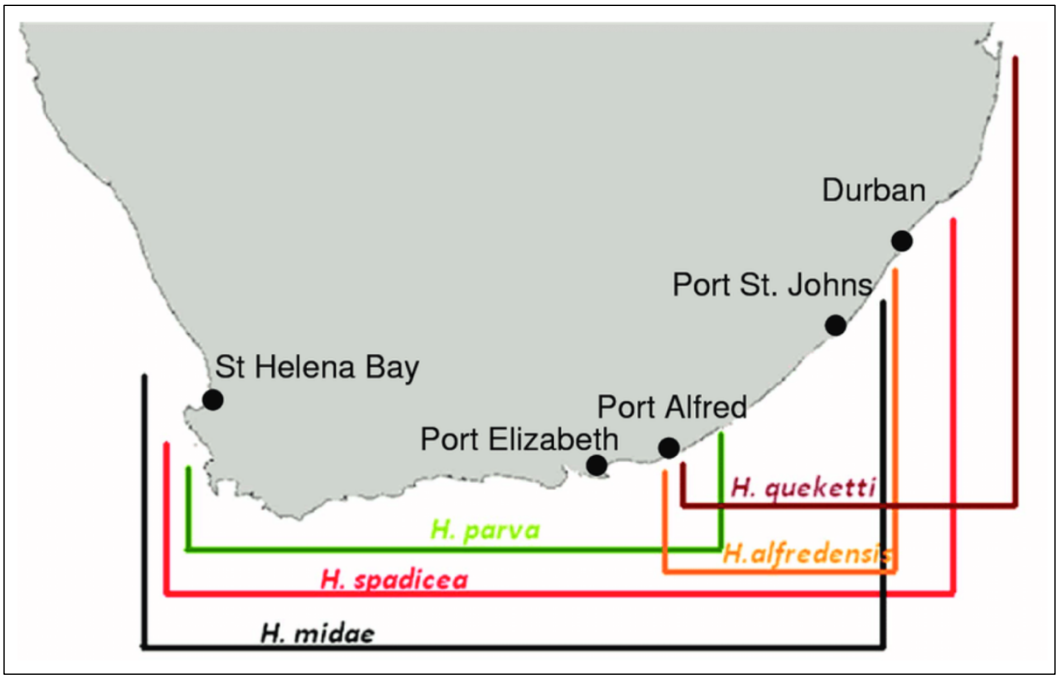

Figure 1.5. Distribution of *Haliotis* spp. along the coastline of South African. Map indicating the natural distribution of the five indigenous abalone species (Bester-van der Merwe et al., 2012).

According to Sales and Britz (2001), *H. midae* requires nearly 30 years in its natural environment to reach a maximum size of 200 mm, as measured by shell length. Even in farmed environments, abalone growth is slow and varies with size and age, but generally requires approximately four years to achieve a marketable size of 100 mm (shell length) before it can be sold for approximately US$ 25 per kilogram on foreign markets (pers. Comm., Nick Loubser, Viking Aquaculture).

Abalone farming in South Africa began in the early 1990s, and the abalone aquaculture industry has grown substantially since then to become the country's main mariculture sub-sector, with most farms situated in the Overberg region of Western Cape Province (DAFF, 2018b). Abalone are mostly cultivated in onshore land-based raceway tank systems that are situated close to the coastline to enable access to a large amount of natural seawater, which is pumped ashore and often pre-treated to improve water quality (FAO, 2010–2020b). Most of the land-based farms (there are 13 abalone farms) operate as flow-through systems (FTS), whereas a smaller number of farms (two at the time of this study) partially recirculate water by using the bioremediation capacity of the macroalga *Ulva lacinulata* that is grown in D-ended paddle-raceway systems linked/integrated with the abalone raceway tanks. There are also at least two other commercial farms that have integrated the cultivation of abalone with *Ulva*, growing *Ulva lacinulata* in abalone effluent to produce supplementary feed for the abalone on the farm and to bioremediate effluent water before it is returned to the sea. These practices improve the economic and environmental sustainability of the farms (Bolton et al., 2016).

There are 13 abalone farms in South Africa located on the Cape's coastline (de Greef, 2017), which is indicative of the colder sea temperatures and accompanying conditions that are suitable for abalone farming. Due to the high temperatures caused by the Indian Ocean water, the KwaZulu-Natal coastline is not suitable for abalone cultivation (Urban-Econ Development Economists, 2018).

Wild abalone are under intense fishing pressure because of the high demand for this sought-after seafood delicacy, particularly from countries in the far east (e.g., China, North Korea,

and Japan). High market demand and decreasing wild stocks have resulted in the expansion of abalone farming across the globe. With over 300 farms and a total production of approximately 4,500 metric tonnes, China is the largest producer of cultured abalone in the world (Troell et al., 2006) and the largest consumer of both wild and cultured abalone (Reddy-Lopata et al., 2006). The South African abalone industry continues to establish itself as a premium brand in Asia and serves as an excellent example of mariculture in a developing nation. The abalone farming industry in South Africa is considered to be newly developed; however, it is a thriving industry with a high production capacity (WCADI, 2021). The ongoing expansion and growth of this industry is considered crucial to the South African economy and society, as it has the potential to address various societal challenges and sustainable development goals (SDGs) faced by many developing countries. These include contributions to economic and enterprise development, job creation, food security, and the potential for the adoption of sustainable mariculture methods (WCADI, 2012). The contributions of the abalone sector to these strategic initiatives are deemed particularly important because most abalone farms in South Africa are located in coastal communities that have previously relied on wild fisheries, where the total allowable catch (TAC) for many wild stocks has been severely reduced or restricted.

1.2.4 Seaweed harvesting and cultivation in South Africa

Seaweeds from natural stocks have long been used in South Africa, with fresh kelp (mainly *Ecklonia maxima* and *Laminaria pallida*) being the most commonly harvested for use as feed for cultured abalone and the extraction or production of growth stimulants for the agricultural sector (Rothman et al., 2020). There is also limited, but sustainable, harvesting of the intertidal red seaweed *Gelidium pristoides* for agar and a declining collection of kelp beach cast for alginate (Anderson et al., 2003, 2010; Rothman et al., 2020). A small amount of *Gracilaria* spp. is also produced on a few abalone farms, mostly as feed for juvenile abalone in hatcheries, with production estimated to be around 600 tonnes per annum. Production of the green seaweed *Ulva* on land-based abalone farms cultivating *H. midae* is by far the success story of seaweed aquaculture in South Africa and the region. The most recent published production figure for *Ulva* is approximately 2000 tonnes (fresh weight) per annum (Rothman et al., 2020). These figures are, however, estimates, as the South African

Department of Forestry, Fisheries, and the Environment does not request official numbers from farms, as requested for other aquaculture products that are sold, since cultured seaweeds (*Ulva* and *Gracilaria*) are almost exclusively used as fresh feed and are not sold (Msuya et al., 2022).

Various names for the farmed *Ulva* have been published in the literature, but a recent DNA study indicates that all farms cultivate the same species, *Ulva lacinulata* (Guiry & Guiry, 2021; Bachoo et al., 2023). Cultivated *Ulva* in South Africa was previously known as either *Ulva armoricana*, *Ulva rigida*, or *Ulva lactuca* (Robert-Anderson et al., 2008; Cyrus et al., 2015). *Ulva* cultivated on South African abalone farms has remained in a vegetative state for many years and does not produce spores or gametes, which is why it has been successfully cultivated for so many years (Bolton et al., 2016). Five farms generate substantial quantities of *Ulva lacinulata* in approximately 30-m-long, D-ended paddle raceway ponds (Bolton et al., 2009). Each paddle raceway produces approximately 1000–2500 kg of *Ulva* every month (Bolton et al., 2009). Some farms cultivate *Ulva* in abalone effluent and practise integrated multi-trophic aquaculture (IMTA) on a commercial scale.

1.3 Problems associated with intensive aquaculture production systems

1.3.1 Biotic and abiotic factors

Aquaculture generally involves the farming of aquatic species at densities that far exceed what is normally encountered in the natural environment. The high stocking densities also mean that these animals are fed large quantities of feed to maintain optimal growth of the species being cultivated. Due to the high stocking densities, feeding rates, routine farming practices, and generally ‘unnatural’ environments of standard farming practices, farmed animals are subject to a range of biotic (biological) and abiotic (physical) stressors that can compromise the health and wellbeing of the animals. Understanding the physiology of abalone as well as the impacts of numerous biotic and abiotic factors on the organism’s growth and health is essential for successfully cultivating the species (Hahn, 1989). Moreover, understanding how the environment impacts the microbiome in the systems and/or the animals has important implications for the management of disease on intensive aquaculture farms.

Abiotic stressors refer to physical elements in the environment, including temperature, pH, oxygen, and salinity; the presence of chemical pollutants; and the handling of animals, which can have a detrimental effect on the organism(s) being cultivated in the system. Biological stressors, on the other hand, include food quality and quantity, competition for food, space, and sexual partners, and the presence of pathogens in the aquaculture system. Frequent occurrences of extreme weather, attributable to both natural and man-made causes, can further exacerbate the physical and biological stressors frequently encountered within most aquaculture farms (Brander, 2007; De Silva & Soto, 2009).

1.3.2 Natural vs. formulated feed

Abalone diets have been shown to significantly impact growth rates (Britz, 1996a, 1996b; Troell et al., 2006). Abalone are typically considered generalist herbivores that quickly adopt a variety of algal diets (Troell et al., 2006; Van Der Merwe, 2009; Van Schalkwyk, 2012). In their natural environments, abalone *H. midae* appear to favour specific macroalgae, including *Ecklonia maxima*, *Laminaria pallida*, *Ulva* spp., and *Gracilaria* spp. (Britz 1996a; Troell et al., 2006; Naylor et al., 2009; Van Der Merwe 2009). These diets contain a significant amount of complex polysaccharides that are not easily digestible, and as a result, enteric bacteria have been shown to significantly contribute to the digestion of these substrates (Erasmus et al., 1997). If the host's intestinal flora lacks the capacity to produce beneficial enzymes, the digestion process will be extremely slow, which will inhibit abalone growth (Moonsamy, 2019). In abalone production systems, abalones are fed either formulated diets or seaweed/kelp, and in some cases, both (DAFF, 2011). The incorporation of seaweed into abalone diets offers several benefits. Seaweeds have been found to improve the palatability of feed, resulting in increased consumption rates and accelerated growth (Naidoo et al., 2006). Moreover, they have a high-quality protein composition, and because of their prebiotic qualities, seaweeds may positively improve abalone health by fostering a balanced gut flora (Naidoo et al., 2006). The incorporation of seaweeds into the diets of abalone can lead to enhanced nutrition and potentially improved health, making it a valuable practice in aquaculture.

Research has also shown slow and inconsistent growth rates for abalone that consume a natural algal diet, whereas growth rates on artificial/formulated diets are comparatively faster and more consistent (Britz, 1996a; Naylor et al., 2009; Van Der Merwe, 2009). This is due to the availability and balance of many nutrients essential for optimal growth in formulated feeds, which are frequently lacking in a monotypic natural-feed diet, in addition to increased protein content in formulated feeds (Mai et al., 1995; Britz 1996a; Britz 1996b; Van Der Merwe, 2009). Feeds need to be designed to meet the nutritional requirements of the animal and formulated to be stable in marine environments. The poorly designed feeds can lead to nutritional stress, reduced growth, poor water quality, and increased susceptibility to disease agents. Poor consumption (as a consequence of poor palatability), and feeds that lack stability, will result in increased leaching of dissolved and particulate organic matter into the system and the surrounding environment. The likelihood of algal blooms and the spread of disease-causing parasites and pathogens increases if waste accumulates because of insufficient husbandry and poor feed digestibility. This will not only lead to nutrient loading (eutrophication) of the surrounding environment but also provide an excellent medium for the proliferation of opportunistic pathogens. Many pathogens are ubiquitous in aquatic environments and are part of the normal flora. These only become problematic when environmental conditions favour their proliferation and/or when animals are stressed and immuno-compromised (Magana-Arachchi & Wanigatunge, 2020). This can be exacerbated by high stocking densities that lead to increased competition for space and food (Ellison et al., 2020).

Abalone cultivation has become a more cost-effective and manageable industry as a result of the introduction of formulated feeds and specialised feeding regimens designed to boost abalone growth (Sales & Britz, 2001). According to the Western Cape aquaculture development initiative, WCADI (2012), abalone fed with a formulated diet demonstrate superior canning properties compared with their wild counterparts, resulting in a potential increase of up to 15% in canning yields. Appropriate feeding techniques for abalone are essential, as diet and the ability of abalone to utilise available food efficiently, resulting in a high feed conversion ratio, have a significant effect on growth rates and growth performance (Naidoo et al., 2006), especially during the early stages of their life (Erasmus et al., 1997).

1.3.3 Temperature

Temperature is a key factor that has a substantial effect on the reproductive cycle of fish and shellfish (Brulé et al., 2022; Rodríguez-Jaramillo et al., 2022). Increasing the temperature in aquaculture can have numerous benefits, such as improved growth rates and shorter production times, more favourable conditions for cultivating fish populations, and increased output through improved health of the cultivated species. Abalone, being ectothermic organisms, are susceptible to the influence of temperature on multiple stages of their growth and maturation (Mccormick et al., 2016). The rate of biological reactions is controlled by temperature, which underpins major physiological processes (Britz et al., 1997; Gilroy & Edwards, 1998; Green et al., 2011). Abalone can be found at water temperatures ranging from 9 to 24°C. However, temperatures ranging between 12 and 20°C are considered physiologically optimal for *H. midae*, and this has direct effects on the rate of metabolic processes and, consequently, growth (Sales and Britz, 2001; Van Der Merwe, 2009). These low temperatures are favourable to *H. midae* growth throughout all phases of its life cycle, whereas high temperatures have been observed to inhibit the survival and developmental success of larvae (Mccormick et al., 2016), and temperatures exceeding 24°C are linked to increased adult mortalities (Jiang et al., 2013a). Elevated temperatures may also lead to increased microbial growth when water quality parameters are not at optimal levels (Jiang et al., 2013a). Conversely, in cases where temperatures are excessively low, there is a possibility of decelerated growth and a delayed larval stage period.

1.3.4 Water quality

There are various physiochemical and biological factors that affect the health of farmed aquatic species (Van Der Merwe, 2009). Aquatic species population/stocking density and water flow affect water quality in intensive aquaculture systems (Colt et al., 2009). Identifying the primary limiting factor for optimal water quality is crucial for optimising the design and management of a specific recirculation system for a specific species, hence improving its capacity for water re-use. Important water quality parameters for optimising growth and survival are salinity, organic waste concentration, nitrogenous waste concentration, alkalinity and hardness, dissolved gasses and the presence of disease-causing microorganisms (Neori et al., 2000; Reddy-Lopata et al., 2006; Van Der Merwe, 2009;

Jarayabhand et al., 2010; Wassnig et al., 2010; Johansen et al., 2011; Salama & Murray, 2011; Naylor et al., 2009; Kim et al., 2013). The water should be of sufficient quality, devoid of impurities (pollutants, chemicals, excess minerals, etc.), and monitored for temperature, pH, dissolved oxygen, and ammonia levels regularly (once to several times a day, depending on system type). The rapid expansion of the abalone industry along the west coast of South Africa has been facilitated by the presence of high-quality coastal water (Troell et al., 2006). Abalone farms in South Africa are located in areas that have higher-quality water, free of any industrial or domestic waste (Troell et al., 2006; Van Der Merwe, 2009).

Juvenile abalone are commonly reared in filtered or UV-treated water due to their extreme sensitivity to water quality issues caused by unwanted microorganisms, pollution, chemicals, or contaminants (Urban-Econ Development Economists, 2018). Typically, flow-through or partial recirculation systems are used in abalone farms around the South African coastline for abalone grow-out. A flow-through system pumps water directly from the ocean into the farm, and the water returns back into the ocean via gravity (Fourie, 2014). The implementation of open flow-through technology allows farm managers to improve water quality parameters by exposing animals to large volumes of natural seawater, but this practice can be detrimental if external conditions are unfavourable (e.g., the occurrence of HABs or pollution events). Similarly, partial recirculation systems pump water onto the farm directly from the ocean, but in the case of a 50% abalone – *Ulva* recirculating system, only 50% of the effluent is released back to the ocean while the remaining 50% is recirculated back to the abalone tanks after bioremediation by *Ulva*. This enables farmers to reduce and lower the nutrient output to the surrounding environment and have more control over water quality variables (Robertson-Andersson, 2006). While various biotic and abiotic factors can compromise the growth, health, and physiology of farmed organisms, the development of integrated aquaculture has emerged as a solution for dealing with excessive nutrients produced by intensive aquaculture, such as organic matter, ammonia, and nitrite (Granada et al., 2016; Tom et al., 2020).

1.4 Integrated multi-trophic aquaculture (IMTA)

1.4.1 IMTA definition and benefits

There is currently no food production sector that is completely energy- and biodiversity-sustainable. All food production sectors require energy and water while producing waste (Diana, 2009; Troell et al., 2017). The global aquaculture sector is no exception, and industrialisation of the sector has led to an increase in water pollution, which has already had significant ecological effects on the surrounding environment (Ottinger et al., 2016). However, some farming practices are more sustainable than others. For instance, cultivating multiple species in the same body of water (polyculture) is regarded as more profitable than cultivating a single species (monoculture). However, polyculture, despite its ability to enhance profitability through product diversification and a reduction of potential hazards associated with monoculture (Chopin et al., 2012.) does not alleviate several of the environmental impacts often associated with large-scale aquaculture.

Integrated multi-trophic aquaculture (IMTA), on the other hand, is increasingly proposed as a more sustainable farming technique that offers numerous benefits. In contrast to polyculture, in which multiple species belonging to the same trophic level are cultured together, IMTA can include species from different trophic levels and can minimize some of the environmental concerns associated with semi- and intensive aquaculture practices while delivering economic benefits, thereby promoting an ecological approach to aquaculture (Troell et al., 2003, 2009; FAO, 2014; Granada et al., 2016). IMTA is characterised as "a practice in which the by-products of one species are recycled as inputs for another" (FAO, 2014). The IMTA concept was introduced by Ryther and colleagues (Ryther et al., 1972) as early as 1972 to mitigate against eutrophication from dissolved and particulate organic and inorganic nutrients released from aquaculture systems. By cultivating fed aquaculture species (e.g., finfish or shrimp) with organic extractive species (e.g., suspension and deposit feeders) and inorganic extractive aquaculture species (e.g., seaweeds), IMTA recycles the by-products (waste) of one species to become inputs (fertilizers, food, and energy) for another (Barrington et al., 2009; Troell et al., 2009). Hence, when the waste or nutrients of one species are turned into resources for another, this prevents the loss of enormous quantities of dissolved

nutrients, which are expensive inputs into any aquaculture system, to the surrounding environment and allows for the co-production of other species (Chopin et al., 2001).

In the past decade, the concept of integrated aquaculture has been used to mitigate the excessive nutrient/organic loading generated by intensive aquaculture production systems, particularly in Asia (Neori et al., 2004). However, in recent years, this practice has gained popularity in western countries as a response to the global demand for seafood, aiding the sustainable expansion of aquaculture in coastal and marine ecosystems (Neori et al., 2004; Troell et al., 2009). In the past fifteen years, countries such as Canada, Japan, Chile, New Zealand, Scotland, and the United States have explored the integration of seaweed(s) with marine fish culture (Sasikumar & Viji, 2015). The use of mussels and oysters as biofilters in fish farming has also been investigated in several other countries. More recently, the offshore migration of numerous coastal finfish farms in Turkey has sparked interest in IMTA, and there is a lot of interest globally in seaweed, bivalve, and crustacean IMTA (e.g., Troell et al., 2009).

Shellfish and seaweeds, among other organic and inorganic extractive species, are essential elements of IMTA, as they make use of wastes produced by the fed aquaculture species and remove suspended small particle organic matter (POM) from the water column, thereby helping to reduce nutrient inputs into surrounding environments (Troell et al., 2003; Chopin, 2006; Barrington et al., 2009; Troell et al., 2009; Van Rijn, 2013; FAO, 2018). For IMTA to work, organisms should be selected on the basis of their functions in the ecosystem and that the selected organisms are able to coexist and contribute to the overall balance and efficiency of the farming system.

1.4.2 IMTA in South Africa

Abalone farming is an aquaculture sector that significantly profits from the use of IMTA through integration with other low-trophic species, such as seaweeds, which are a natural food source for abalone and have been demonstrated to have several benefits when integrated with abalone (Bolton et al., 2016). South Africa is ranked as the world's third-largest producer of cultured abalone (Gordon & Cook, 2004) and has been practise IMTA by integrating the production of abalone (*H. midae*) with the green seaweed *Ulva* for more than

two decades (Bolton et al., 2016). Most of the farms in South Africa practicing IMTA cultivate *Ulva* in abalone effluent and have been successfully practicing full commercial-scale IMTA for several years. There are five commercial abalone farms in South Africa, including Irvin and Johnson (I&J) Cape Abalone located at Danger Point (34°37'35.1" S 19°17'47.6" E), Abagold (Pty) Ltd. in Hermanus (34°26'02.8"S 19°13'17.7" E), and Buffeljags Abalone near Bredasdorp (34°45'14.7"S 19°36'51.9" E) located on the southwest coast, Western Cape Province. Of the remaining two farms, Diamond Coast Aquaculture (29°39'59.8"S 17°03'03.2" E) is located along the northwest coast in the Northern Cape province, and Wild Coast Abalone (Pty) Ltd. (32°45'04.3"S 28°16'27.9" E) is located in the Eastern Cape region close to a remote settlement called Haga Haga (Fig. 1.5). The motivation for cultivating *Ulva* on the aforementioned farms differs. For instance, Wild Coast Abalone primarily cultivates *Ulva* as a supplementary feed for abalone because they do not have access to large quantities of the natural seaweed *Ecklonia maxima* because the farm is located just north of the natural distribution range of *E. maxima*. Conversely, Buffeljags Abalone Farm primarily cultivates *Ulva* to allow for partial (50%) water recirculation on their farm to save on pumping (electrical) costs, which is estimated to save up to 40% on electrical costs (Nick Loubser, pers. comm.), but also uses the IMTA-grown *Ulva* as a supplementary feed for abalone (saving a further 20% on feed costs). In addition to the five main *Ulva* producers listed above, a few farms cultivate *Ulva* in smaller amounts as a supplement for animal feed (Bolton et al., 2009).

Ulva grown in IMTA systems has proven to be a better candidate for large-scale production than many other macroalgal species. This is because *Ulva* is easy to grow, and many *Ulva* spp. are considered 'weeds' as they often proliferate in coastal waters, especially in sheltered bays with high nitrogen contents (Bolton et al., 2016). Moreover, *Ulva* is easy to harvest, especially when compared with growing microalgae, and selected strains of *Ulva* can remain vegetative consistently when grown at high densities, avoiding disintegration when spores are produced for reproduction (Bolton et al., 2009; Bolton et al., 2016; Rothman et al., 2020). One of the many advantages of using IMTA-grown *Ulva* as a supplementary feed for cultured abalone is that it is highly effective as a biofilter in IMTA systems (Bolton et al., 2009; Bolton et al., 2016). Moreover, its high surface area to volume ratio and high affinity for nutrients, particularly nitrogen and phosphorus, promote rapid nutrient uptake, which results in high growth and

production rates and a massive accumulation of biomass that has a higher protein content compared with seaweeds collected from the wild, such as fresh kelp, *Gracilaria*, and others (Neori et al., 2004; Leston et al., 2011; Robertson-Andersson et al., 2011). These qualities constitute a useful resource in IMTA systems, and hence, seaweeds are often utilised as extractive species for dissolved inorganic nutrients, serving as bio-remediators of farm effluent water and providing a range of other benefits to aquaculture systems and the organisms co-cultured within these systems (Chopin et al., 2001; Neori et al., 2004; Zhou et al., 2006; Sanderson et al., 2008). The majority of *Ulva* is cultivated in large paddle raceways using nitrogen-rich abalone effluent; however, because of biosecurity concerns, it is also cultivated on some farms in paddle raceways utilising fertilised seawater (Bolton et al., 2009; Neveux et al., 2018). There is also considerable socioeconomic pressure on the South African government to produce more jobs in a region with significant unemployment and poverty (Troell et al., 2006). In remote coastal areas, the potential for this industry to grow and create jobs is particularly attractive. Thus, there is strong support for this method of co-cultivating seaweed with abalone from the government, industry, and the general community.

Figure 1.6. Shallow seaweed (*Ulva* spp.) raceways receiving effluent water from the adjacent the abalone (*Haliotis midae*) raceway tanks (covered tanks on left) at Haga Haga Abalone farm, on the southeast coast of South Africa. Photo by R.J. Anderson.

1.4.3 Benefits and potential risks associated with IMTA systems integrated with seaweeds

In addition to making significant contributions to the primary production of estuarine ecosystems, seaweeds also play a role in nutrient cycling by converting inorganic forms of energy into biomass, which can then move up the food chain (Torres et al., 2008; Leston et al., 2011). The bioremediation capacity of seaweeds cultivated in abalone effluent water permits water recirculation and lowers nutrient output to the surrounding environment (Robertson-Andersson, 2007). Irvin and Johnson Cape Abalone began growing *Ulva* and abalone in an integrated system on an experimental basis in 2006, demonstrating that approximately 50% of effluent water can be recirculated back to abalone in raceway tanks after *Ulva* removed the majority (more than 70%) of the dissolved ammonia (Bolton et al., 2009). Robertson-Andersson et al. (2008) further demonstrated that *Ulva* could be grown in integrated systems with abalone, removing ammonia, allowing for partial water recirculation, and reducing pumping costs while having no negative effects on abalone health or seaweed growth rate. The recirculation of water through the shallow *Ulva* raceways has also been shown to raise the water temperature (by approximately 1˚C), which can be beneficial to abalone growth at certain times of the year (Chopin et al., 2008).

Full (100%) water recirculation has also been demonstrated to be possible for short periods of time (2–3 days) when abalone effluent water is recirculated through *Ulva* ponds on an abalone farm (De Prisco, 2019). Full recirculation can provide protection to abalone during adverse environmental events, such as harmful algal blooms or red tides (as they are often called locally when caused by blooms of *Alexandrium catenella*) and oil spills, as it can allow the land-based facility to isolate itself for short periods from the surrounding environment until conditions improve (Robertson-Andersson, 2007; Bolton et al., 2009; Rothman et al., 2020). When molluscs are integrated with seaweeds, the seaweeds not only remove nutrients, but also take up carbon dioxide (CO_2) excreted by the molluscs, produce O_2 during photosynthesis, and help to buffer seawater pH. Therefore, seaweeds essentially provide a double buffer for shellfish farming by reducing both nutrient and CO_2 concentrations and possibly mitigating the consequences of eutrophication and ocean acidification (when cultivated on a large scale in open oceans or bays).

Traditional recirculating aquaculture systems may not be appropriate for abalone production; however, partial recirculation (50% and 75%) aquaculture methods are beneficial. Depending on which species are cultured, the accumulation of contaminants may represent a risk, and if these contaminants are present, direct consumption of farmed invertebrates or algae may constitute a risk to human health. One of aquaculture's most detrimental environmental effects is the release of large amounts of polluted effluents containing dissolved nutrients, uneaten feed, and faeces (Amirkolaie, 2008; Wang et al., 2020). Organic enrichment from the release of large amounts of nutrients, especially nitrogen and phosphorous, degrades the environment of receiving water bodies and sediments (Marinho-Soriano et al., 2011) and can lead to the growth of unwanted organisms, such as opportunistic and pathogenic bacteria and harmful algae. Therefore, improving aquaculture waste management is important for reducing potential environmental and economic impacts caused by eutrophication and disease transmission and will also be important for farmers wanting to recirculate water and improve overall circularity on their farms (Chávez-Crooker & Obreque-Contreras, 2010). As such, for these systems to function properly and to manage them better, a thorough understanding of the microbiome within these systems is important.

Biosecurity measures are applied to prevent the accidental introduction of pathogens, parasites, and non-native pests to avoid the introduction and spread of disease. It is essential to execute appropriate biosecurity measures for protecting consumer health, the sustainability of the industry, and environmental preservation. The concept of biosecurity is not new, yet most seaweed-producing countries pay little attention to it. Recently, researchers have begun to investigate seaweed diseases and infections, their routes of infection, and potential treatments. Typically, seaweed infections are caused by the presence of causative agents at an infected spot, which triggers the seaweed's defence mechanism to create hydrogen peroxide. Prolonged infection alters the physiology of seaweed, weakening its external structure and increasing its susceptibility to subsequent infection by additional opportunistic pathogens (Hurtado et al., 2019).

1.5 Microorganisms in integrated aquaculture systems

1.5.1 Impacts of microorganisms on the health of aquaculture species

The abalone-*Ulva* integrated system likely harbours diverse microbial communities that interact across several system components, playing critical roles in system health and productivity. In this system, the water microbiomes in the abalone and *Ulva* tanks help with nutrient cycling and water quality, whilst the leaf-associated microbiome on *Ulva* surfaces aids in nutrient uptake and protects against pathogens (Bolton et al., 2009; Kang et al., 2011; Aníbal, et al., 2014; Hurd et al., 2014). Moreover, some microorganisms are known to be associated with various abalone organs, including the digestive tract, promoting digestion and immunological function (Corthier & Doré, 2010; Honda & Littman, 2012). Collectively, these microbial communities are essential for balancing nutrients, promoting the health of *Ulva* and abalone, and ensuring the long-term sustainability of the integrated system.

The development of disease in aquaculture facilities and natural aquatic environments is a complicated interplay between the host species, disease agents, and the environment (Moreira et al., 2021). In an aquaculture setting, the vulnerability of the hosts, the virulence of the infectious agent, and the environmental conditions (biotic and abiotic factors) have a significant impact on disease outbreaks and progression (Dillon & Meentemeyer, 2019). In intensive and semi-intensive production systems characterised by high stocking densities, intensive feeding, poor water exchange and quality, and poor farm management practices, the occurrence of disease may be promoted or exacerbated (Wanja et al., 2020). Many of the microorganisms that have been documented to cause disease in wild and farmed organisms are ubiquitous in aquatic environments and generally only cause disease when there is a shift in the equilibrium between the host, the disease agent, and the environment, favouring the growth of the pathogen(s) or susceptibility of the host(s) (Pillay, 2004). Previous studies have shown that disease progression is heavily influenced by a system's microorganisms rather than a single aetiological agent (Bass et al. 2019). The pathobiome concept suggests that disease in animals is caused by interactions between host-associated prokaryotes, eukaryotes, viruses, hosts, and the environment, rather than a single pathogen (Bass et al. 2019). Emerging diseases, such as those prevalent in farmed shrimp (Kooloth Valappil et al. 2021), highlight the need to understand the microbiomes of aquaculture species and their

environment. Pathogenic microorganisms can influence the microbial ecosystem by outcompeting beneficial microbes or triggering immune responses in cultured animals. This can alter the balance of microbial communities, leading to dysbiosis and decreased system efficiency.

Disease management strategies, which include sound biosecurity practices, are therefore an integral component of any animal production system to ensure optimal health and welfare of the animals within the system. However, in aquatic environments, the close contact between microorganisms (bacteria, fungi, viruses, and other parasites) and their host, as well as the use of open production systems, can exacerbate this challenge (Olafsen, 2001). Multiple diseases have emerged as significant economic or ecological challenges in aquaculture and provide a substantial barrier to the industry's continued growth and expansion (Verschuere et al., 2000; Macey, 2005). Control of endemic diseases incurs significant annual expenses, with annual global disease-related losses to the aquaculture industry estimated to be between US$ 6 billion (Cain, 2022). Abalones are no exception and have been shown to be sensitive to several opportunistic marine pathogenic bacteria, such as *Vibrio parahaemolyticus*, *Vibrio anguillarum*, and *Vibrio carchariae*, as well as prokaryotes such as *Candidatus Xenohaliotis californiensis* (CXc) and viruses such as Haliotid herpesvirus-1 (HaHV-1) (Nicolas et al., 2002; Bower, 2003; Macey & Coyne, 2005; Corbeil, 2020; Cruz-Flores & Cáceres-Martínez, 2020). In other aquaculture industries, when pathogenic agents, such as bacteria, viruses, and other parasites, are detected, farmers usually administer antimicrobial agents to feed and/or rearing water (Gram et al., 2001). However, excessive and indiscriminate use of antimicrobials has led to the emergence of bacterial resistance and a fear of the spread of antimicrobial resistance genes to human pathogens (Liu et al., 2017; Schar et al., 2020). Due to these concerns, the European Union has set stricter regulations on the use of antibiotics in aquaculture (Ronson & Medina, 2002).

Certain industries, such as the Norwegian salmon farming industry, have drastically reduced the number of antimicrobials they use and have adopted more sustainable strategies, such as the use of vaccines (Jeong et al., 2020). Because antimicrobials are often administered as in-feed treatments, which are expensive and ineffective because sick animals generally have

a poor appetite, the residues often end up in the environment, where they can have an adverse impact on ecosystem health (by altering the microbiomes of the receiving environment) (Macey, 2015; Liu et al., 2017; Schar et al., 2020). In addition, antimicrobial residues can accumulate in the tissues of animals and, when consumed by humans, can lead to allergies and/or direct toxicity (Liu et al., 2017). These residues can alter the microbiome of the gastrointestinal tract of aquatic species, causing dysbiosis (changes in the make-up and function of microbiota in a specific environment) and a range of other adverse physiological impacts (Kim et al., 2019; Sumithra et al., 2022). Therefore, the indiscriminate use of antibiotics is harmful to the health of aquatic host species, the environment, and food consumers (Moriaty, 1999), and alternative, more sustainable strategies for disease management are required. These include strategies such as vaccines, the use of natural immuno-stimulants, pre- and probiotics, and the use of natural plant products such as seaweeds, the latter either administered as a feed supplement, an ingredient added to formulated feed(s), or by co-culturing seaweeds with the primary aquaculture organism. Seaweeds play a critical role by inhibiting the proliferation of certain bacterial pathogens (Pang et al., 2006; EL-Sayed et al. 2023), thereby contributing to the overall health and stability of the aquaculture system. Current studies demonstrate the potential of microbiome-based interventions, such as probiotics, to enhance aquaculture sustainability and fish health (Amenyogbe, 2023). The nature of the aquaculture system can have significant impacts on microbiomes of finfish (Minich et al., 2020, 2021; Uren Webster et al., 2020) and invertebrates, as demonstrated for oyster (Arfken et al., 2021) and shrimp (Tepaamorndech et al., 2020), but more research is needed to establish effective practices and address analytical challenges in order to fully integrate microbiome insights into aquaculture management.

1.5.2 Diseases in seaweed aquaculture and their economic effects

The economic ramifications of disease outbreaks in commercial seaweed farms have been observed to be severe, particularly for farmers in developing nations. The growing seaweed culture industry has resulted in an increase in the prevalence of diseases and aquaculture pests since the early 2000s. The countries that produce the most commercial seaweed, such as China, Indonesia, and the Republic of Korea, are also the nations hardest hit by seaweed

disease outbreaks (FAO, 2018). These outbreaks have resulted in a substantial decrease in hydrocolloid production, and the seaweed farming industry could collapse if no effective treatments or mitigation techniques are implemented (Kim et al., 2014; Ward et al., 2019). For instance, a 2014 study revealed that the disease epidemic in China caused an estimated 25–30% loss of harvested brown algae, *Saccharina japonica* (kombu) (Wang et al., 2014; Ward et al., 2020). In Korea, *Pyropia* sp. (nori) farms saw a loss of up to 20% between 2011 and 2013, whereas in the Philippines, a 15% decline in *Kappaphycus alvarezii* productivity resulted in a loss of approximately US$ 300 million (Kim et al., 2014; Cook et al., 2016). These statistics clearly demonstrate how severely the economy has been impacted by seaweed diseases and how important it is to have a better understanding of beneficial and pathogenic disease agents (microbiome) associated with seaweeds, particularly if seaweeds are to be integrated with other species, as is the case for the integration of *Ulva* with abalone in the South African abalone farming industry. The loss of *Ulva* in an abalone-*Ulva* IMTA will not only compromise the functionality of the system and the health of the animals in the IMTA (due to the loss of bioremediation capacity); however, feeding unhealthy or diseased effluent-grown *Ulva* back to abalone can be an additional biosecurity risk. Improved knowledge of the microbiome of the seawater and *Ulva* in an abalone-*Ulva* IMTA with partial recirculation is therefore fundamentally important for proper disease and risk management on abalone farms.

1.5.3 Roles of microorganisms in nutrient cycling

In addition to disease-causing microorganisms, there are several other microbial species in marine and aquaculture environments that collectively contribute to the cycling of nutrients within the systems. Microbial nitrification and denitrification by microorganisms in the water column or associated with tissues and mucosal surfaces of the cultured organisms, as well as in the sediments in tanks and below cages, play a vital role in the bioremediation of farm effluents (Chavez-Crooker & Obreque-Contreras, 2010; Marinho-Soriano et al., 2011). Biological nitrification occurs under aerobic conditions when bacteria such as *Nitrosomonas* and *Nitrococcus* convert ammonium to nitrite and *Nitrobacter* oxidize nitrite to nitrate (NH_3 – NO_2 – NO_3), using a substantial amount of oxygen that can reduce dissolved oxygen levels (Prosser, 1986). In contrast, biological denitrification occurs under conditions of low oxygen,

and it is the transformation of fixed nitrogen into N_2 gas that returns to the atmosphere (Skiba, 2008). Numerous other microorganisms, including various bacteria, archaea, and eukaryotes, are capable of denitrification reactions. Another method of denitrification is through anaerobic ammonium oxidation. As the volume of waste streams becomes more manageable and various treatment options, including recirculation loops (typically found in outdoor settings), IMTA, special reactors under anoxic conditions, and others are developed, the implementation of water recirculation systems and the application of these biological filters in land-based aquaculture facilities will become more common (Chavez-Crooker & Obreque-Contreras, 2010; Van Rijn, 2013).

As previously stated, a few commercial abalone farms in South Africa employ integrated aquaculture techniques, whereby the macroalgae *Ulva* is cultivated in effluent water or fertilised seawater and thereafter utilised as a supplementary feed for abalone. Although the use of this method has proven effective in South Africa over an extended period, there remains a lack of knowledge regarding the microbial populations associated with *Ulva* cultivated in effluent systems, their benefits and roles in nutrient cycling, and their potential capacity to transmit pathogenic microorganisms to cultured abalone. Nonetheless, it has been observed that certain farms have successfully cultivated *Ulva* in abalone effluent water and utilised effluent grown *Ulva* as feed for abalone for over 15 years, without any consequence of disease-related implications (Bolton et al., 2009).

1.6 Methods for monitoring microbial communities in complex aquaculture systems

1.6.1 Culture-based vs culture-independent techniques

There are several direct and indirect methods for detecting and quantifying environmental bacteria, fungi, oomycetes, and other environmental microorganisms, each with advantages and disadvantages (Lee et al., 2013; Bursle & Robson, 2016). Culture-based detection approaches are the most widely used, although they usually underestimate the number of target species in the environment and often miss unculturable microorganisms (Figdor & Gulabivala, 2008). Isolation, cultivation, maintenance, and subsequent observation of morphological features and/or characteristics of colonies are the foundations of culture-based approaches for detecting and identifying fungal and bacterial species. However, these methods are time-consuming and laborious and pose a serious threat to misidentification

because of intra- and interspecific variation in morphological characteristics (Figdor & Gulabivala, 2008). Consequently, the use of culture-based techniques alone for disease management could be problematic. Molecular techniques, on the other hand, provide an outstanding tool for the detection, identification, and characterization of microorganisms involved in various environmental and food ecosystems (Bursle & Robson, 2016).

Within the last two decades, molecular biology techniques that offer precision and high sensitivity have revolutionised the detection, identification, and quantification of microbes in complex ecosystems. These methods are not dependent on the culturability or viability of the target organism and typically involve DNA or RNA isolation followed by polymerase chain reaction (PCR) amplification of specific gene regions, which has become a central technique in biochemistry and molecular biology and currently resides at the forefront of molecular diagnostic technology. Sanger sequencing has been extensively used in traditional molecular diagnostic methods (Sanger et al., 1977). Although, it is efficient for sequencing short DNA fragments, it is time-consuming and ineffective for large sequence fragments and large numbers of samples. More recently, metabarcoding has been used to study all small-scale organisms in a specified environmental niche (Handelsman et al., 1998). This culture-independent technique has evolved to gain more insights into the microbial diversity present in various natural environments, including but not limited to soil, marine water, and the gastro-intestinal tracts of both vertebrates and invertebrates, through the application of next-generation sequencing techniques (López-García & Moreira, 2008).

1.6.2 Gene regions for bacterial, fungal and oomycete identification using next-generation sequencing

The small-subunit ribosomal RNAs (rRNA) (16S in prokaryotes and 18S in eukaryotes) are commonly used as targets for bacterial identification as they are abundant (Mitreva, 2017; Hassler et al., 2022), have enough evolutionary information for inference of close as well as distant phylogenetic relationships, and are flanked by conserved sequences that can be used as primer-binding sites for PCR prior to sequencing (Kapustina et al., 2021). The 16S (1541 bp), 23S (2930 bp), and 5S (190 bp) are among the gene regions present on the prokaryotic ribosome. The 16S gene has been extensively studied primarily because of its relatively

shorter length, which facilitates the process of amplification (Clarridge, 2004). The length of the 16S rDNA gene region is estimated to be approximately 1,500 base pairs (bp), encompassing nine hypervariable regions (V1–V9) that are interspersed between conserved regions (Cardenas & Tiedje, 2008). The V4 region has been identified as being particularly useful in classifying bacteria because it achieves good domain specificity, higher coverage, and a broader spectrum in the Bacteria domain (Mizrahi-Man et al., 2013; Zhang et al., 2018a). In contrast to bacteria, the discovery of gene targets in pathologically significant oomycetes and fungi is still a work in progress. Coding and non-coding spacer regions comprise the rDNA of fungi (De Filippis et al., 2017; Raja et al., 2017). The coding region consists of the 18S, 5.8S, and 28S units, as well as several noncoding regions composed primarily of internal transcribed spacers (ITS) and intergenic sequences. The ITS1 and ITS2 variable regions have been commonly used as the primary gene targets for fungal identification.

The introduction of next-generation sequencing (NGS) technologies has substantially improved the ability to identify microorganisms in complex ecosystems/ environments and characterise metabolic and regulatory systems by which hosts and microbes interact to define a healthy or diseased state in the host organism (Malla et al., 2019). This technology is crucial for understanding the composition of microbial communities and their functional and metabolic features (Malla et al., 2019). The first step in performing NGS is the extraction of genomic DNA from test samples, PCR amplification, library preparation, and sequencing on the researcher's sequencing platform of choice (Buermans & den Dunnen, 2014). Even though each manufacturer offers quality ratings and estimations of accuracy, there is no consensus that a 'quality base' from one platform is equivalent to that from another (Metzker, 2009).

Currently, the most popular NGS platforms include Illumina, Ion Torrent, and Nanopore sequencing platforms. Illumina technology is one of the most widely used next-generation sequencing (NGS) technologies, with scalable choices to fit a wide range of study designs, sequencing prices, and data usage needs (Voelkerding et al., 2009; Buermans & den Dunnen, 2014). Illumina offers a method for selecting an optimum sequencing platform via its

sequencing platform comparison tool and offers sequencing platforms such as NovaSeq, NextSeq, and HiSeq. While the Ion Torrent platform is similar to Illumina in terms of speed and quantity, it detects newly synthesised nucleotides using semiconductor technology rather than fluorescent labelling. It measures hydrogen ions released during DNA polymerization with solid-state pH meters, resulting in shorter sequencing run times. However, concerns exist regarding sequencing error rates, particularly with long homopolymer sequences (Buermans & den Dunnen, 2014; Heather & Chain, 2016; Besser et al., 2018). Moreover, Ion Torrent supports applications such as whole genome sequencing (WGS), panel gene sequencing (PGS), whole exome sequencing (WES), and molecular clinical studies. Complete Genomics technology developed by Beijing Genomics Institute (BGI) and MGI Tech Co. Ltd. uses sequencing by ligation, PCR-free rolling circle amplification (RCA), and DNA nanoball (DNB) nanoarrays (Goodwin et al., 2016; Fehlmann et al., 2016). BGI/MGI's NGS platforms are used for a variety of sequencing applications including WGS, WES, PGS, transcriptome sequencing, microbial sequencing, epigenetics, and clinical applications. Their sequencers are comparable in performance, quality, and throughput to other NGS technologies, including Illumina (Fehlmann et al., 2016; Zhu et al., 2018). The ongoing efforts to further improve DNA sequencing quality, length, efficiency, and cost-effectiveness will help ease the implementation of NGS technology.

Multiple factors, such as sample storage (Choo et al., 2015), DNA extraction techniques, selection of the gene region of interest (Teng et al., 2018), sequencing platforms (Fouhy et al., 2016), and bioinformatics pipelines, influence microbiome results (Nelson et al., 2014; Allali et al., 2017). To alleviate some of these challenges, microbiome investigations should incorporate mock communities, which are a specified mixture of DNA or cells of bacterial strains at specific ratios that allow for the optimisation, validation, and benchmarking of microbiome studies (Bokulich et al., 2016). The utilisation of high-throughput sequencing (HTS) technology, such as NGS, is essential for the comprehensive study of aquaculture microbiomes. Currently, the complete characterisation of the microbiome and its roles within the aquaculture environment is still in its early stages.

1.7 Concluding remarks, aims and objective of the study

Infectious and non-infectious diseases have accompanied the rise of commercial shellfish cultivation. Numerous significant microbial diseases can be caused by microorganisms that are normally present in the culture system or surrounding environment that manifest themselves when environmental conditions favour their growth and cause stress in the host (aquaculture species). A metagenomics approach could provide an in-depth understanding of the microbiome and improve our understanding of microbial diversity in aquaculture operations operated under varying conditions and contribute towards improved management of aquaculture systems. Little is known about the microbial communities associated with partially recirculating integrated abalone – *Ulva* systems. Therefore, the aim of this study is to characterise the microbial and fungal communities associated with the seawater and *Ulva* obtained from an integrated abalone – *Ulva* system with 50% water recirculation, which will be achieved through the following objectives:

Objective 1 (Chapter 2): Optimisation of methods for sample preparation, DNA extraction, selection of molecular (DNA) markers and PCR conditions, and bioinformatics pipelines. This objective evaluated different methods of extraction of genomic DNA from seawater samples for improved detection of bacteria, fungi, and oomycetes in marine environments. ITS2 primers for the amplification of the ITS2 gene region from the extracted fungal and oomycete DNA were assessed, and a comparison of bioinformatic pipelines was performed for bacterial and fungal microbiome data analysis.

Objective 2 (Chapter 3): Characterisation of the bacterial community associated with seawater from abalone – *Ulva* IMTA system, where 50% of the water is recirculated. The microbiome of the IMTA system was compared with that of an *Ulva* raceway system that does not receive abalone effluent and is not recirculated (non-IMTA). Bacterial communities of IMTA and non-IMTA systems were characterised using culture-based methods and next-generation sequencing of the V4 hypervariable region of the 16S rDNA gene.

Objective 3 (Chapter 4): An assessment of the fungal and oomycete microbiome associated with seawater from *Ulva* systems receiving abalone effluent using next-generation

sequencing of the ITS2 gene region. The aim of this work was to assess the fungal and oomycete diversity and composition in an integrated abalone – *Ulva* system where 50% of the water is recirculated (IMTA) and to compare it with an *Ulva* raceway system that does not receive abalone effluent and is not recirculated (non-IMTA).

Chapter 2: Evaluation of DNA isolation methods and comparative analysis of 16S rDNA and ITS Sequencing Pipelines.

2.1. Introduction

Macroalgae, such as the green seaweed *Ulva*, are known to play an important role in marine ecosystems and are increasingly being utilised in a variety of aquaculture activities, particularly for integrated multi-trophic aquaculture (IMTA) systems to produce high-value products such as abalone. In South Africa, *Ulva* is used as a biofilter by several commercial abalone farms to bioremediate abalone effluent, which can allow for recirculation of water, and the co-produced *Ulva* is often employed as a supplementary food source for the abalone (Robertson-Andersson, 2003; Bolton et al., 2009). Characterising, monitoring, and potentially regulating the microbiome in IMTA systems could result in higher production outputs and creative ways for expanding and enhancing the aquaculture sector (Bentzon-Tilia et al., 2016; Dittmann et al., 2017). When in a state of balance, the microbiome inhibits pathogen colonisation and serves as a reservoir for host-associated microbiomes. These communities contribute to the metabolic and immunological health of animals and plants.

Advances in DNA sequencing technologies have revolutionised microbiome studies and our ability to examine the makeup and dynamics of microbial communities in complex and dynamic environments (Knight et al., 2018). Despite the increase of scientific data on sequencing the ITS region for fungi and the 16S rRNA gene for bacteria, the microbial composition of the marine environment is highly diverse and poses challenges for interpreting and comparing multiple studies. The effective study of microbiomes in complex ecosystems is dependent on and impacted by several factors, including the initial experimental design and the development, optimisation, and validation of sample collection and processing techniques (including sample storage, filtration, and DNA extraction methodologies). The choice of molecular sequencing and analysis technology, as well as the methods for data analysis, including the choice of reference database and statistical analyses, are equally important factors to consider when conducting microbiome studies (Kim et al., 2017; Knight et al., 2018; Bokulich et al., 2020; Bharti & Grimm, 2021). As with any detection system, precision, accuracy, and sensitivity must be balanced; however, due to the high variability in the physical

and chemical characteristics of the studied environments, especially marine environments, there are limited guidelines on the optimal protocols for microbiome monitoring studies.

The choice of filter paper is an essential first step in the recovery of microbial DNA from water samples (Hinlo et al., 2017). They vary in type, composition, and pore size, with filters ranging from 0.22 µm to 3 µm being commonly used in marine DNA studies (Olson et al., 2012; Barnes et al., 2014; Kelly et al., 2014; Andruszkiewicz et al., 2017; Collins et al., 2018). However, 0.22 µm filters are most commonly used for microbial studies (Byappanahalli et al.2021). Filter paper materials such as cellulose nitrate (CN), glass fiber (GF), polyethersulfone (PES), nylon, polycarbonate (PC), and cellulose acetate (CA) filters have been widely used in marine environmental DNA studies (Goldberg et al., 2016). The type of filter material that filter papers are composed of can influence how microbial cells and/or DNA bind to each type of filter paper during the filtration steps. The inherent properties of filter papers, including depth and binding capacity, may influence DNA binding affinities to different materials (Hinlo et al., 2017).

Selection of the right DNA isolation method is another important step to consider in a microbiome study because it could introduce biases during PCR and sequencing, which in turn have a significant impact on subsequent analyses and ultimately the predicted composition of the microbiome. It is important to ensure that the extraction method effectively captures all microorganisms targeted in this study. The most commonly used measure of method quality is DNA yield (concentration), with the notion that a higher yield is more likely to be representative of the community under study (Lu et al., 2015). The type of extraction method has a significant impact on the representativeness and reproducibility of extraction methods. Harsher, crude methods may yield better results but may degrade sensitive organisms, whereas excessively gentle methods may fail to extract DNA from gram-positive organisms with thick cell walls (Salonen et al., 2010; Wesolowska-Andersen et al., 2014).

For bacterial and fungal sequencing studies, choosing a variable region of the 16S rRNA gene and ITS regions of the ribosomal rRNA gene is another important step that needs to be considered. Primers used for amplification can bind to regions that are not entirely conserved

across all bacteria and do not have the same affinity for all possible DNA sequences. The V4 region (250 bp) of the 16S rRNA gene is considered a viable choice for taxonomic evaluation because it yields results that are most comparable and least biased across various bioinformatic pipelines (Caporaso et al., 2011; D'Amore et al., 2016). On the other hand, considerable variation has been reported in the literature for the gene regions targeted for fungi and oomycetes for metagenomic studies (De Filippis et al., 2017). The ITS region of the ribosomal RNA gene is the formal DNA barcode marker for fungi (Hibbett et al., 2011; Schoch et al., 2012), although there is a lack of consensus about which ITS (ITS1 or ITS2) gene to use for the identification of fungal genera or species in different environments (Dentinger et al., 2011; Bazzicalupo et al., 2013). Moreover, there is a lack of consensus on which ITS primer sets have the highest resolution for fungal diversity (Blaalid et al., 2013; Mello et al., 2011; Agler et al., 2016). Consequently, the true diversity of fungi remains largely unknown, particularly in marine environments (Grossart et al., 2016; Hibbett, 2016; Lücking & Hawksworth, 2018).

To investigate complex microbial communities, bioinformatics tools have been used to efficiently process the vast amounts of data produced by amplicon sequencing to generate a taxonomic overview. To analyse the 16S rRNA gene sequence data, a variety of bioinformatic tools are available, including QIIME2 (Bolyen et al., 2019), mothur (Schloss et al., 2009), Bioconductor (Callahan et al., 2016b), and USEARCH (Edgar, 2010). Pipelines for processing fungal ITS amplicon datasets include QIIME2 (Boyle et al., 2019), PIPITS (Gweon et al., 2015), PipeCraft (Anslan et al., 2017), CLOTU (Kumar et al., 2011), CloVR-ITS (White et al., 2013), PlutoF (Abarenkov et al., 2010), and ITScan (Ferro et al., 2014), of which only a few are web-based tools. In addition, there also specialised sequence databases that provide taxonomy annotations, such as Greengenes (DeSantis et al., 2006) and SILVA (Pruesse et al., 2007) for 16S rRNA and UNITE (Nilsson et al., 2015) for fungal ITS data.

Despite recent advances in sequencing technologies, unstandardised laboratory and computational methods produce biases that may lead to non-comparable results and/or disparities between studies (Bharti & Grimm, 2021). Thus, microbiome studies should carefully consider and optimise sample collection, preservation, filtration and DNA extraction

methods, as well as the choice of sequencing platform, database selection, and bioinformatic pipelines to generate and interpret data that is reliable, informative, and comparable with other studies (Kazantseva et al., 2021). In this study, to assess microbial diversity dynamics in IMTA systems, seawater and *Ulva* were sampled. In this chapter, each of these sample types were also spiked with known amounts of bacteria and fungi to assess the DNA extraction method's efficiency and accuracy. In IMTA systems, water is recirculated, and *Ulva* serves as a source of feed, highlighting the importance of biosecurity. By including both sample types, this study can validate microbial detection methods to ensure accurate results, and provide insights into the microbial composition of both seawater communities and *Ulva*-associated microorganisms. Therefore, the aim of this chapter was to optimise methods for sample preparation, sequencing, and bioinformatics analysis of bacterial and fungal next-generation sequencing data. This was achieved by the following objectives: *(1)* evaluate different methods for the extraction of high-quality bacterial and fungal genomic DNA from seawater and *Ulva* samples, with the performance of each method evaluated through the addition of known amounts of specific bacterial and fungal species prior to extraction; *(2)* optimise amplification of the ITS2 gene region from the extracted fungal and oomycete DNA; and *(3)* compare two commonly used pipelines for 16S rDNA (QIIME2 and mothur) and ITS2 (QIIME2 and PIPITS) using only forward reads vs. merged reads for raw sequence processing using a subset dataset. Through these objectives and the validation of extraction methods, gene regions, and bioinformatic pipelines, the results from this chapter will inform best practices and methods to apply in subsequent chapters that will focus on the characterisation of the microbiome of an integrated abalone-*Ulva* aquaculture system.

2.2 Materials and methods

2.2.1 Preparation of pure cultures of *Vibrio anguillarum* and *Halioticida noduliformans* for sample processing optimisation

2.2.1.1 Bacterial colonies

The bacterium used in this study was a strain of *Vibrio anguillarum* transfected with a stable *Vibrio*-derived plasmid (plasmid pEVS146; provided by Prof. Eric Stabb, University of Georgia) coding for chloramphenicol (Cm) and kanamycin (Kan) antibiotic resistance (Knapp et al., 2019). Bacterial colonies from a glycerol stock of *V. anguillarum* were aseptically transferred

and plated on a tryptic soy agar (TSA) plate. The plate was inverted and placed in the incubator overnight to grow at 30°C. Following incubation, a small amount of bacteria was transferred to a 15 mL sterile Hawk tube containing 5 mL of tryptic soy broth (TSB) media using a flamed loop and vortexed thoroughly until all the bacteria were suspended and clumps were no longer visible. The solution was adjusted to an optical density (OD) of 0.1 ± 0.005 at a wavelength of 540 nm, which has previously been determined to be equivalent to a bacterial concentration of 4×10^7 colony-forming units per mL ($CFU.mL^{-1}$) (Knapp et al., 2019).

2.2.1.2 Media preparation

The TSA and TCBS plates were prepared as per the manufacturer's instructions and supplemented with 2.0% and 1.5% NaCl (w/v), respectively, as described by Burgents et al. (2005), Macey et al. (2008), and Knapp et al. (2019). The UA plates consisted of 0.1% (w/v) yeast extract, 0.5% (w/v) Ulvan extract (described below), and 15 $g.L^{-1}$ of bacteriological agar in 1000 mL of 0.22 µm filtered seawater. The Ulvan was extracted from dry *Ulva*, obtained from Buffeljags Cape Abalone Farm, according to the method described by Jaulneu et al. (2010). Briefly, *Ulva* was dried in an oven at 60°C for 18 hours. The 50 g of dried *Ulva* was homogenised into coarse (<1 cm) pieces in a food processor before adding the homogenised *Ulva* to 450 mL of Millipore water and autoclaving at 120°C for 15 mins. After partial cooling, the algal suspension was filtered through a sieve (0.25 mm) and centrifuged at 5,000 × g for 5 mins. The pellet was discarded, and 2.5 volumes (1.125 mL) of 70% ethanol were added to precipitate the Ulvan from the supernatant. After one hour, the white, dough-like Ulvan precipitate was removed and washed with 96.9% ethanol. The precipitate was centrifuged at 5,000 × g for 5 mins, washed with 96.9% ethanol, and vacuum dried at 30°C for 4 hours. The dried Ulvan was ground into a fine powder using a mortar and pestle, and aliquots stored in 1.5 mL microcentrifuge tubes at -20°C until needed. On average, 50 g of dried *Ulva* yielded 4.612 g of Ulvan (9.2% dw).

2.2.1.3 Fungal cultures

The fungal-like organism (oomycete) used in this study was *Halioticida noduliformans*, the causative agent of abalone tubercle mycosis in the South African abalone *Haliotis midae* (Macey et al., 2011). Pure cultures of *H. noduliformans* were maintained on peptone-yeast-glucose-saline (PYGS) agar (w/vol., 0.125% yeast extract, 0.125% peptone, 0.3% D-glucose,

and 1.2% bacteriological agar made up in autoclaved seawater (ASW)) without antibiotics at a temperature of 20°C, and routinely sub-cultured once every 2 to 3 weeks. To obtain substantial quantities of mycelia for the purpose of isolating genomic DNA, agar plugs measuring 2 to 3 mm in diameter were obtained from the growing edge of an actively growing *H. noduliformans* culture (ca. 7-day-old cultures). Each agar plugs was obtained from actively growing mycelia that were 7 days old. Each agar plug was then transferred into individual sterile glass test tubes pre-filed with 5 mL of PYGS broth (sterilised using an autoclave). Tubes were placed in an incubator set at a temperature of 20°C, and the mycelia were allowed to grow for a period of 10 to 14 days. Following incubation, the mycelia were rinsed three times using sterile ASW before transferring the washed cells to an empty sterile 100 mm petri dish and separating the washed mycelia from the agar plug prior to DNA isolation.

2.2.2 DNA Isolation

To optimise DNA extraction conditions, known quantities of the bacterium *V. anguillarum* (100 µL of a 4×10^7CFU.mL^{-1} bacterial solution) and the washed oomycete *H. noduliformans* mycelia (1 mg of fungal hyphae) were added to 250 – 1000 mL volumes of ASW (250, 500, 1000 mL), mixed thoroughly, and aseptically filtered through 0.22 µm pore-size filter nitrocellulose membrane filters (47 mm diameter, Millipore Corp., Bedford, Mass). The time taken to filter each of the seawater samples was carefully recorded. The filter was then briefly vortexed to dislodge the bacteria, followed by centrifugation. DNA was subsequently extracted from the resulting pellet. Similarly, known concentrations of bacteria and fungi were also added to 15 g of healthy vegetative *Ulva* thalli before grinding to a fine powder using liquid nitrogen.

Four DNA isolation kits were selected based on their common usage for similar types of samples (Demkina et al., 2023). The kits were tested to extract genomic DNA from the spiked seawater and *Ulva* samples, namely the Stratec RTP Bacteria kit (Stratec ®, Cat No: 1040500200), the Qiagen Fast Stool kit (Qiagen, Cat. No. 51604), the Qiagen QIAamp Micro kit (Qiagen, Cat. No. 56304) and the Qiagen Plant Mini kit (Qiagen, Cat. No. 69104), following manufacturer recommendations for the isolation of bacterial DNA where applicable. Similarly, fungal and oomycete DNA from spiked seawater and *Ulva* samples was isolated using the following four kits/methods: Heat lysis method, Qiagen Fast Stool kit (Qiagen, Cat. No. 51604),

Qiagen QIAamp Micro kit (Qiagen, Cat. No. 56304) and Qiagen Plant Mini kit (Qiagen, Cat. No. 69104). Each DNA isolation method was tested across six samples (N = 6), and the data was recorded as the mean DNA concentration ($ng.\mu L^{-1}$) ± standard error for each method.

2.2.3 DNA quantification and quality assessment

To avoid repeated freeze-thaw cycles, the DNA was separated into 10 µL aliquots and kept at 20°C. Since DNA extracts can contain impurities such as proteins and other organic compounds that can interfere with downstream PCR amplification, DNA purity was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA) at absorbance ratios of 260/280 and 260/230. Samples exhibiting a 260/280 ratio ranging from about 1.7 to 2.0, as well as a 260/230 ratio ranging from approximately 2.0 to 2.2, were deemed of sufficient quality in this study.

To verify the integrity of the isolated DNA for each extraction method, a real-time quantitative polymerase chain reaction (qPCR) was conducted targeting the Kanamycin (Kan) resistance gene and the LSU rDNA gene (Table 2.1) to quantify differences in the recovery of *V. anguillarum* and *H. noduliformans* DNA, respectively. For *V. anguillarum*, a 129 bp fragment of the Kan resistance gene was amplified using 400 nM forward primer RTKnF, 400 nM reverse primer RTKnR, and 200 nM TaqMan probe (Table 2.1). The amplification for *Vibrio* was caried out with a 15 min of incubation at 95°C, followed by 50 cycles of denaturation at 95°C for 30 s and a combined annealing and elongation step at 60°C for 45 s.

For *H. noduliformans,* reactions were carried out in a volume of 25 µL containing 1 µL genomic DNA (12.5 µL of 1 × Promega GoTaq® SYBR Green master mix (Promega, Catalog #A6001)), 0.5 µL of each primer (400 nM), and 10.5 µL Millipore water). The amplification was carried out with an initial denaturation of 95°C for 5 min, annealing at 59 °C for 45 s and an elongation at 72°C for 45 s. Amplifications were monitored using a Bio-Rad CFX96™ real-time PCR detection system on a C1000™ thermal cycler. Data collection and real-time analysis occurred at the annealing step of each cycle and melt curve data collection and analysis occurred at each increment in the latter 70 cycles. Each of the DNA samples (N = 6) per method was tested in

triplicate, and the data was recorded as the mean (± SE) quantification cycle (Cq) number for each method. The lower the Cq value, the higher the recovery of each respective species.

Table 2.1. Summary of primers used in this study to amplify the V4 hypervariable region of the 16S rRNA gene and the nuclear ribosomal internal transcribed spacer 2 (ITS2) with overhang adapter sequence underlined.

	Primer	Oligonucleotide Sequence (5'-3')	Target	Fragment size	Annealing temperature	Reference
16S rRNA	515F	TCGTCGGCAGCGTCAGATGTG TATAAGAGACAG GTGYCAGCMGCCGCGGTAA	16S (V4)	250 bp	65°C	Caporaso et al.,2011
	806R	GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG GGACTACNVGGGTWTCTAAT)				
ITS2	ITS3_KYO2F	TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGGATGAAGAACGYAGYRAA	ITS2	700 bp	47°C	Toju et al., 2012
	ITS4_KYO3R	GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAGCTBTTVCCKCTTCACTCG				
V. anguillarum	RTKnF	TGATGCGCTGGCAGTGTT	Kan	129bp	60°C	Macey et al., 2008
	RTKnR	CTCGCATCAACCAAACCGTTA				
	Probe	TGCGCCGGTTGCATTCGATTCCTGT				
H. noduliformans	HN.LSU-F	CGTCATAGTCAGTTTGTAT	LSU rDNA	141 bp	59°C	Greeff et al., 2012
	HN.LSU-R	ATCCCATCAATACCCTTA				

2.2.4 ITS2 PCR amplification optimisation

There are three significant criteria for primer selection in fungal DNA barcoding NGS of environmental samples. Firstly, to effectively assess the diversity and community structures of fungal species in environmental samples, it is essential for the DNA barcoding primers to amplify sequences of a wide range of fungal taxa. This feature serves as a basic prerequisite for the primers employed in biodiversity studies. Secondly, selective amplification of fungal sequences and, thirdly, amplicon length are also factors to be considered when choosing barcoding primers (Toju et al., 2012). In the context of ecological and microbiological studies, DNA barcoding of the ITS2 region employing the ITS3_KYO2 and ITS4_KYO3 primer sets (Toju et al., 2012) with read lengths less than 700 bp would be sufficient. The ITS2 primers were tested to assess whether they would sufficiently detect the presence of fungal and oomycete DNA that might be present in the system, with *Pythium*, *Halioticida*, *Saprolegnia*, *Achlya*, and *Aphanomyces* being included in the analysis. PCR cycling conditions were optimised to enhance the specificity of fungi and oomycete detection. The PCR reactions were carried out in a volume of 25 µL containing 2 µL genomic DNA at a concentration of 1 ng/mL for each fungus, respectively. These fungi and oomycetes included *Fusarium* sp., *Halioticida noduliformans*, *Pythium*, *Saprolegnia ferax*, *Saprolegnia parasitica*, *Saprolegnia autralis*,

Aphanomyces astici, and *Achyla bisexual*. The remainder of the PCR volume consisted of 12.5 µL of 1 × Promega GoTaq® SYBR Green master mix (Promega, Catalog #A6001) and 0.5 µL of each primer. The PCR was caried out with ITS2 primers (ITS2_KY02 and ITS3_KYO4) under a temperature profile of 95°C for 10 min, followed by 35 cycles at 94°C for 20 s, 47°C for 30 s, and 72°C for 20 s followed by 72°C for 7 min. The concentrations of $MgCl_2$, dNTPs, PCR primers, and the template DNA in the reaction buffer were 1.5 mM, 200 mM, 0.5 mM, and 1 ng/mL of each fungus mentioned above, respectively. The PCR product quality was assessed using UV light after gel electrophoresis on a 1% agarose gel containing ethidium bromide (EtBr).

2.2.5 Bioinformatics pipelines for bacterial identification (16S rDNA sequence data)

2.2.5.1 Mothur 16S bioinformatics

In order to assess the performance of bioinformatics pipelines in analysing microbiome data from samples collected from abalone effluent water (AEW) and seawater (SW) systems, the study evaluated two commonly used bioinformatic pipelines, namely QIIME2 and mothur, and two reference databases (Greengenes and SILVA) for the 16S gene analysis. Analyses conducted in mothur followed the 16S metagenomic standard operating procedure (SOP) provided by Kozlch et al. (2013) at Schloss Lab (Miseq_SOP: http://www.mothur.org/wiki/Miseq_SOP). The raw fastq forward and reverse read files were used to construct contigs with the use of the *make.contigs* command to generate individual fasta files. Duplicate reads and reads with ambiguous base calls were removed, and sequences were trimmed to 300 bp using the *screen.seqs* command for the V4 gene region. Any redundancies created were removed using the *unique.seqs* command, and to further de-noise sequences, *pre.cluster* was used to split sequences by group and sort them by abundance, allowing one difference for every 100 bp. An alignment was generated using the *align.seqs* command and the SILVA alignment template. The alignment was cleaned using the *screen.seqs* and *filter.seqs* commands to make sure that the sequences overlapped in the same region and to remove overhangs. The *pre.cluster* command was used to merge any reads that were within a two-base pair similarity of a more abundant read, producing ASVs for downstream analysis. Chimeras were detected using remove.*vsearch* and removed using *remove.seqs* command. Reads were assigned a taxonomic classification using the *classify.seqs*

command and a Naïve Bayes classifier that was trained on the SILVA (release 132) (Quast et al., 2013) and Greengenes (gg-13-8-99-nb) (DeSantis et al., 2006) 16S reference databases, respectively. Taxonomic-level identification was based on 99% identity between the reference database and amplicon sequence variants (ASVs) (Edgar, 2018).

2.2.5.2 QIIME2 16S bioinformatics

Demultiplexed paired-end fastq reads were imported into the Quantitative Insights into Microbial Ecology 2 program (QIIME2- 2020.11) using the Casava input format. QIIME2 is an open-source software that was used to assess raw data through the 16S Metagenomics workflow. The Divisive Amplicon Denoising Algorithm 2 (DADA2) software package (Callahan et al., 2016a), wrapped in QIIME2, was used to quality filter, trim, de-noise, and merge the data, as well as remove chimeras using the "consensus" method. Specifically, DADA2 was used to remove low-quality reads (phred score < Q20), reads below 150 bp in length, and reads with a low abundance (n < 10 counts). Resultant feature sequences representing the number of sequences that belong to different taxa (ASVs) and the summary statistics were summarised and annotated using the QIIME2 feature–classifier plugin (https://github.com/qiime2/q2-feature-classifier).

Two reference databases for the identification of taxa were tested in the current study. Firstly, a Naïve Bayes classifier was pre-trained on the 16S V4 sequences in the SILVA reference database (Quast et al., 2013). Secondly, the classifier was pre-trained on the 16S V4 sequences in the Greengenes database (DeSantis et al., 2006). Contaminating mitochondrial, chloroplast, archaea, and eukaryote sequences were filtered out of the resulting feature table. The QIIME2 *feature-classifier classify-sklearn* confidence threshold value was set to 70% (QIIME2 recommended default) (Bokulich et al., 2018).

2.2.6 Bioinformatics pipeline optimisation for fungal/oomycete identification (ITS2 sequence data)

2.2.6.1 Challenges associated with ITS2 data

The bioinformatics workflows that are commonly used to process bacterial 16S rRNA gene sequencing data do not sufficiently address the challenges posed by fungal amplicon

sequence data, as stated by Diaz et al. (2017) and Halwachs et al. (2017). Most notably, the variability of fungal ITS sequence length (of ca. 700 bp) poses a challenge when merging paired-end Illumina reads, as the resulting sequences may exceed the maximum merged read length and consequently be excluded from the analysis. Conversely, there are instances where the opposite holds true, as evidenced by the current study, wherein sequences prove to be poor due to their lack of overlap, necessitating additional joining steps. To avoid the possibility of taxa being excluded, a comparative analysis was conducted between bioinformatic processing of forward and merged forward and reverse reads, respectively, with the aim of improving the fungal and oomycete classification using PIPITS and QIIME2 software.

2.2.6.2 Curating a fungal and oomycete ITS2 reference database

RESCRIPt was implemented as a free, open-source QIIME2 (Boylen et al., 2019) plugin. A UNITE database FeatureData [Sequence] and a FeatureData [Taxonomy] artifact were created in QIIME2. An accession metadata file was created by downloading the accession file from the National Centre for Biotechnology Information (NCBI) (nucleotide database) after searching query "ITS2 and oomycete". *Halioticida noduliformans* was specifically added to this database (due to its importance and relevance to the abalone system) and another sequence.qza and taxonomy.qza file was created manually. The artifacts were merged for reference sequences (databases before mapping/classifying) and for corresponding taxonomy files. The resulting database was used for taxonomic identification of ASVs for the ITS2 dataset.

2.2.6.3 PIPITS/QIIME2 ITS2 analysis with merged reads

The raw fungal ITS2 reads were processed using PIPITS (Gweon et al., 2015). Since the pre-processing steps in PIPITS are specifically made for paired end, overlapping sequence reads, additional joining steps were first required for non-overlapping reads using the AMPtk (amplicon toolkit) package to first trim forward and reverse primer sequences, then merge the paired end reads using VSEARCH (Palmer et al., 2018). The resulting assembled reads were quality filtered (FASTX-Toolkit), which discarded sequences where 80% of the bases had a phred score lower than 20. The resulting merged reads were further analysed in QIIME2 (Bolyen et al., 2019), where DADA2 (Callahan et al., 2016a) was used for error correction, quality filtering, chimera removal, and sequence variant calling of the Illumina amplicon

sequences. Trimming of the ITS2 region of interest was done using ITSxpress (Rivers et al., 2018) for the identification of exact sequence variants. The taxonomic assignment of sequences was performed using the Naïve Bayes classifier, with reference databases including the UNITE reference database and a curated oomycete ITS reference database from the present study. Notably, the latter database contained a sanger sequence of *H. noduliformans*. This was done because the ITS sequence for *H. noduliformans* is not available in the NCBI database.

2.2.6.4 QIIME2 ITS2 analysis with merged reads

Demultiplexed paired-end fastq reads were imported into the Quantitative Insights into Microbial Ecology 2 program (QIIME2-2020.11) using the Casava input format. *Computations were performed using facilities provided by the University of Cape Town's ICTS High Performance Computing team* (hpc.uct.ac.za) The Divisive Amplicon Denoising Algorithm 2 (DADA2) software package wrapped in QIIME2 was used to quality filter, trim, de-noise, and merge the data, including the removal of chimeras, using the "consensus" method. Specifically, DADA2 was used to remove low-quality reads (phred score < 20). Forward reads were trimmed to a maximum read length of 228 bp, and reverse reads were trimmed to a maximum length of 161 bp (phred score < 20). The curated UNITE fungal and oomycete ITS reference databases were used as the reference database, and taxonomic assignments were carried out for the ASVs using the QIIME2 q2-feature-classifier (https://github.com/qiime2/q2-feature-classifier.

2.2.6.5 QIIME2 ITS2 analysis with forward reads

After sequencing, forward reads were processed using the QIIME2 pipeline (version 2022.2; *http://qiime2.org/). Computations were performed using facilities provided by the University of Cape Town's ICTS High Performance Computing team* (hpc.uct.ac.za). Demultiplexed paired-end FASTQ files were imported into QIIME2 using the Casava input format. The DADA2 (Callahan et al., 2016a) plugin was used for error correction, quality filtering, chimera removal, and sequence variant calling of the Illumina amplicon sequences. Forward reads were trimmed to a maximum read length of 228 bp at a phred score < 20. Trimming of the ITS region of interest was done using ITSxpress (Rivers et al., 2018), which is available as a QIIME2 plugin,

to retain the ITS region of choice. The UNITE database (Nilsson et al., 2018) combined with a custom oomycete database that included the *H. noduliformans* sequence, curated using NCBI, was used as a reference database for ITS2 sequences. Taxonomic assignments were carried out for the ASVs using the QIIME2 q2-feature classifier (https://github.com/qiime2/q2-feature-classifier) and a Naïve Bayes classifier.

2.2.7 Statistical analysis on a subset of data

This chapter's analysis was conducted on a subset of samples obtained from the abalone effluent water (AEW) system. These samples included *Ulva* samples denoted with "U", incoming water denoted with "In", and outgoing water denoted with "Ou" (In_P4C4_2, U_P2_1, U_P2_2, U_P4_1, Ou_P2C2_2, Ou_P1C1_3, In_P1C1_2, In_P1C1_3, and Ou_P6C6_4) from the AEW system. The aim of this statistical analysis on a subset of samples was to assess differences in diversity and taxonomic identification across the respective bioinformatic pipelines and to assess the taxa in the IMTA system (see Chapter 3 and 4 for a detailed description of the experimental design and sampling). The assessment of alpha (within sample) diversity was conducted using the R *phyloseq* package (McMurdie & Holmes, 2013) and the *Vegan* package (Dixon, 2003), as implemented in MicrobiomeAnalyst (Dhariwal et al., 2017; Chong et al., 2020). Species richness was estimated using the Chao1 index, which accounts for rare species/ASVs to provide a measure of diversity as it represents the total number of ASVs detected across the representative sample types. An analysis of variance (ANOVA) was then conducted on the Chao1 indices to determine if there were statistically significant differences in species richness between cohorts. MicrobiomeAnalyst was also used to perform beta (between-sample) diversity analysis using the same R packages, implementing a multivariate non-metric multidimensional scaling (NMDS) analysis utilising Bray-Curtis dissimilarity indices. Statistical significance ($p < 0.05$) was assessed using a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001). Taxonomic abundance was evaluated by constructing an abundance table to assess the differences in the top five most abundant taxa identified using different bioinformatics pipelines.

2.3 Results

2.3.1 DNA quantity, purity, and integrity

Following the filtration of varying amounts of seawater (250, 500, and 1000 mL) using 0.22 µm filters, it was demonstrated that it was only practical to filter a maximum amount of 500 mL of seawater. Larger volumes (1000 mL) of seawater took too long to filter (> 30 min per sample), which may compromise the integrity and viability of microbiological cells as well as the yield and quality of DNA, and the filters became clogged when filtering larger volumes (data not shown). Furthermore, it was found that the choice of DNA extraction method significantly influenced both the yield and the purity and quality of the DNA. The DNA extracted from autoclaved seawater spiked with *V. anguillarum* was assessed using four DNA isolation methods (Fig. 2.1). The Qiagen Fast Stool kit yielded the highest concentration of DNA (5.2 ng.µL^{-1}), followed by the Stratec RTP Bacteria kit with 3.2 ng.µL^{-1}, whereas the Qiagen Plant Mini kit yielded the lowest concentration of DNA (1.4 ng.µL^{-1}). The A260/230 and A260/280 absorbance readings of samples obtained using the different kits were compared using a NanoDrop spectrophotometer to determine the purity of the isolated DNA (Table 2.2). For a sequencing platform, the recommended A260/280 ratio of isolated DNA should be is 1.8 – 2.0 for the A260/280 ratio and 2.0 for the A260/230 ratio (Usman et al., 2014; Hassan et al., 2015; Lucena-Aguilar et al., 2016). The DNA extracted from seawater spiked with *V. anguillarum* using the Stratec RTP Bacteria kit and the Qiagen QIAamp Micro kit met these quality criteria and recommendations. Conversely, the Qiagen Plant Mini kit did not pass the purity thresholds, while the Qiagen Fast Stool kit passed the purity threshold for the A260/280 ratio but failed for the A260/230 ratio, indicating a high degree of contamination with polysaccharides and salts.

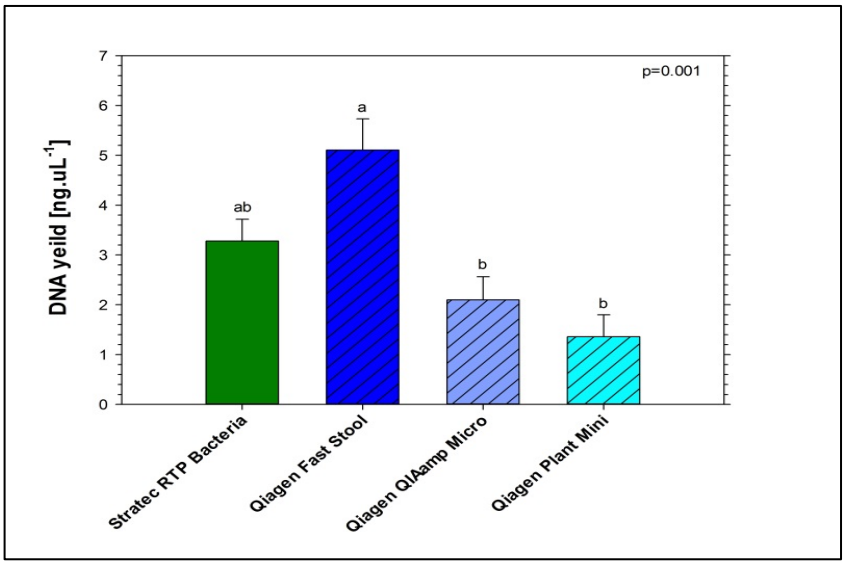

Figure 2.1. Comparison of DNA extraction methods (Stratec RTP Bacteria, Qiagen Fast Stool kit, Qiagen QIAamp Micro and Qiagen Plant Mini kit). The mean (± SE) yield of genomic DNA (ng. μL^{-1}) isolated from 0.22 µm filters containing cells obtained from filtering 500 mL of autoclaved seawater spiked with a known amount of *Vibrio anguillarum* (4×10^6 CFUs). Different letters indicate significant differences (One-Way ANOVA, $p < 0.05$) between treatments. A total of 6 samples were tested for each DNA isolation method.

Table 2.2. Quality of gDNA isolated from 0.22 µm filters containing cells derived from 500 mL autoclaved seawater spiked with *V. anguilarum* (4×10^6 CFUs) using various extraction methods. Recommended purity of DNA for next-generation sequencing is 1.8 - 2.0 for A260/280 ratio and ~2.0 for A260/230 ratio.

Absorbance ratios	StratecRTP Bacteria	Qiagen Fast Stool kit	Qiagen QIAamp Micro kit	Qiagen Plant Mini kit
A260/280	1.82 ± 0.05*	1.89 ± 0.12	1.80 ± 0.05*	1.67 ± 0.03
A260/230	2.01 ± 0.03*	1.61 ± 0.21	2.1 ± 0.08*	0.80 ± 0.07

Note: DNA that passed the purity threshold are marked with an asterisk*

When assessing seawater samples spiked with *H. noduliformans*, the Heat lysis method produced the highest DNA yields (9.8 ng.μL^{-1}) compared with the other extraction methods (Fig. 2.2). For all the other DNA extraction methods, the obtained DNA yields were lower than 10 ng.μL^{-1} with the Qiagen Fast Stool kit yielding the lowest DNA yield (0.2 ng.μL^{-1}). Even though the Qiagen QIAamp Micro kit and Qiagen Plant Mini kit produced DNA yields less than 10 ng.μL^{-1}, these were the only two methods/kits that yielded DNA that met the quality and purity criteria recommended by the NGS platforms, indicating low contamination with proteins. On the other hand, the DNA extracted using the Heat lysis protocol and the Qiagen

Fast Stool kit did not yield DNA that passed the quality criteria in any of the samples tested (Table 2.3).

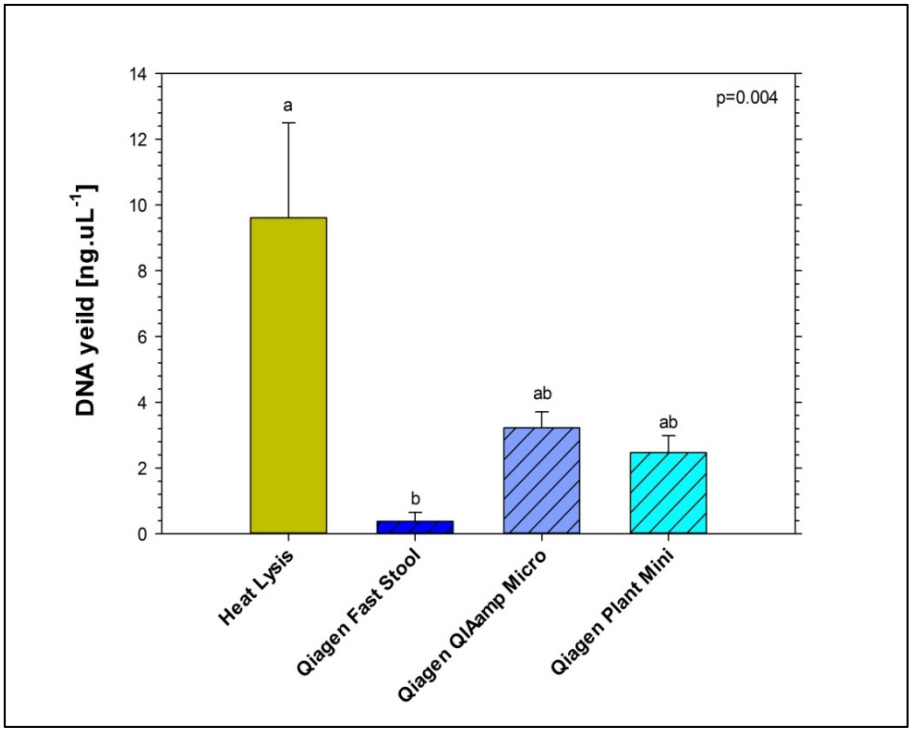

Figure 2.2. Comparison of DNA extraction methods (Heat lysis, Qiagen Fast Stool kit, Qiagen QIAamp Micro kit, and Qiagen Plant Mini kit). The mean (± SE) yield of genomic DNA (ng. μL^{-1}) obtained from seawater (500 mL) samples spiked with 1 mg of *Halioticida noduliformans* mycelia extracted using four different methods/kits. Different letters indicate significant differences (One-Way ANOVA, $p < 0.05$) between treatments. A total of 6 samples were tested for each DNA isolation

Table 2.3 Quality of gDNA from 0.22 µm filters containing cells derived from 500 mL autoclaved seawater spiked with *H. noduliformans* using various extraction methods. Recommended purity of DNA for next-generation sequencing is 1.8 - 2.0 for A260/280 ratio and ~2.0 for A260/230 ratio.

Absorbance ratios	Heat lysis	Qiagen Fast Stool kit	Qiagen QIAamp Micro kit	Qiagen Plant Mini kit
A260/280	1.45 ± 0.08	1.57 ± 0.15	1.92 ± 0.04*	1.98 ± 0.03*
A260/230	0.69 +0.04	1.61 ±0.21	2.0 ± 0.14*	2.02 ± 0.03*

Note: DNA that passed the purity threshold are marked with an asterisk*

Upon conducting a more in-depth analysis on the genomic DNA extracted using the different kits, by utilising *V. anguillarum*-specific primers in a real-time PCR assay to quantify the recovery or amplification of the *V. anguillarum* kan gene in the spiked seawater samples, it was observed that the Qiagen Fast Stool and Stratec RTP Bacteria kits exhibited the highest Cq values (as depicted in figure 2.3), indicative of poor amplification or low recovery of the kan gene from the spiked samples. The results indicate that while the latter two kits yielded the highest amounts of genomic DNA, the quality of the isolated DNA appears to be lower as the amount of DNA from each of the methods yielded higher Cq values, indicating a lower recovery of *V. anguillarum*.

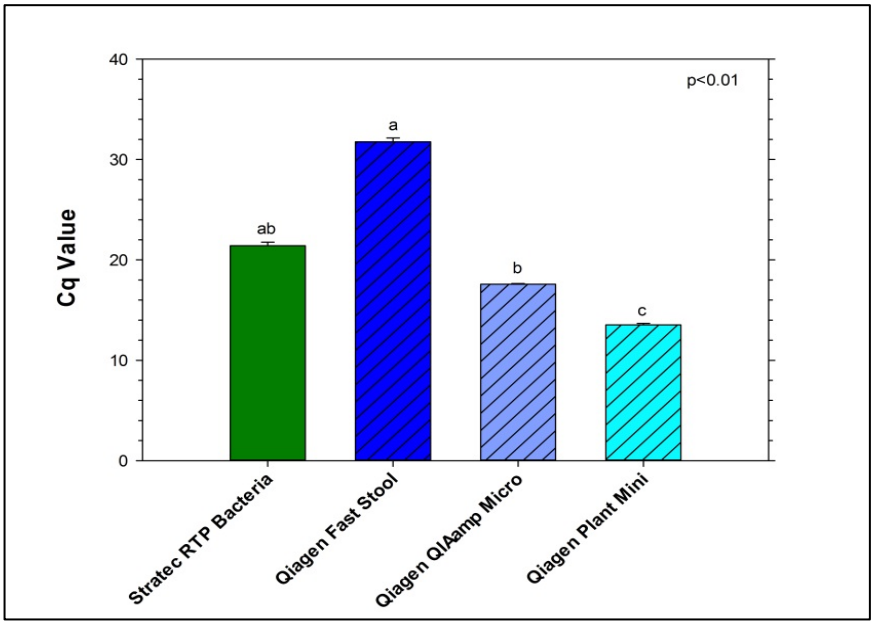

Figure 2.3. Quantification cycle (Cq) threshold values obtained following real-time PCR amplification of the *V. anguillarum* kan resistant gene on an equivalent amounts of total genomic DNA obtained from using *Vibrio anguillarum* spiked seawater samples using four different genomic DNA isolation kits. The data represents the mean (± SE) quantification cycle (Cq) number (N = 6) for each method. Different subscripts denote a significant difference (One-Way ANOVA, $p < 0.05$) between methods.

Upon evaluating the recovery of *H. noduliformans* DNA from seawater spiked with 1 mg of mycelia using a real-time PCR assay with *H. noduliformans*-specific primers, it was observed that each method yielded Cq-values that were similar and not statistically significantly different from one another (one-way ANOVA; $p > 0.05$) (Fig. 2.4). Due to the low yield of DNA (0.2 ng.µL^{-1}) obtained through the utilisation of the Qiagen Fast Stool kit, the DNA samples

acquired through this particular method were omitted from the real-time PCR analysis. The Qiagen QIAamp DNA Micro kit was selected for the extraction of total genomic DNA from 500 mL seawater samples that were filtered using 0.22 µm-pore-size filter membranes (47 mm diameter) in all subsequent experiments, based on the results obtained from the bacterial and fungal optimisation experiments mentioned above.

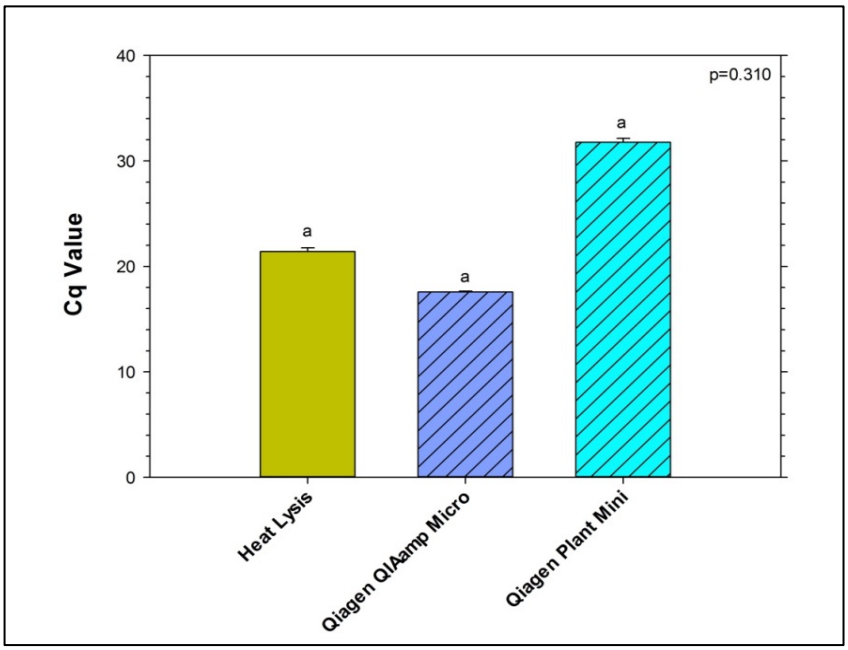

Figure 2.4 Quantification cycle (Cq) threshold values obtained following real-time PCR on an equivalent amount of *Halioticida noduliformans* genomic DNA isolated using three different genomic DNA isolation methods/kits. The data represents the mean (± SE) quantification cycle (Cq) number (N = 6) for each method. No significant difference was observed between methods (One-way ANOVA; $p > 0.05$).

2.3.2 ITS2 amplification optimisation

The ITS2 primer pair chosen for testing in this study was ITS3_KYO2/ITS4_KY03 to amplify the 700 bp of the ITS2 gene (Fig. 2.5). For this primer pair, single bands were observed for the amplification of *Fusarium* sp., *H. noduliformans*, *Pythium* sp., *S. ferax*, *S. parasitica*, *S. australis*, *A. astaci*, and *A. bisexual* (Fig. 2.5). The results obtained from the PCR amplification demonstrated the ability of ITS2 primers to amplify DNA from fungi and oomycetes that could potentially be present in the abalone effluent water (AEW) and seawater (SW) system of Buffeljags abalone farm. The ITS3_KYO2/ITS4_KY03 primer pair was selected for subsequent analysis due to the production of clean bands of the expected size that were generated for each of the samples below.

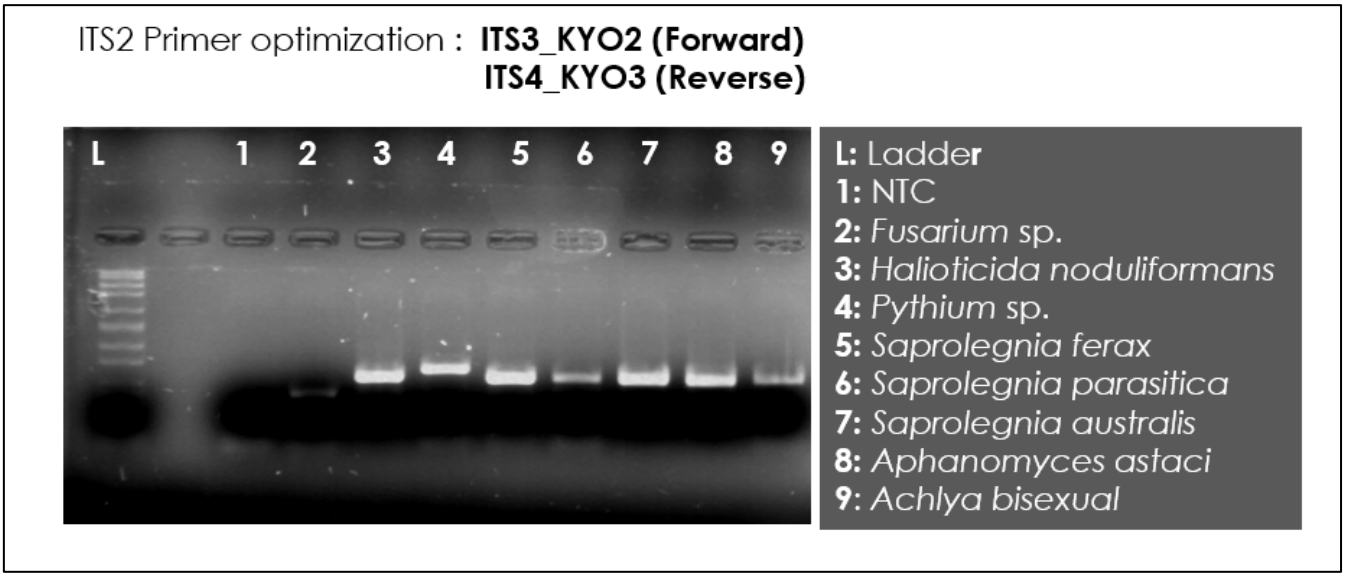

Figure 2.5. Gel image showing PCR amplification of a portion of the ITS2 [700bp] gene. **Lane L**: DNA ladder (1kb), **Lane 1**: non template control (ddH20), **Lane 2**: *Fusarium* sp., **Lane 3**: *H. noduliformans*, **Lane 4**: *Pythium* sp., **Lane 5**: *S. ferax*, **Lane 6**: *S. parasitica*, **Lane 7**: *S. australis*, **Lane 8**: *A. astaci* and **Lane 9**: *A. bisexual.*

2.3.3 Bioinformatics optimisation for 16S rDNA and ITS2

2.3.3.1 16S rDNA bioinformatics pipelines

To evaluate the performance of the two reference databases, Greengenes and SILVA, the percentage of amplicon sequence variants (ASVs) successfully assigned to the genus level for each database was determined. It is important to note that the sequence processing steps were identical up until the taxonomic classification step, where different reference databases were used. Overall, the SILVA reference database consistently detected 10% more genera (mean 85.817% vs. 73.75%, respectively) at the genus level when compared with the Greengenes database. Moreover, the SILVA database detected a total of five genera that were not identified by Greengenes. Conversely, Greengenes was able to detect one genus that was missed by SILVA. Moreover, the two commonly used bioinformatic tools (software) for analysing 16S rRNA gene sequences generated from NGS platforms, namely QIIME2 and mothur were compared against SILVA reference database. A total of 2 856 295 and 2 426 395 raw reads from a subset data (N=9) was used for the comparative analysis of the QIIME2 and mothur pipelines, respectively. The differences in pre-QC read counts observed between QIIME2 and mothur could be attributed to quality score filtering, primer and adapter removal, chimera detection, contaminant or low-abundance sequence removal. The mothur pipeline

resulted in a higher proportion of unclassified reads (Table 2.4). The number of genera detected using QIIME2 and mothur was 359 and 340, respectively. A difference was also observed between the two pipelines with respect to the number of reads assigned to taxa, with the reads processed in QIIME2 exhibiting a 16.28 % higher mapping rate than mothur. Analysis of the 60 samples took approximately five days (computational time) when using QIIME2 and approximately three weeks when using the mothur pipeline (Table 2.4). Furthermore, QIIME2 showed the best overall performance, combining high sensitivity with excellent specificity based on the number of input reads detected and the number of reads assigned to identity. A difference was observed in the number of genera detected between the two pipelines. Both pipelines showed a general agreement in the identification of the genera, with the proportion of *Vibrio* varying from 11% (QIIME2) to 10.5% (mothur), *Pseudalteromonas* from 8% (QIIME2) to 6% (mothur), *Leucothrix* from 5% (QIIME2) to 4% (mothur), and the Arcrobacteraceae genus from 4% (QIIME2) to 3% (Mothur) (Table 2.5). Based on the computational time, the number of reads assigned to taxonomy, and the number of unclassified reads, QIIME2 was chosen for the analyses of 16S the rRNA gene sequence in the current study.

Table 2.4 Comparison of analysis with QIIME2 and mothur.

	QIIME2	**mothur**
Approximate analysis time	5 days	3 weeks
Number of reads pre-QC	2 856 295	2 426 395
Number of unique ASVs assigned identity	1023	936
Number of unclassified reads at phylum (%)	3	3.8
Number of unclassified reads at family (%)	27	30
Number of unclassified reads at genus (%)	46	58
Number of genera identified	359	340

Table 2.5. The five most abundant bacterial genera detected by QIIME2 and mothur.

QIIME2	**mothur**
Vibrio (11%)	*Vibrio* (10.5%)
Psuedoalteromonas (8%)	*Psuedoalteromonas* (6%)
Rhodobacteraceae genus (7%)	Rhodobacteraceae genus (7%)
Leucothrix (5%)	*Leucothrix* (4%)
Arcrobacteraceae genus (4%)	*Arcrobacteraceae genus* (3%)

Observed alpha diversity using the Chao1 richness estimator showed statistically significant difference between cohorts for QIIME2 and mothur ($p < 0.05$) (Fig. 2.6). The AEW_in and AEW_out cohorts had a higher Chao1 diversity when compared to AEW_*Ulva* for both QIIME2 and mothur. The permutational multivariate analysis of variance (PERMANOVA) was performed using a Bray-Curtis dissimilarity matrix to evaluate differences between the bioinformatics pipelines. Samples from AEW_in and AEW_out cohorts were grouped closer to one another (Fig. 2.7), while AEW_*Ulva* cohort had its own separate cluster. This clustering pattern was observed for both QIIME2 and mothur.

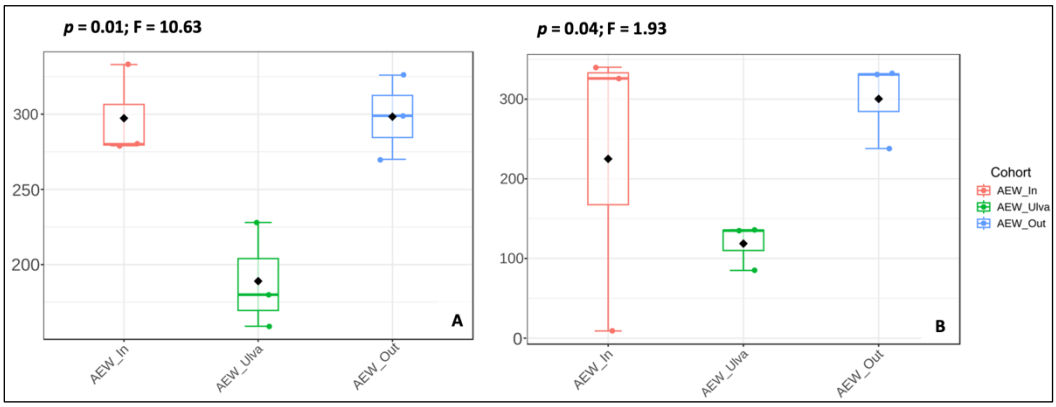

Figure 2.6. Average alpha diversity measures (Chao1) of QIIME2 (A), mothur (B) from bacterial samples at genus level where the minimum, maximum and mean as well as ANOVA F-values and p-values are indicated for each cohort. Samples are from the AEW (IMTA) system. *Ulva* samples are denoted by "*Ulva*", inlets are denoted by (In), and outlets are denoted by (Out).

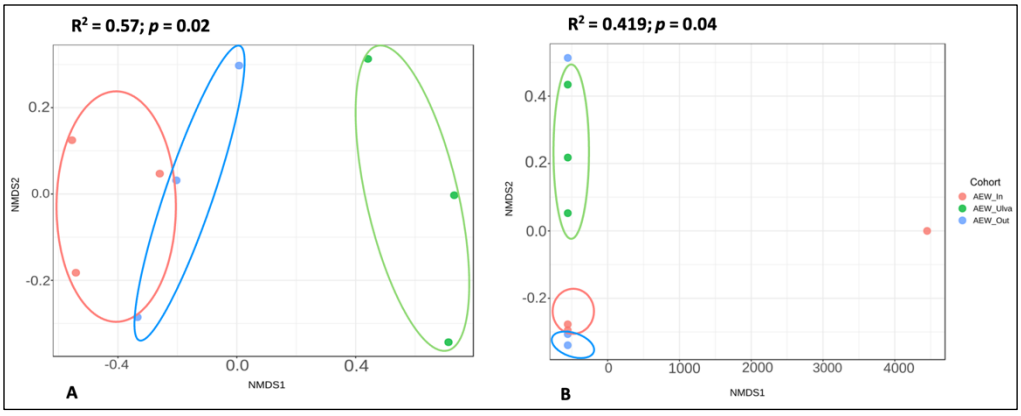

Figure 2.7. Non-metric multi-dimensional scaling analysis (NMDS) from bacterial samples at genus level showing the difference in pipelines of QIIME2 (A) and mothur (B). Samples are from the AEW (IMTA) system. *Ulva* samples are denoted with "*Ulva*", inlets are denoted with (In), and outlets are denoted with

2.3.3.2 ITS2 bioinformatics pipelines

When comparing the use of different bioinformatics pipelines on the ITS2 sequences from a subset of data against a custom database that included UNITE and *H. noduliformans* sequences, it was shown that QIIME2 (forward reads) retained 30% more reads than PIPITS (merged reads) and QIIME2 (merged reads) post-QC (Table 2.6). To verify the adequate species richness of the mycobiome, rarefaction analysis of the two pipelines was performed. For all rarefaction analyses, raw counts were used for a subset of samples in QIIME2 (forward reads) (Fig. 2.8), QIIME2 (merged reads) (Fig. 2.9) and PIPITS (merged reads) (Fig. 2.10), with QIIME2 (forward reads) exhibiting a higher species richness than QIIME2 merged reads. QIIME2 (forward reads) consistently identified more ASVs than merged reads, detected more ASVs per sample, and returned generally higher alpha diversity metrics as a result.

Table 2.6. Number of sequences reads and ASV number for merged and single end forward reads against UNITE reference database.

	Forward reads (QIIME2)	**Merged (QIIME2)**	**Merged reads. PIPITS/ QIIME2**
Minimum read count	7109	6425	2807
Number of reads pre-QC	1 210 914	374 586	365 008
Number of ASVs	169	10	179
ASVs with > 2 counts	16	9	12
Average read count per samples	134 546	41 620	40 556
Unclassified sequences	739 287	208 946	190 287

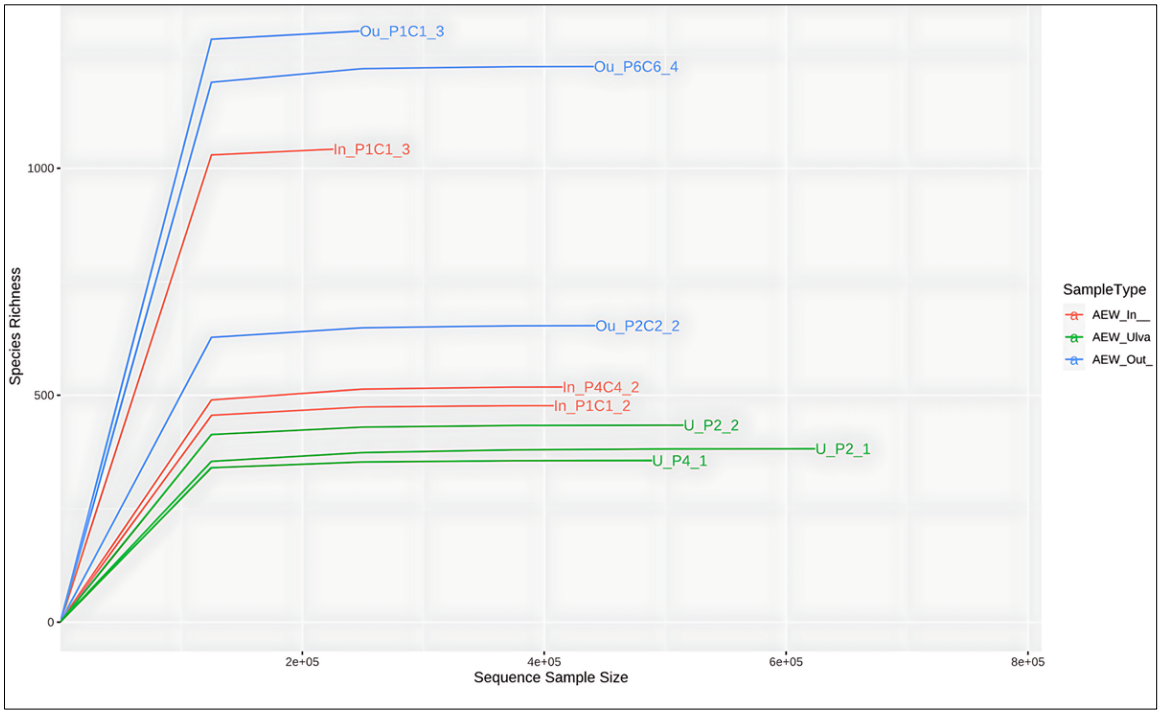

Figure 2.8. Rarefaction analysis across three groups of subset data samples for QIIME2-processed ITS2 forward read sequences. *Ulva* samples are denoted by "U", Inlets are denoted by (In), and outlets are denoted by (Ou).

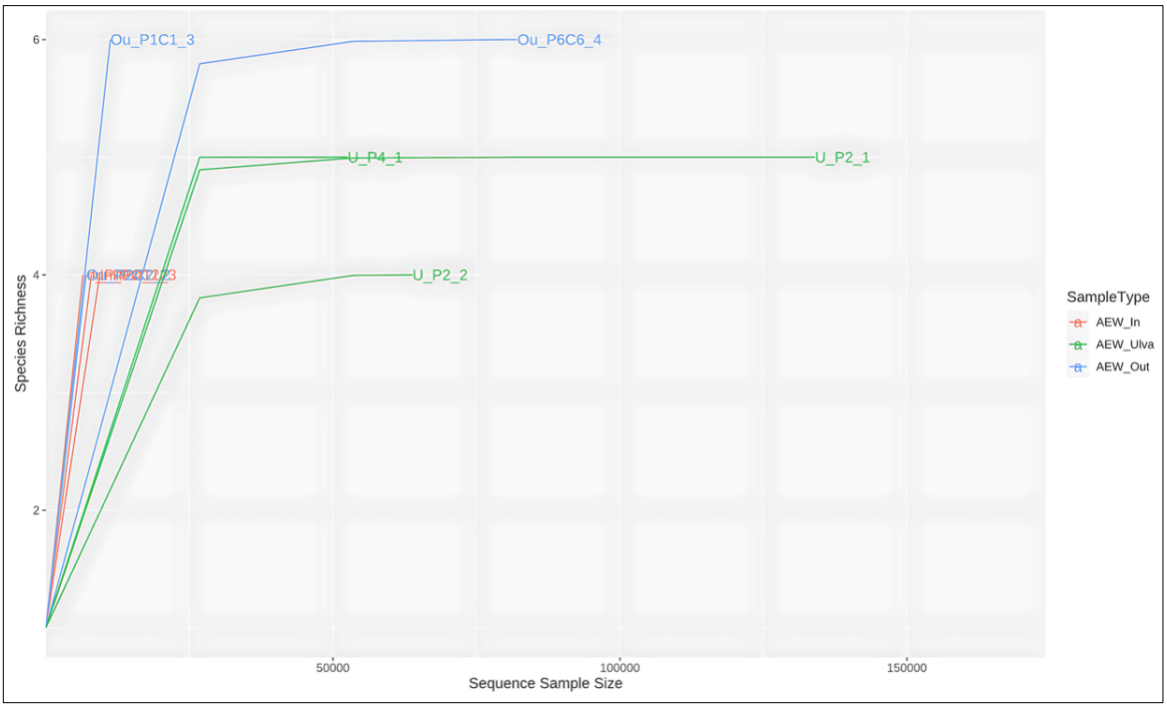

Figure 2.9. Rarefaction analysis across three groups of subset data samples for QIIME2 merged ITS2 read sequences. *Ulva* samples are denoted by "U", Inlets are denoted by (In), and outlets are denoted by (Ou).

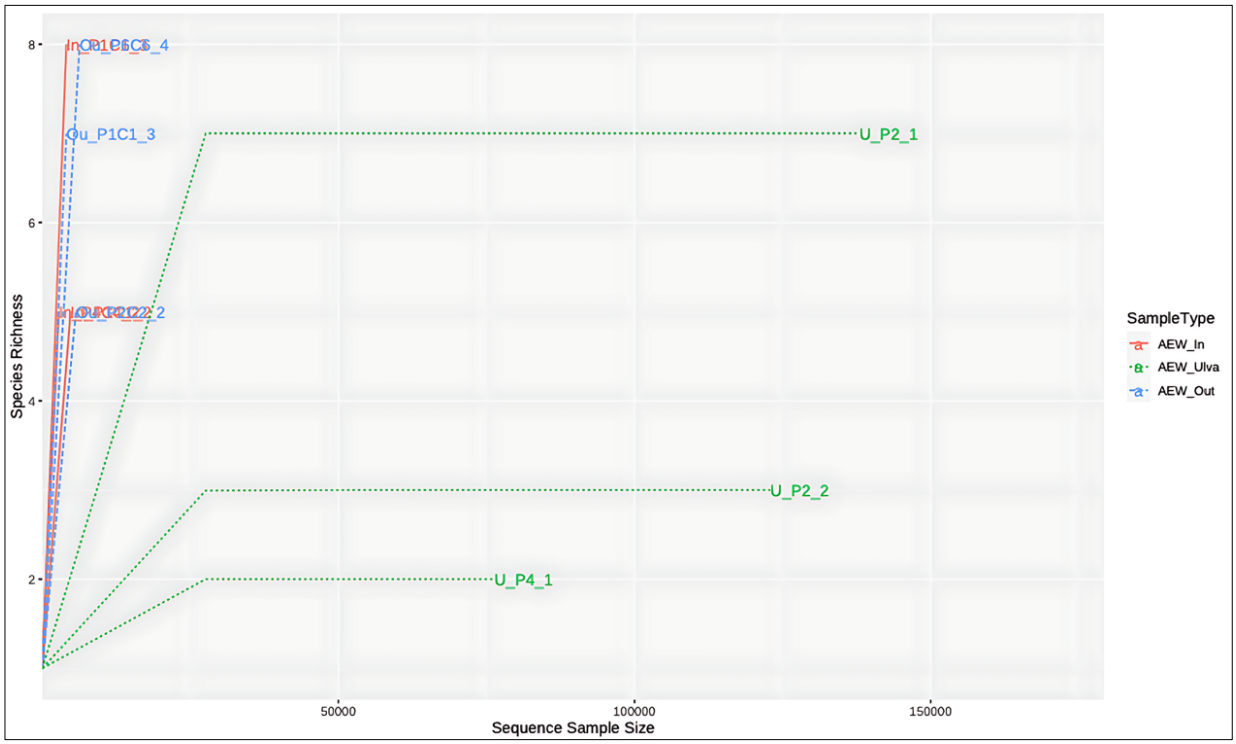

Figure 2.10. Rarefaction analysis across three groups of subset data samples for PIPITS/QIIME2 merged ITS2 read sequences. *Ulva* samples are denoted by "U", Inlets are denoted by (In), and outlets are denoted by (Ou).

Observed alpha diversity using the Chao1 richness estimator showed no significant difference between cohorts for forward reads ($p > 0.05$) (Fig. 2.11). Similarly, the alpha diversity of merged reads showed no significant difference between the cohorts ($p > 0.05$) (Fig. 2.11). The alpha diversity patterns between forward and merged reads were similar for QIIME2 (forward) and PIPITS (merged reads). The permutational multivariate analysis of variance (PERMANOVA) was performed using a Bray-Curtis dissimilarity matrix to evaluate differences between the bioinformatics pipelines. Samples were grouped according to their corresponding experimental groups (Fig. 2.12), regardless of which bioinformatic tool was used.

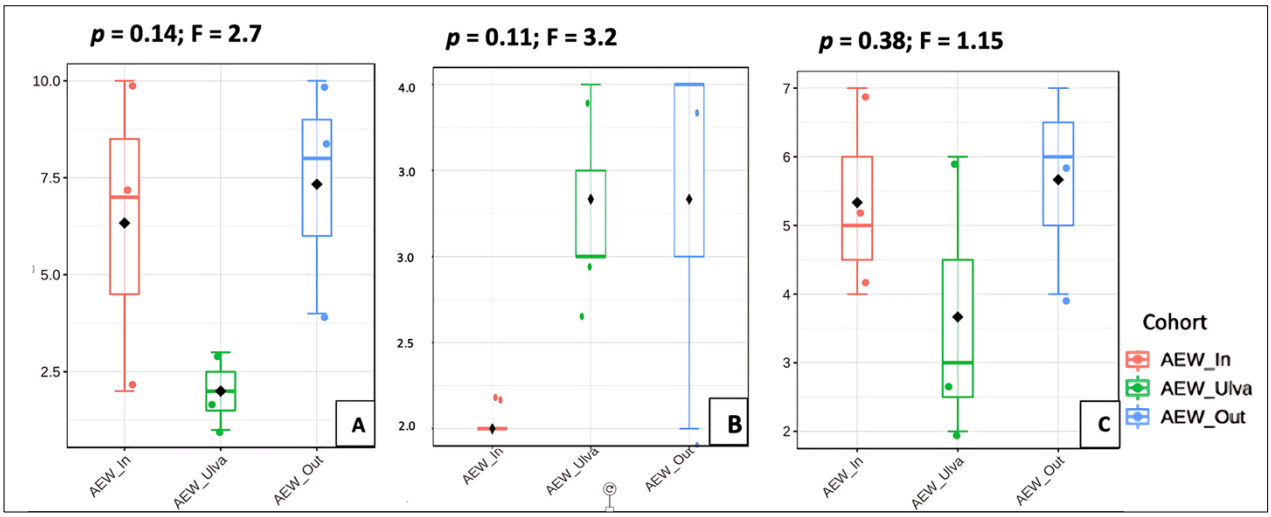

Figure 2.11. Average alpha diversity measures (Chao1) of QIIME2 forward reads (A), QIIME2 merged reads (B) and PIPITS merged reads (C) at genus level where the minimum, maximum and mean as well as ANOVA F-values and p-values are indicated for each cohort. Samples are from the AEW (IMTA) system. *Ulva* samples are denoted by "*Ulva*", inlets are denoted by (In), and outlets are denoted by (Out).

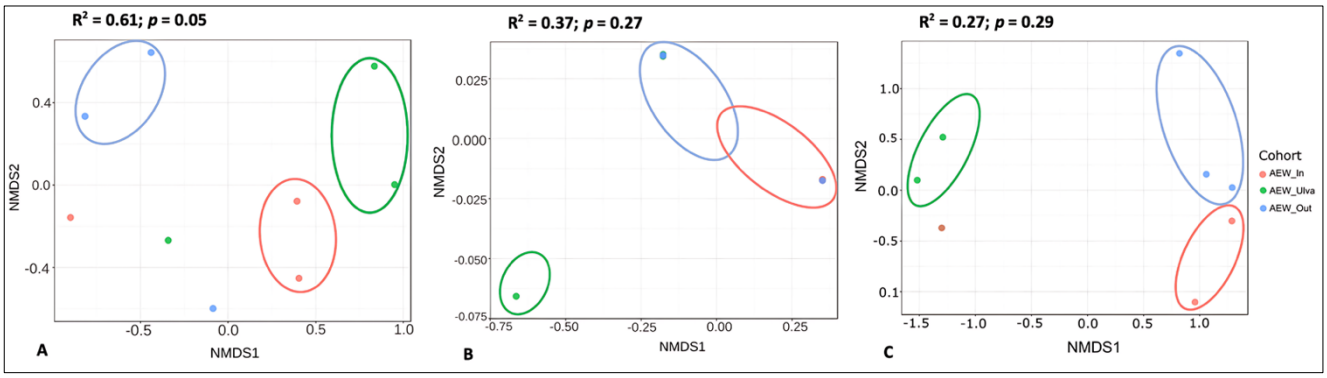

Figure 2.12. Non-metric multi-dimensional scaling analysis (NMDS) at genus level showing the difference in pipelines of QIIME2 forward reads (A) and QIIME2 merged reads (B) and PIPITS merged reads (C). Samples are from the AEW (IMTA) system. Ulva samples are denoted with "*Ulva*", inlets are denoted with (In), and outlets are denoted with (Out).

The pipelines showed variation in the identification and abundance of various genera, with the proportion of members belonging to the Pezizomycotina varying from 92% (Merged) to 99% (Forward) and the Ascomycota genus from 5% (Merged) to 0.02% (Forward) (Table 2.7). The QIIME2 merged reads at genus level were assigned to fungi (98%), but were largely unidentified, with some assignment to Chytridiomycota (1.5%) and Basidiomycota (0.19%).

Table 2.7. The five most abundant fungal genera detected by QIIME2 and PIPITS against UNITE reference database.

Forward (QIIME2)	**Merged (QIIME2)**	**Merged (QIIME2/PIPITS)**
Pezizomycotina genus (99%)	Fungi (98%)	Pezizomycotina (92%)
Oomycota genus (0.11%)	Chytridiomycota genus (1.5%)	Ascomycota genus (5%)
Byssochlamys genus (0.03%)	Basidiomycota (0.19%)	Chytridiomycota genus (2.5%)
Saccharomyces genus (0.02%)		Stemphylium (0.15%)
Ascomycota genus (0.02%)		Cladosporium genus (0.03%)

2.4 Discussion

The application of genomics-based research in the analysis of complex microbiomes has led to a better understanding of the composition and functioning of microbial communities in various areas such as human, animal, and plant health, infectious diseases, environmental pollution, agriculture, and food safety. Given that infectious agents inhabit numerous ecological environments, DNA must be extracted from various samples in a timely and cost-effective manner. Using the same standard operating procedure (SOP) to process various samples is advantageous. This study examined how different DNA extraction kits affect the quality of DNA for bacterial and fungal communities. It also compared common bioinformatics pipelines for the 16S rDNA gene (QIIME2 vs. mothur) and ITS2 (PIPITS vs. QIIME2) to determine how these pipelines can change taxonomic classification and the diversity analyses that follow.

Optimisation of genomic DNA extraction methods for the recovery of large quantities of (>10 ng/µL) high-quality genomic DNA is crucial for accurate and sensitive characterisation of taxa using next-generation sequencing (NGS). This is especially important for complex matrices such as environmental samples and plant tissues. Therefore, the first objective of this chapter was to identify optimal DNA extraction methods/kits for isolating total genomic DNA from seawater and *Ulva* samples collected from abalone – *Ulva* integrated systems. Four methods were assessed for DNA isolation from bacteria, including the Stratec RTP Bacteria kit, the Qiagen Fast Stool kit, the Qiagen QIAamp Micro kit, and the Qiagen Plant Mini kit (these four kits were chosen as they are frequently reported in the literature).

While the Qiagen Fast Stool kit had the highest concentration of DNA, the purity assessments showed that it did not meet the required standards, potentially due to the presence of polysaccharides and salts. This may inhibit further downstream analyses, such as PCR amplification, as previously reported (Michiels et al., 2003; Križman et al., 2006; Schrader, 2012). The Qiagen Fast Stool kit has been extensively employed in microbiome research, including the human microbiome project (Knudsen et al., 2016), because of its ability to isolate substantial amounts of DNA from a variety of sample types. However, the kit was specifically developed for extracting DNA from stool samples, which are known to contain a significant

quantity of microbial DNA as well as various other compounds that could inhibit downstream genomic applications. The reason(s) for the low DNA recoveries with the Qiagen QIAamp Micro kit are unknown; however, it may be a result of template DNA loss through degradation or trapping of nucleic acids in the filter paper during filtration. The Qiagen QIAamp Micro kit passed the A260/280 nm ratio, which is generally accepted as indicative of pure DNA (Olson & Morrow, 2012), and the A260/230 nm ratio indicated a low degree of contamination with polysaccharides and salts. Moreover, the Cq values obtained from the Qiagen QIAamp Micro kit also yielded low Cq values (Fig. 2.3), indicating a high recovery of *V. anguillarum* DNA.

In comparison to bacteria or mammalian cells, the process of extracting DNA from fungi is typically more challenging because of the inherent difficulty in breaking down the fungal cell wall using traditional methods of extraction used for bacteria or viruses. This is because fungi have a thick, complex cell wall, which results in poor DNA release. This study demonstrated that the Heat lysis method yielded significantly higher quantities of DNA than the other fungal DNA isolation techniques (as illustrated in figure 2.3). However, this method did not meet the quality and purity standards recommended by most NGS platforms. Conversely, the Qiagen QIAamp Micro kit and Qiagen Plant Mini kit were found to generate a lower yield of DNA than the Heat lysis method. Griffiths et al. (2006) conducted a study in which they employed a Qiagen kit for DNA extraction, employing a bead beating technique in conjunction with lysis buffer. Their findings indicate that this method resulted in a higher yield of DNA than alternative approaches. Van Burik et al. (1998) also demonstrated that DNA could be more effectively extracted from filamentous fungi when glass beads were vortexed for long periods of time. Moreover, a study by Nawrot et al. (2010), in which five commercial kits were compared for the extraction of DNA from *Aspergillus fumigatus* spores, demonstrated that the highest DNA yield was obtained using the QIAamp DNA Mini kit with mechanical cell disruption. In the current study, the Qiagen QIAamp Micro kit alone yielded DNA that was amplifiable and met the recommended standards, as evidenced by the A260/A280 and A260/230 ratios, which indicated purity and a low level of protein contamination. However, the addition of bead-beating with the Qiagen QIAamp Micro kit might yield higher DNA and should therefore be considered for future studies. Although no single method was found to be universally effective in addressing all challenges, the Qiagen QIAamp Micro kit was selected

for use in the remainder of the study because it yielded sufficient quantities of DNA, exhibited high quality in terms of DNA purity and stability, and was able to adequately extract bacterial and fungal DNA spiked into matrices representative of the samples to be tested in the remainder of the study. The criteria for DNA extraction methods have previously included DNA yield, quality, and success of PCR amplification, as demonstrated by Yu & Morrison (2004) and Zhang (2006). A combination of the aforementioned criteria suggests that the Qiagen QIAamp Micro kit is the optimal approach for DNA extraction from microorganisms associated with seawater and seaweed. However, it is important to note the DNA extraction process for *Ulva* was carried out directly from the frozen *Ulva* tissue. Consequently, the resulting DNA includes *Ulva* DNA, together with microbial DNA from intracellular and surface microorganisms associated with the sampled *Ulva.*

Primer selection, such as DNA extraction kits, is a crucial element to consider in the variation and detection of microbial communities. The ITS2 region exhibits significant variability at the species level, as reported by Nilsson et al. (2009). Therefore, the primers chosen for amplifying this region must enable the identification and analysis of various fungal as well as oomycete species that one would expect to find in the samples to be tested, as suggested by Amend et al. (2010) and Jumpponen & Jones (2010). In the context of ecological and microbiological research, DNA barcoding of the ITS2 region using ITS3_KYO2 and ITS4_KYO3 primers and sequencers with read lengths less than 700 bp would be sufficient for sequencing microbiological studies. The present study aimed to evaluate the suitability of DNA barcoding of the ITS2 region using ITS3_KYO2 and ITS4_KYO3 primers for the identification of fungi and oomycetes in marine environments. This approach has been previously demonstrated to be informative in the context of fungal microbiome studies (Toju et al., 2012). The results of the PCR demonstrate that the primers are suitable for cataloguing and identifying oomycetes under standard PCR conditions, as illustrated in figure 2.5.

For the assessment of different bioinformatics tools to process raw sequences in microbiome studies, a subset of samples was used to compare 16S rDNA and ITS2 gene sequence data using the analysis pipelines QIIME2, mothur, and PIPITS. Samples collected from the abalone effluent water system at the Buffeljags abalone farm were used in this analysis. The

emergence of 16S rRNA-based classification has vastly improved our understanding of bacterial taxonomy and diversity in various samples and environments. It is now commonly used for bacterial identification due to its accuracy and speed (Reller et al., 2007; Srinivasan et al., 2015). The 16S rRNA gene, approximately 1.5 kb in length, is a valuable molecular target as it is found in all bacteria, either as a single copy or in multiple copies, as it is highly conserved over time and within a species (Reller et al., 2007; Sabat et al., 2017). Although 16S rRNA gene sequencing is a valuable tool for understanding bacterial communities, it has some limitations. Due to high sequence similarities between some bacteria, this method may not always identify bacteria to the species level (Deurenberg et al., 2017). The generation of chimeras (artifact sequences formed by two or more biological sequences incorrectly joined together) and the inherent error rate of sequencing are important factors to consider. Moreover, sequencing 16S rRNA often involves one (or more, depending on cost) of the gene's variable regions (Yang et al., 2016). Short sequencing reads (about 300 – 500 bases) from 16S rRNA may not be suitable for bacterial species resolution. While short reads from NGS platforms are considered accurate, studies have demonstrated that long-read sequencing (10,000 – 15,000 bases) produces better taxonomic classification at the genus and species levels (Nygaard et al., 2020; Pearman et al., 2020). To address some of the aforementioned technical limitations, algorithms that are used to identify and remove chimera sequences (e.g., DADA, UCHIME) as well as sequencing errors (e.g., denoising) have been developed (Jo et al., 2016). In addition, taxonomic assignment is dependent on reference databases, and the results may differ depending on the choice of reference database. Once amplified, 16S rRNA gene sequences are curated and can be accessed in publicly available databases, such as Greengenes (DeSantis et al., 2006) and SILVA (Quast et al., 2013), which can then be accessed by other researchers. Using 16S rDNA gene sequencing technologies, bacteria colonising macroalgae and abalone have been effectively identified in previous studies (Burke et al., 2009; Lachnit et al., 2009). In the current study, the SILVA database was shown to perform better than the Greengenes database because the Greengenes database predicted fewer genera than the SILVA database and was therefore chosen as the reference database for the remainder of this study. One of the reasons for the poor performance of the Greengenes database may be the fact that it has not been updated

since 2013, meaning that it is missing many of the novel bacterial sequences that have been discovered since 2013.

This chapter showed that little difference exists between the QIIME2 and mothur pipelines when analysing 16S rRNA sequence data with respect to taxonomic classifications, as shown in Table 2.4. Similar abundance patterns of specific bacterial genera were observed between the two pipelines. The most abundant genus was *Vibrio*, with 11% and 10.5% relative abundance recorded for QIIME2 and mothur, respectively, followed by members of the genus *Pseudoalteromonas* with 8% and 6% relative abundance, respectively. In addition, QIIME2 assigned more ASVs to identity (16.28%) than mothur. The primary difference between mothur and QIIME2 is in their methods for OTU clustering and taxonomic classification. For ASVs, QIIME2 uses a denoising-based approach to ASV assignment, using algorithms as implemented in the DADA2 plugin to resolve sequences down to the level of single-nucleotide differences across the sequenced gene region, whereas for OTU clustering in mothur, sequences with less than 3% variance from each other are binned into the same OTU (Marizzoni et al., 2020). The denoising approach of QIIME2 offers a more accurate and reproducible representation of microbial diversity compared to the clustering approach of mothur, which can obscure subtle sequence variations due to its reliance on similarity thresholds. Especially with the increased accuracy of sequencing technology, researchers are now favouring ASVs over OTUs. For the analysis of fungi, differences were observed with regard to the number of unclassified sequences identified by the two pipelines (Table 2.6), where forward reads from QIIME2 exhibited a lower number of unclassified reads than merged reads and PIPITS/QIIME2 (merged reads). Moreover, differences were observed in the taxonomic classification and relative abundance of ASVs between the two pipelines (Table 2.6). Members of the Pezizomycotina genus dominated the taxa identified by the QIIME2 (forward reads) and QIIME2/PIPITS (merged) pipelines, with 99% and 92% abundance, respectively, whereas QIIME2 (when using merged reads) was dominated by ASVs assigned to fungi (98%) at the genus level. Differences were observed between the pipelines in terms of the amount of time taken to analyse samples and ease of use; however, this could be reduced by the use of a high-performance computing (HPC) cluster, which was only used for the analyses conducted in QIIME2. Based on computational time, user friendliness, number

of reads assigned to taxonomy, and number of unclassified reads, QIIME2 was used for all subsequent data processing in this study (Chapter 3). Overall, the results produced by QIIME and mothur are comparable and when analysing seawater samples, a reliable, high-level overview of sample composition is likely to be produced using either of the pipelines for the analysis of 16S rRNA gene sequencing data.

The application of next-generation sequencing in previous studies on fungal metabarcoding has resulted in the development of several bioinformatic pipelines (White et al., 2013; Bálint et al., 2014; Gweon et al., 2015). Nevertheless, no standard method exists for analysing fungal sequencing data, which has resulted in many sequences still being classified as uncultured fungi. For this reason, two fungal pipelines were evaluated to determine how much each affected taxonomic classification. PIPITS represents command-line-based platforms and offers a limited number of tools, but data analysis is easily performed with a straightforward pipeline. In particular, PIPITS uses the ITSx tool (Bengtsson-Palme et al., 2013) to improve the accuracy of ITS-based analysis of fungi and other eukaryotes by removing conservative flanking genes for precise clustering. While QIIME2 incorporates DADA2's accurate quality filtering method, ITSxpress was used in the current study to extract ITS subregions for more accurate species identification (Bengtsson-Palme et al., 2013). Differences were observed with regard to the number of unclassified sequences identified by the two pipelines (Table 2.6), where forward reads from QIIME2 exhibited a lower number of unclassified reads than merged reads (QIIME2) and PIPITS/QIIME2 (merged reads). Moreover, differences were observed in the taxonomic classification and relative abundance of ASVs between the two pipelines (Table 2.6). Members of the Pezizomycotina genus dominated the taxa identified by the QIIME2 (forward reads) and QIIME2/PIPITS (merged) pipelines, with 99% and 92% abundance, respectively, whereas QIIME2 (when using merged reads) was dominated by ASVs assigned to fungi (98%) at the genus level. Higher reads in QIIME2 (forward reads) were preferred because less information was lost. Fewer ASVs were identified in total for merged reads, and fewer ASVs may indicate that QIIME2/PIPITS (merged reads) are missing variants or exclude reads that could not be merged. Based on these results, QIIME2 and the Qiagen QIAamp DNA Micro kit arguably showed the best overall performance, combining high sensitivity with excellent specificity, and were both used for subsequent analyses.

2.5 Conclusion

This chapter evaluated the efficiency of extracting high-quality DNA from five established DNA extraction methods without any modifications. An important conclusion of this study is that the Qiagen QIAamp DNA Micro kit extraction method was more effective than the other isolation methods tested in this study for both bacterial and fungal isolation. Proper handling of seawater samples and reproducible and reliable DNA extraction methods are crucial for marine microbiome studies. Furthermore, moderate differences in sensitivity and specificity were observed between different bioinformatic pipelines. QIIME2 demonstrated superior sensitivity and resolution compared with PIPITS and mothur for the analysis of ITS and 16S rRNA, respectively. The key differences were in the number of reads retained and ASVs detected. However, on a broad scale, datasets analysed using different combinations of techniques showed comparable high abundances of ASVs and taxonomic classifications. Microbiome datasets are very challenging to verify, especially for poorly characterised marine fungi. Bacterial and fungal analyses in subsequent chapters (Chapter 3 and Chapter 4) were conducted using QIIME2 to assess the bacterial and fungal community dynamics in an abalone-*Ulva* IMTA system.

Chapter 3: Bacterial communities associated with recirculating abalone-*Ulva* aquaculture systems

3.1 Introduction

Aquaculture systems are complex ecosystems that house various bacterial populations. These bacteria play an important role in the health and functionality of these systems, as they are closely linked with productivity and nutrient cycling, as well as the nutritional status and health of the animals cultured within these systems (Cornejo-Granados et al., 2017). Several water quality parameters, such as phosphate, nitrate, nitrite, ammonia, and dissolved oxygen (DO) concentrations, are influenced by microbial processes and can also influence the composition of the microbiota in the water (Crab et al., 2007). Dissolved and particulate organic loads, particularly from self-pollution by inorganic nutrients, fish faeces, and uneaten feed, can cause a significant increase in bacterial levels (Butterworth, 2010), but can also cause problems in the surrounding environment where high nutrient effluent may stimulate eutrophication.

In comparison to traditional monoculture systems, integrated multi-trophic aquaculture (IMTA) systems have a higher diversity of cultured species (Chopin et al., 2012). In IMTA, organisms occupying different trophic levels use the same limited physical space or occupy neighbouring areas, and one or more species' co-products (organic and inorganic nutrients) become resources for a lower farmed trophic level. The cultivation of more high-value species within the system is frequently assumed to improve the economic viability of the IMTA process as well as its societal acceptability (Chopin, 2010). In IMTA systems, there is often an exchange between water bodies (and the nutrients that come with them) via water movement or artificial current systems (inland basins or ponds). Seaweeds are often integrated into marine aquaculture systems to take up nitrogen in the form of ammonium, produce oxygen, and remove carbon dioxide through photosynthesis. *Ulva* spp. have been successfully grown in integrated systems in South Africa since 2002, where *Ulva* is grown in abalone effluent water and sometimes fed back to the abalone as supplementary feed, thus recycling nutrients received from feed and excreted by the animals (Bolton et al., 2009, 2016).

The bioremediation capacity of certain seaweeds, such as *Ulva* spp., means that the use of seaweeds in abalone culture could allow for the recirculation of water, reduce pumping costs, and reduce the impact of an abalone farm on the surrounding environment by bioremediating farm effluent water (Bolton et al. 2016). Additionally, seaweeds have been shown to not only reduce bacterial levels in integrated systems but can also modulate and/or reduce levels of known pathogenic organisms such as *Escherichia coli, Pseudomonas aeruginosa, Vibrio alginolyticus, Aeromonas hydrophila*, and *Staphylococcus aureus* (Lean et al., 2015; Charoensiddhi et al., 2017), while other seaweeds have been shown to improve the immune response of certain shellfish and finfish species (Setyawan et al., 2018; Cantelli et al., 2019; Thépot et al., 2021). However, many abalone farmers in South Africa are still reluctant to adopt this technology (IMTA) because there could be potential biosecurity risks associated with the use of effluent-grown *Ulva* as a supplementary feed or feed additive, as well as risks associated with growing more than one species and recirculating water within the land-based systems. This has prevented the wider adoption of IMTA, a more sustainable farming practice, and has highlighted the need for an improved understanding of the microorganisms associated with integrated abalone – *Ulva* systems with recirculation.

Land-based aquaculture practices often include highly engineered recirculating aquaculture systems (RAS). These systems are constructed to optimise water use, often achieving a 90%–99% reduction in water consumption compared to more conventional methods (Ebeling & Timmons, 2012). However, if not managed carefully, recirculating water can result in decreased water quality, primarily through the accumulation of dissolved and particulate organic waste and uneaten feed (Olsen & Olsen, 2008). Recirculating aquaculture systems, often used in fish farming, rely on microorganisms to maintain healthy water quality, nutrient cycling, animal welfare, and disease control (Rurangwa & Verdegem, 2015a). However, many daily operations on aquaculture farms (e.g., stocking, cleaning, feeding) may affect the microorganisms community composition as well as cause stress in the farmed animals and can create a favourable environment for opportunistic pathogens. Most of these concerns are also applicable to IMTA systems.

The vast majority of bacterial pathogens found on aquaculture farms are ubiquitous in marine systems and threaten the well-being of many aquaculture species (Sudheesh et al., 2012; Sanches-Fernandes et al., 2022; Irshath et al., 2023). In particular, bacteria belonging to the *Vibrio* genus are major obstacles for fish and molluscan culture, with several *Vibrio* species being identified as causal agents for mass mortality events in abalones, as well as other aquaculture establishments. For instance, in Taiwan (Liu et al., 2000) and China (Cai et al., 2007), *V. parahaemolyticus* caused large stock losses of *Haliotis diversicolor supertexta*. Infections of *V*. *carchariae*/*harveyi* have been observed in *Haliotis tuberculata* along the French coast (Nicolas et al., 2002) and in *H. discus hannai* in Japan (Sawabe et al., 2007), whereas *V. splendidus* was isolated from moribund *H. rubra* and *H. laevigata* during disease outbreaks in Australia (Handlinger et al., 2005). Furthermore, *V. splendidus* has been linked with pustulate disease in juvenile abalone, which is characterised by small areas of tissue necrosis affecting the *H. iris* aquaculture industry (Bingham, 2012; Georgiades et al., 2016). In New Zealand, bacterial infections are similarly associated with poor health in commercial culture facilities (Diggles et al., 2002; Diggles & Oliver, 2005), and stress factors such as high temperatures, grading trauma, use of anesthetics, and altered salinity typically exacerbate such diseases.

Microbial communities are also important constituents of aquatic ecosystems. In aquaculture production, they are crucial for nutrient recycling, degradation of organic matter, and control of diseases (Zeng et al., 2017). Specific microbes can be used to purposely steer microbial colonisation to improve system performance or animal health (Rurangwa & Verdegem, 2015b). One method of preventing pathogen colonisation is to use probiotic bacteria that compete for nutrients, space, produce growth inhibitors, or quench cell communication (quorum sensing) and inhibit the formation of biofilms by unwanted bacteria (Defoirdt et al., 2007; 2008; Kesarcodi-Watson et al., 2008). In South Africa, Macey and Coyne (2005) demonstrated that the bacterium *Vibrio midae* SY9 improves the nutrition and health of small (20 mm shell length) and large (67 mm shell length) *H. midae* and showed that this bacterium can effectively colonise the digestive tract of abalone. A study by Erasmus et al. (1997) also showed that enteric bacteria of the South African abalone *H. midae* could enhance the growth

rate of the host (Macey & Coyne, 2005), through the production of polysaccharolytic enzymes that aid in the digestion and assimilation of complex polysaccharides found in seaweeds commonly fed to abalone on farms. The potential of probiotics to increase abalone aquaculture production has been demonstrated in various studies. Host – microbiome interactions influence metabolism and contribute to the host's ability to adapt to changing environments (Woodhams et al., 2020). It has been demonstrated that growth rates, digestive health, carbohydrate assimilation, and disease resistance of farmed abalone are all significantly improved by bacteria found on ingested macroalgae and within the abalone's digestive tract (Erasmus et al., 1997; Macey & Coyne, 2005; 2006; Daume, 2006). For instance, Hadi et al. (2014) demonstrated that feeding juvenile *H. iris* (20 – 30 mm) with probiotic-supplemented diets significantly enhanced growth and decreased mortality.

Host – microbiome interactions play a critical role in abalone health and physiology (Danckert et al., 2021), as well as in macroalgal development. In *Ulva* spp., algal growth- and morphogenesis-promoting factors are produced by the host-microbiome complex (Califano et al., 2020). The host – microbiome complex constitutes microorganisms that reside within and on the surfaces of hosts and have an impact on their evolutionary, immunological, and ecological processes (Woodhams et al., 2020b). Bacteria are not only essential for the development of *Ulva* but also for the settlement of its zoospores (Patel et al., 2003; Tait et al., 2005; Joint et al., 2007). In some cases, the growth, cell differentiation, and morphogenesis of *Ulva* are influenced by the microbial community, which is specific to different species (Lachnit et al., 2009), but in other cases, *Ulva* belonging to the same species (*Ulva australis*) showed differences in their microbial profiles when collected from sampling sites very close to one another (Burke et al., 2011a). This observation prompted the latter authors to propose a "competitive lottery model" to explain the process of bacterial community assembly on macroalgal surfaces (Burke, et al., 2011b; Ghaderiardakani et al., 2017). The competitive lottery model suggests that communities are formed randomly die to their surrounding environment (Chesson & Warner, 1981). In contrast, another theory known as the hologenome theory suggests that there is a core community of bacteria that has remained

stable over time and functions as a driving force for the evolution of the host (Zilber-Rosenberg & Rosenberg, 2008).

Currently, the understanding of microbial community dynamics within IMTA systems is scarce, particularly for abalone – *Ulva* IMTA systems. This presents an obstacle for pro-active system management and wider adoption of IMTA on abalone farms in South Africa because of the perceived biosecurity concerns associated with this practice. In the past, culture-dependent methods were the method of choice for studying bacterial diversity in environmental systems. Although useful, this approach is time-consuming and provides limited information due to the selectivity of nutrient media and culture conditions, which favours the growth of only a fraction of the microbes that are present in the community (normally less than 2%) (Al-Awadhi et al., 2013). Therefore, the primary drawback of this technique is that it grossly underestimates the quantity and diversity of microorganisms in the samples being studied. In contrast, culture-independent approaches, such as high-throughput next-generation sequencing (NGS) of the bacterial 16S hypervariable ribosomal RNA (rRNA) gene, have made it possible to study complex ecosystems, such as aquaculture environments, using an "all-encompassing systems biology approach," resulting in an understanding of the microbial composition of complex systems that can also be used to predict the putative functional roles of the microbial taxa (Bentzon-Tilia et al., 2016). This chapter aims to improve understanding of the benefits and potential disease risks associated with bacteria in integrated multi-trophic aquaculture (IMTA) systems by characterising the bacterial microbiome of the effluent water in an abalone-*Ulva* system with 50% water recirculation and comparing it to a non-integrated system (non-IMTA) that does not recirculate seawater or receive effluent. This chapter assesses the stability of microbial communities spatially (across different system compartments) and temporally (across seasons) to evaluate the impact of co-cultivated *Ulva* spp. on microbial dynamics. It is hypothesised that the IMTA system will have a more diverse bacterial microbiome than the non-IMTA system, and that the presence of *Ulva* spp. will favourably influence microbial dynamics, reducing potentially harmful bacteria and improving the system microbiome overall.

3.2 Materials and Methods

3.2.1 Site and system description

Water (N = 40) and *Ulva* (N = 20) samples were collected from Buffeljags Abalone Farm, which is a subsidiary of Viking Aquaculture (https://www.vikingaquaculture.co.za/abalone/farming/) and is situated near the remote settlement of Buffeljags in the Bredasdorp district on the Cape South Coast of South Africa (34.7550° S, 19.6154° E) (Fig. 3.1A). Both *Ulva* and water samples were collected from two separate *Ulva* production system types on the farm across four seasons: autumn (March 2020), winter (August 2020), spring (October 2020), and summer (January 2021). One system consisted of *Ulva* raceway tanks that received seawater directly from adjacent coastline, hereafter referred to as the seawater (SW) or non-IMTA system, whereas the other systems were comprised of *Ulva* raceways receiving abalone effluent-water, hereafter referred to as the abalone effluent-water (AEW) or IMTA systems. The *Ulva* samples were collected from within each *Ulva* paddle-raceway system, whereas the water samples were collected from the effluent water flowing from the abalone tanks back into the *Ulva* raceway, henceforth referred to as the inlet, and the water flowing out of the *Ulva* paddle-raceways back into the abalone tanks, henceforth referred to as the outlet of each system. The seawater *Ulva* raceway system (Fig. 3.1B) is a non-IMTA system that provides seawater to the adjacent abalone hatchery and nursery on Buffeljags Abalone Farm. The raceway receives fresh seawater that is pumped directly from the ocean in front of the farm. The paddle-raceway contains mesh grids secured to a wooden frame that is attached to the tank, at the end of the D-ended raceway where water leaves the system, to prevent *Ulva* washout (Fig. 3.1B). The tank is occasionally drained for cleaning, and then restocked with *Ulva* that was removed from the tank. *Ulva* grown in this system is also used to feed juvenile abalone in the nursery. There is only one SW raceway on Buffeljags Abalone Farm.

The abalone effluent water raceways (Fig. 3.1C) are IMTA systems that contain *Ulva* that is used as a supplementary feed for abalone and for the bioremediation of effluent water to allow for 50% water recirculation on the farm. The farm currently has seven modular abalone-*Ulva* IMTA systems, called platforms, which are each composed of four clusters that each

consist of one *Ulva* paddle-raceway and several abalone tanks (six rows each made of seven abalone raceway tanks) (Fig. 3.1C; D). The *Ulva* raceways of platforms 1, 2, 4, and 6 (Fig. 3.1D) were sampled at Buffeljags to assess variation across raceways (spatial variation) and across seasons over the course of one year (temporal variation). All clusters on the farm are isolated from each other, and each cluster has its own sump (Vol. ca. 25 000 L) and D-ended *Ulva* paddle-raceway (ca. 300 000 L). Effluent water from the abalone tanks in each cluster flows into the *Ulva* paddle-raceway, and ca. 50% of this bioremediated water is then recirculated back to the abalone tanks after mixing in the sump with 50% fresh seawater, pumped directly from the sea in front of the farm. Water flow from the sump to the abalone tanks and each row of abalone raceways has a single effluent pipe, which discharges into the adjacent *Ulva* raceway. The *Ulva* raceway therefore receives water from all the abalone tanks in the cluster and discharges approximately half of this volume as bioremediated effluent to the sea.

Figure 3.1.(A) Map depicting the location of the Buffeljags abalone farm along the Cape south coast of South Africa. **(B)** The seawater *Ulva* paddle-raceway system providing water to the hatchery and nursery at Buffeljags abalone farm. **(C)** *Ulva* raceways, each linked with one abalone effluent water cluster at Buffeljags abalone farm, receiving effluent water from the adjacent abalone raceways. **(D)** Aerial photograph of Buffeljags abalone farm showing the general outlay of the farm with the seven modular abalone-*Ulva* IMTA systems, called platforms, which are each composed of four clusters that each consist of one *Ulva* paddle-raceway and several abalone tanks (6 rows each made of 7 abalone raceway tanks).

3.2.2 Collection of water and *Ulva* samples

This study examined the spatio-temporal variation of the microbiome in the abalone-*Ulva* systems at Buffeljags abalone Farm by sampling *Ulva* and water from multiple raceways during spring, summer, autumn, and winter between March 2020 and January 2021. At each sampling time, water samples were collected to measure the physiochemical parameters of water within the systems. Four nutrient uptake experiments were conducted on water samples collected from inlet, outlet and centre of the systems at the farm to provide preliminary quantification of the biofiltering capacity of *Ulva* at the site. Total ammonium nitrogen (NH_4^+), soluble reactive phosphate (PO_4^{3-}) and nitrite (NO_2^-) were measured in triplicate according to the manual methods described by Grasshoff et al. (1983) and nitrate (NO_3^-) following the cadmium reduction method of Nydahl (1976).

Healthy vegetative *Ulva lacinulata* thalli (approximately 15 g) were collected from the SW (N = 4) and AEW (N = 16) systems, one sample per season from each *Ulva* raceway, and immediately transferred into separate pre-labelled sterile 50 mL conical centrifuge tubes. Only healthy *Ulva* blades with no necrotic white tissue were used for microbiological analysis. The freshly collected *Ulva* was rinsed with 10 mL of autoclaved seawater (ASW) via inversion for 20 seconds to remove any loosely attached organisms and debris. Thereafter, the rinsed samples were placed in a portable fridge set to 4˚C and transferred within 3 hours to the Department of Forestry, Fisheries, and the Environment (DFFE) Marine Research Aquarium (MRA) in Cape Town, South Africa (33°92′05′′S; 18°38′11′′E), where they were processed further. At the research facility, a portion of the rinsed *Ulva* (approximately 20 mg) was used to quantify culturable bacteria (details provided below). The remaining *Ulva* was ground to a fine powder using a mortar and pestle with liquid nitrogen, and the ground samples were stored at -80°C until needed for DNA extraction. Inlet and outlet water samples (1000 mL) were collected in triplicate from the SW and AEW systems in sterile 1000 mL Schott bottles and immediately refrigerated at 4°C. Samples were transferred to the MRA in Cape Town, as described above. At the MRA, water samples were processed for the enumeration of culturable bacteria and NGS, as described below.

3.2.3 Enumeration of culturable bacteria in seawater and *Ulva*

A culture-based approach was implemented by utilising a standard plate-counting technique to quantify changes in the number of total culturable bacteria (TCB), total *Vibrio* species (TV), and *Ulva*-specific bacteria (UB) growing on the *Ulva* and in the seawater from the SW and AEW systems at Buffeljags Abalone Farm. Three selective media were used for the culture-based approach: Ulvan Agar (UA; Jaulneau et al., 2010), Tryptic Soy Agar (TSA; Difco), and Thiosulfate Citrate Bile Sucrose Agar (TCBS; Sigma Aldrich) (as described in Chapter 2). Ulvan agar is a minimal medium supplemented with Ulvan, the primary carbohydrate of *Ulva*. The Ulvan served as the primary carbon source of the media, facilitating the isolation and evaluation of *Ulva*-specific bacteria. Tryptic Soy Agar is regarded as a general media and is routinely used for the isolation of marine bacteria, providing an indication of the total culturable bacteria within the community (Lemos et al., 1985; Romanenko et al., 2008). Lastly, TCBS medium is routinely used for the selective isolation and cultivation of several enteropathogenic *Vibrio* species (Kobayashi et al., 1963), providing an overview of *Vibrio* dynamics within the systems.

3.2.3.1 Enumeration of bacteria in seawater

For the isolation and enumeration of culturable bacteria from seawater, the 1000 mL water samples collected from the SW and AEW systems (both inlet and outlet) were aseptically filtered through 0.22 µm-pore-size filter membranes (47 mm diameter, Millipore Corp., Bedford, Mass.). Thereafter, to loosen the bacterial cells, each membrane filter and its accompanying cells were transferred into separate sterile 15 mL centrifuge tubes containing 10 mL of sterile 1× phosphate-buffered saline (PBS) (7.3 mM monosodium phosphate, 180 mM disodium phosphate, 0.15 M sodium chloride, pH 7.2) supplemented with 0.1% Tween 20 and 0.05% Antifoam A. Preliminary experiments indicated that supplementing PBS with Antifoam A and Tween 20 dramatically improved the recovery of bacteria and did not interfere with any downstream applications, such as cultivation and polymerase chain reaction (PCR). Tubes were vortexed for 5 mins to remove any bacteria attached to the membrane before making tenfold serial dilutions in sterile autoclaved seawater. An aliquot (100 µL) of each dilution (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) was plated in triplicate onto TSA, TCBS, and UA plates.

Immediately following plating, petri dishes were sealed with Parafilm, and the TSA and TCBS plates were incubated at 21°C for two days, whereas UA plates were incubated at 21°C for up to 7 days (accounting for slow growth) and monitored daily for growth. Bacterial colonies were counted using a dissecting microscope and recorded for each plate following the respective incubation periods. The remaining 9.9 mL of ASW-containing bacteria (from each sample) was centrifuged for 10 mins at 11,000 × g to concentrate the bacterial cells. Following centrifugation, the supernatants were carefully discarded, and the tubes containing the cell pellet were stored at -80°C until required for DNA extractions and NGS.

3.2.3.2 Enumeration of bacteria on *Ulva*

Bacteria were isolated from fresh vegetative thalli of *Ulva lacinulata* using a modified version of the method described by Nakanishi et al. (1996). Briefly, algal thalli were gently rinsed by inversion for 20 sec in autoclaved seawater (ASW) to detach any loosely associated debris, after which approximately 0.5 g of thallus was vortexed vigorously in 10 mL of ASW for 10 mins to detach the bacteria associated with *Ulva*. The thallus was removed, and the remaining supernatant was tenfold serially diluted in sterile autoclaved seawater. An aliquot (100 µL) of each dilution (10^0, 10^1, 10^{-2}, 10^{-3}, and 10^{-4}) was plated onto TSA, TCBS, and UA plates in triplicate. Plates were incubated and enumerated as described above in Section 3.2.

3.2.3.3 Statistical analyses for colony forming unit (CFU) data

SigmaPlot 12.0 (Systat Software, San Jose, CA, V, 2011) was used to plot bar graphs, and JASP software (Version 0.16.4; JASP Team, 2022) was used to perform all statistical analysis. All data were tested for normality (Shapiro-Wilk test) and equal variance (Levene's test). An independent sample t-test was performed to assess whether there were statistically significant ($p < 0.05$) differences between the number of culturable bacteria between the inlet (water entering the *Ulva* raceway) and outlet (water leaving the *Ulva* raceway) water within and between the AEW/IMTA and SW/non-IMTA systems. A t-test was also performed to assess if the number of culturable bacteria differed on *Ulva* (CFU.g^{-1} of *Ulva*) obtained from the IMTA and non-IMTA systems. A one-way analysis of variance (ANOVA) was performed to assess whether there were statistically significant ($p < 0.05$) difference in the number of culturable

bacteria between seasonal samples (AEW (N=8), SW (N=2) and *Ulva* (N=5)). Tukey's honest significant difference (HSD) post hoc test was used to test the differences among means for significance between samples. An independent t-test was conducted to determine whether bacterial numbers were significantly different in the system receiving effluent water from abalone raceways (IMTA) when compared with the *Ulva* raceway receiving seawater (non-IMTA). Data values on all figures refer to the means ± standard error (SE) of the mean.

3.2.4 Microbiome of *Ulva* and seawater

3.2.4.1 DNA isolation

Microbial genomic DNA was isolated from the frozen ground *Ulva* samples and bacterial pellets obtained from the seawater samples (described above in Section 3.2.3.2) using the QIAamp® DNA Micro kit (Qiagen, Cat. No. 56304) following the manufacturer's instructions. The entire bacterial pellet obtained from each SW (N = 8) and AEW sample (N = 32) was used for genomic DNA isolation, whereas approximately 5 mg of each frozen ground *Ulva* sample collected from the SW (N = 4) and AEW (N = 16) systems at Buffeljags abalone farm was used for DNA extraction. DNA extractions were performed in duplicate, and a negative control, containing only Millipore water, was included to account for possible contamination during the extraction process and/or from the columns of the extraction kit. Post-DNA extraction, DNA concentration and integrity were determined using a NanoDrop spectrophotometer (Genova Nano, Jenway, Bibby Scientific Ltd., UK). The DNA quality was assessed spectrophotometrically by determining the A260/A280 ratio and the A260/A230 ratio, with a ratio of 1.8 indicative of DNA that is pure and free of protein contaminants or organic compounds (Lucena-Aguilar et al., 2016). DNA integrity was further assessed following 0.8% agarose at 80 V for 45 min gel electrophoresis to ensure the presence of high molecular weight DNA in all the samples.

3.2.4.2 16S PCR amplification

To characterise the bacterial microbiome of the *Ulva* and seawater samples, the V4 hypervariable region of the 16S ribosomal rRNA gene (ca. 250 bp) was amplified using the forward primer 515F and reverse primer 806R (Caporaso et al., 2011), with an added Illumina

adapter overhang nucleotide sequence (Table 3.1). Polymerase chain reactions were performed by the Centre of Proteomics and Genomics Research (CPGR) in Cape Town (South Africa). Each 25 μL polymerase chain reaction (PCR) consisted of 2.5 μL of 5 ng/μL genomic DNA, 12.5 μL 2 × KAPA HiFi HotStart ReadyMix (Roche; Cat# 07958935001), 0.5 μL of each primer, and 9 μL ddH_2O. The 'touchdown' PCR protocol described by Maapea et al. (2021) was utilised for the amplification of the V4 hypervariable region of the 16S ribosomal rRNA gene as it was shown to improve PCR specificity and avoid the occurrence of satellite bands that were evident following the first round of PCR when using the standard Illumina protocol. PCR amplification cycling conditions consisted of an initial denaturation at 95°C for 5 mins, followed by 10 cycles of touchdown PCR (30 sec at 95°C, 30 sec at 65°C, with a 1°C per cycle decrement, and 30 sec at 72°C), followed by an additional 25 cycles of PCR (30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C), and a final extension step for 10 min at 72°C. Negative controls, containing all components excluding DNA templates, were run in parallel to ensure that the reagents were not contaminated and that there was only amplification of bacterial genomic DNA across the samples included in this study. The ZymoBIOMICS Microbial Community Standard, consisting of equal concentrations of eight bacterial and two fungal stains (Zymo Research, Cat # D6300), was included as a positive control. Amplified PCR products, including the negative and positive controls, were electrophoresed on 0.8% (w/v) Tris-Acetate-EDTA (TAE) agarose gels to confirm successful amplification, reaction specificity, and fragment size (~250 bp fragment expected). PCR products (1μL of each) were further analysed on an Agilent 4200 TapeStation System using D1000 Reagents (Agilent; Cat# 5067-5583) and D1000 ScreenTape (Agilent, Cat# 5067-5582) to verify amplicon sizes.

Table 3.1. Primers used for amplification and sequencing of the V4 hypervariable region of the 16S rRNA gene with added Illumina adapter overhang nucleotide sequence bolded portion and with underlined sequence being the locus specific V4 primers.

Primer	Oligonucleotide sequence (5'- 3')	Targeted region	References
Forward, *515F*	**GTGYCAGCMGCCGCGGTAA** TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	V4 region (~250 bp)	Caporaso et al., 2011
Reverse; *806R*	**GGACTACNVGGGTWTCTAAT** GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG		

3.2.4.3 Library preparation and next-generation sequencing

Indexing, library preparation, and Illumina next-generation sequencing (NGS) of the 16S rRNA genes were performed at CPGR. Briefly, PCR products were purified using AMPure XP beads (Beckman Coulter; Cat # A63881; Lot 17970000) as per the manufacturer's instructions, and the V4 amplicons were eluted in 52.5 µL of 10 mM Tris, pH 8.0. Thereafter, dual indices and Illumina sequencing adaptors were attached to the purified PCR products using the Nextera XT index Kit v2 (Illumina; Cat # 15052163; Lot 20400443) and the KAPA HiFi HotStart ReadyMix. A second PCR purification step was carried out to purify the indexed sequencing libraries using AMPure XP beads. After purification of the individual libraries, the concentration of dsDNA in each library was quantified using the fluorometric Qubit 1 × dsDNA HS Assay (Thermo Fisher Scientific; Cat# Q33231). Library sizes were verified using the D1000 reagents and ScreenTape on the 4200 TapeStation System. The DNA concentration in each of the V4 libraries was normalised to 20 ng/µL, and 100 ng of each V4 library was pooled for sequencing. The concentration of amplifiable Illumina adapter-ligated molecules in the final library pools was confirmed by qPCR using the KAPA Illumina Library Quantification Kit (Roche; Cat# 07960204001). A library dilution series of 1:10 000, 1:100 000, 1:1000 000 and 1:10 000 000 of each library pool was quantified. The overall fragment sizes of the pooled V4 libraries were determined using the D1000 Assay on the Agilent 4200 TapeStation system. The size adjusted-library concentrations were calculated, and the libraries were diluted to 4 nM. The 4 nM pooled sequencing libraries were denatured using 0.2 M NaOH and diluted to 5 pM. The libraries were combined with the denatured phage PhiX internal sequencing control (Illumina, Cat# 15017666) at a spike-in concentration of 10% v/v, as advised by the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Illumina, 2013). The sequencing libraries were heat denatured at 95°C for 2 mins and placed on ice for 5 mins. The denatured libraries were loaded on the Illumina MiSeq instrument and sequenced using the Illumina MiSeq Reagent Kit v2 (500 cycles), consisting of the buffer cartridge (Illumina; Cat # 15033625) and the flow cell and incorporation buffer (Illumina; Cat# 15033626). The sequencer was programmed to perform a paired-end, dual-indexed 2 × 250 cycle sequencing run for each library pool. FASTQ files were automatically generated at the end of each run

and saved on the MiSeq onboard computer. The quality of the sequence run was analysed using Illumina Sequence Analysis Viewer (Version 2.4.5).

3.2.4.4 Raw data processing

The open-source software Quantitative Insights into Microbial Ecology 2 (QIIME2) (version 2022.2; *http://qiime2.org/*) (Caporaso et al., 2020) was used to import demultiplexed paired-end FASTQ files using the Casava input format. Analysis of sequence data was carried out using the Divisive Amplicon Denoising Algorithm 2 (DADA2) QIIME2 plug-in (Callahan et al., 2016). DADA2 infers an Illumina sequencing error profile to resolve true sequences from noise and quantifies the number of each amplified sequence variant (ASV). Forward reads were trimmed to a maximum read length of 232 bp, and reverse reads were trimmed to a maximum length of 228 bp (phred score < 20). The remaining reads were denoised, merged, screened for chimeric sequences, which were subsequently removed, and assigned as distinct ASVs using DADA2. Taxonomic classification of ASVs was carried out using the SILVA reference taxonomy v132 (Quast et al., 2013). Known contaminants, including mitochondrial and chloroplast sequences, were removed.

3.2.5 Bacterial microbiome data analysis

3.2.5.1 Data filtering and normalisation

A rarefaction curve analysis was used to indicate whether coverage was sufficient to capture the bacterial diversity present within each sample. Data was filtered in MicrobiomeAnalyst (Dhariwal et l., 2017; Chong et al., 2020) for low-count reads (at a default of 20% prevalence across all samples), where features (ASVs) containing only or mostly zeros were removed to account for possible sequencing errors. A filtered dataset was used to calculate alpha (within sample) diversity statistics and to estimate overall ASV abundance. Uneven sequencing depth, under-sampling, and data sparsity were all corrected for using the relative log expression (RLE) transformation (Hawinkel, 2015) for non-zero cell counts as follows:

$$\frac{c_{ij}}{(\prod_{j=1}^{m} c_{ij})^{1/m}}$$

where a mean (m) across samples is used as a pseudo-reference sample. The median of this measure across all ASVs is used as a scaling factor (j) for each sample, where each ASV is denoted as (i). This method scales the raw read counts in each sample through a sample-specific factor based on the median and mean of the number of reads for each sample. The normalised dataset was used for multivariate beta diversity (between samples) tests and univariate differential abundance analysis. Low variance reads (at a default variance of 10% based on standard deviation) were removed to reduce the effect of multiple testing in the differential abundance analysis.

3.2.5.2 Alpha diversity

The within sample (alpha) bacterial number (i.e., the mean diversity of species in different sites or habitats within a local scale) was determined in MicrobiomeAnalyst using the R *Phyloseq* (McMurdie & Holmes, 2013) and *Vegan* packages (Dixon, 2003). Several alpha diversity measures were calculated, including the Chao1 (Chao, 1984), Shannon (Shannon, 1948), and Simpson (Simpson, 1949) indices to assess species richness (number of different taxa present), evenness (uniformity of the abundance of each of the taxa present), and uniqueness, respectively. The statistical significance of cohort-wise differences for each alpha diversity measure (Choa1, Shannon, and Simpson) was assessed using an ANOVA ($p < 0.05$) at family and genus level. Moreover, to further analyse seasonal samples, the systems were plotted separately for an in-depth analysis of diversity and abundance, more specifically to assess the difference between inlet and outlet. However, only the AEW system was plotted, as this was not possible for the SW system due to sample number limitations.

3.2.5.3 Beta diversity

Beta (between-sample) diversity was also assessed in MicrobiomeAnalyst using the same R packages previously listed. Non-metric multidimensional scaling (NMDS) analysis using a Bray-Curtis dissimilarity matrix was used to plot the sample dissimilarity. The hierarchical relationship between samples was assessed through a sample-based clustering analysis based on a Bray-Curtis distance matrix and Ward clustering algorithm and was visualised through dendrograms. Corresponding statistical significance ($p < 0.05$) was evaluated using a

permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001), a permutational analysis of multivariate dispersions (PERMDISP; Anderson, 2006), and an analysis of similarities (ANOSIM; Clarke, 1993).

3.2.5.4 Differential abundance

Overall ASV abundance was assessed by creating bar graphs at genus level based on relative (%) abundances (N < 10 counts are merged and denoted as "others") in MicrobiomeAnalyst. The samples were collapsed into their respective cohorts based on the systems (AEW and SW) they were collected from and the components of the systems (inlet, outlet, and *Ulva*) across different seasons. The effect size was calculated using linear discriminant (LefSe) analysis to test for significant associations between cohorts and bacterial communities, and the 25 ASVs that most likely (Kruskal-Walls rank sum test; significance: $p \leq 0.05$; LDA score > 2) explain differences between the cohorts were identified. Following this, the significant ASVs ($p \leq 0.05$) were retained, and these were used in a linear discriminant analysis to assess the effect size of the significant ASVs. The univariate method, DESeq2 (Love et al. 2014), was used to identify differentially abundant ASVs at the genus and species level across cohorts. Using the normalised dataset, the mean, variance, and mean dispersion estimates for each ASV were calculated to identify ASVs with means that exceeded the threshold calculated for that ASV (statistical significance: $p < 0.05$). (Hawinkel, 2015). Furthermore, the false discovery rate (FDR) was calculated to adjust *p*-values for multiple comparisons to reduce the possibility of type I errors (false positives) (Benjamini & Hochberg 1995). Boxplots were constructed for the top 20 differentially abundant ASVs at the genus and species level.

3.2.4.5 Functional profiling

The putative metabolic functions of bacteria were assessed using the Tax4Fun package (Aßhauer et al., 2015) with the representative sequence ASV abundance table, which transforms the SILVA-based ASVs into KEGG gene orthologs (KO) counts across all samples. The resultant KO table was input into the Shotgun Data Profiling (SDP) using KEGG (Kyoto Encyclopedia of Genes and Genomes) (Lu et al., 2023). To improve the accuracy and reliability of the KEGG pathways, MicrobiomeAnalyst was used to remove extremely low abundance

and variant KOs (based on < 4 KO filtration) from each sample (Dhariwal et l., 2017; Chong et al., 2020). The inferred metagenomic functions were assigned using the Kyoto Encyclopedia of Genes and Genomes database (KEGG). The associations between functional categories and the experimental factors (sample type) were tested using the global test algorithm (Goeman et al., 2004), which is a robust test to identify whether particular gene sets (i.e., KEGG pathways) are significantly associated with the phenotype shifts on the basis of their abundance profiles.

3.3 Results

3.3.1 Water parameters

The average seasonal measurements of physiochemical parameters in the abalone effluent water including temperature, nitrogen compounds (NO_2^-, NO^-_3, NH_4^+, $PO4^{3-}$) are presented on average (+SEM) for the AEW system and SW systems in table 3.2 and 3.3, respectively. The water temperature ranged from 14.69°C (±0.24 °C) in winter to 17.7°C (± 0.38 °C) in summer. The pH levels ranged from 7.74 (± 0.04) in winter to 8.3 (± 0.17) in summer. Nitrite levels were higher during winter 1.27 mg/L (± 0.22) and autumn 1.23 mg/L (± 0.33) compared to other seasons. Ammonium appeared higher during winter and summer, 10.51 mg/L (± 2.51) and 8.69 mg (± 2.45), respectively, and lower in spring (3.36 mg/L ± 0.9) whereas nitrate levels were highest in autumn 18.99 mg/L (± 2.80) and winter 13.52 mg/L (± 2.65) and lowest in summer -1.77 mg/L (± 0.7). The phosphate levels were slightly higher in summer, and spring compared to other seasons, ranging from 2.82 mg/L (± 0.93) in winter to 4.59 mg/L (± 0.59) in summer. Overall, the nitrogen compounds and phosphate were lower in the SW system, compared to the AEW system. ANOVA analysis revealed statistically significant differences across seasons for all environmental parameters, except for nitrite and ammonium in the SW system and ammonium and phosphate in the AEW system.

Table 3.2. Seasonal average variations in physicochemical parameters (temperature and pH), nitrogen compounds (nitrate—NO3, nitrite— (nitrate—NO^-_3, nitrite—NO^-_2, ammonium—NH_4^+, and phosphate $PO4^{3-}$) in the abalone effluent water (AEW) aquaculture farming systems. Values represent the average (±SEM) of all seasonal measurements from the four aquaculture systems.

Seasons	Temp (°C)	NO_2^- (mg/L)	NO^-_3 (mg/L)	NH_4^+ (mg/L)	$PO4^{3-}$ (mg/L)
Summer	17.6 ± 0.38	0.25 ± 0.06	-1.77 ± 0.7	8.69 ± 2.45	4.59 ± 0.59
Autumn	17.05 ± 0.48	1.23 ± 0.33	18.99 ± 2.80	6.23 ± 2.71	2.84 ± 0.61
Winter	14.69 ± 0.24	1.27 ± 0.22	13.52 ± 2.65	10.51 ± 2.51	2.82 ± 0.93
Spring	15.94 ± 0.36	0.69 ± 0.1	13.49 ± 3.2	3.36 ± 0.9	3.27 ± 0.4
ANOVA	$p<0.05$	$p<0.05$	$p<0.05$	$p>0.05$	$p>0.05$

Table 3.3. Seasonal average variations in physicochemical parameters (temperature and pH), nitrogen compounds (nitrate—NO3, nitrite— (nitrate—NO^-_3, nitrite—NO^-_2, ammonium—NH_4^+, and phosphate $PO4^{3-}$) in the seawater (SW) aquaculture farming systems. Values represent the average (±SEM) of all seasonal measurements from the four aquaculture systems

Seasons	Temp (°C)	NO_2^- (mg/L)	NO^-_3 (mg/L)	NH_4^+ (mg/L)	$PO4^{3-}$ (mg/L)
Summer	16.3 ± 0.01	0.083 ± 0.28	-1.86 ± 0.3	0.78 ± 2.9	1.73 ± 0.38
Autumn	12.8 ± 0.03	0.27 ± 0.04	16.78 ± 0.65	0.95 ± 0.21	0.72 ± 0.35
Winter	15.6 ± 0.33	0.23 ± 0.14	4.74 ± 0.39	-0.26 ± 0.2	0.39 ± 0.2
Spring	14.5 ± 0.23	0.22 ± 0.2	4.77 ± 0.2	0.19 ± 0.22	0.91 ± 0.1
ANOVA	$p<0.05$	$p>0.05$	$p<0.05$	$p>0.05$	$p<0.05$

3.3.2 Enumeration of culturable bacteria in seawater and on *Ulva*

Systems receiving effluent water from abalone raceways (IMTA) had higher bacterial counts than seawater (non-IMTA) *Ulva* raceways receiving seawater directly from the surrounding coastline (p = 0.02) (Fig. 3.2). Across both system types, there was a decline in the total number of general marine bacteria from the inlet to the outlet, as indicated by the growth on TSA plates, with the mean of the inlet group from the IMTA system (M = $3.311x10^5$, SD = 113364.850) significantly different from that of the outlet (M = $1.173x10^5$, SD = 49096.110) (p < 0.001). A greater reduction in marine bacteria between the inlet and outlet of water samples was observed in the effluent water system (IMTA) when compared to the seawater system (non-IMTA) (Fig. 3.2A). In both systems, the macroalga *Ulva* had a strong inhibitory effect on the total number of *Vibrio* species within the water column, as indicated by the significant

reduction in bacteria growing on TCBS from the inlet to the outlet ($p < 0.02$) (Fig. 3.2B), more specifically during summer and spring, indicating a modulatory effect by *Ulva*, while winter and autumn exhibited the opposite pattern. Moreover, the Ulvan-specific bacteria exhibited a similar trend to that of TCBS, where there was a decrease in the number of bacteria from the inlet to the outlet of both systems during the summer and spring seasons. The bacterial species cultivated on TSA had a higher abundance when compared to both TCBS and Ulvan in the inlet and outlet water samples of effluent water systems. Seasonally, a high abundance of Ulvan-specific bacteria and *Vibrio*'s growing on TCBS in both systems was observed in the spring ($p < 0.05$), which decreased over the course of the summer. There was also a shift observed from the inlet to the outlet of Ulvan-specific bacteria and *Vibrio*'s during spring and summer. In autumn, a high abundance of general marine bacteria was observed in the non-IMTA system ($p < 0.001$). The IMTA system exhibited a high abundance of TSA-specific bacteria across all seasons, which decreased from the inlet to the outlet (Fig. 3.2).

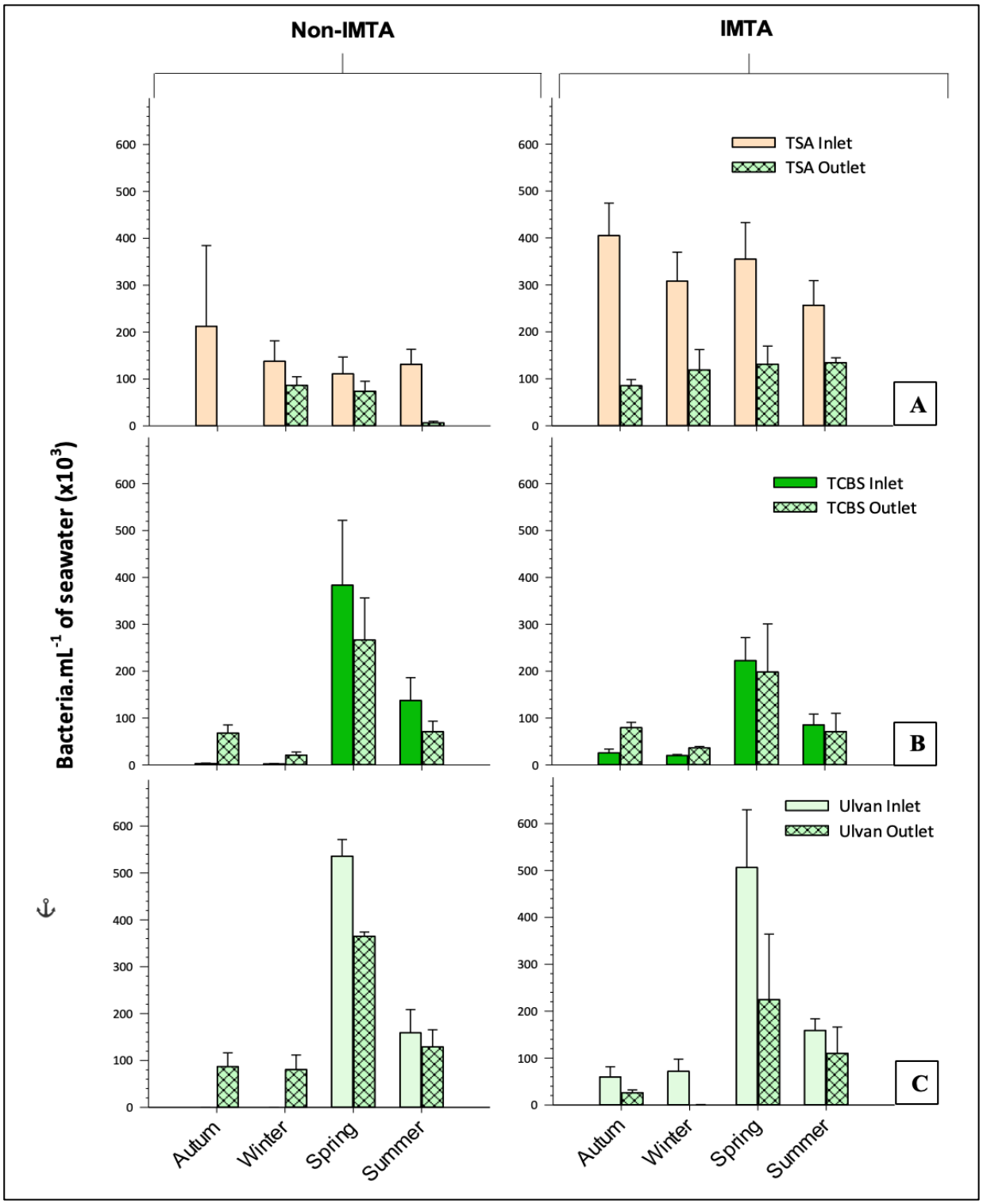

Figure 3.2. Average number of culturable bacteria in IMTA (AEW) and non-IMTA (SW) seawater sampled across seasons growing on three selective media (N = 10 per season); Tryptone soy agar (TSA) (A), Thiosulfate Citrate Bile Sucrose Agar (TCBS) (B) and Ulvan (C).

On all three types of selective media, the bacterial abundance of the *Ulva* cultured in the effluent system was greater than that of the *Ulva* cultured in seawater system (non-IMTA) (Fig. 3.3). During autumn and winter, the IMTA *Ulva* had a high bacterial abundance when compared to spring and summer. This pattern is opposite to the one observed for the water samples.

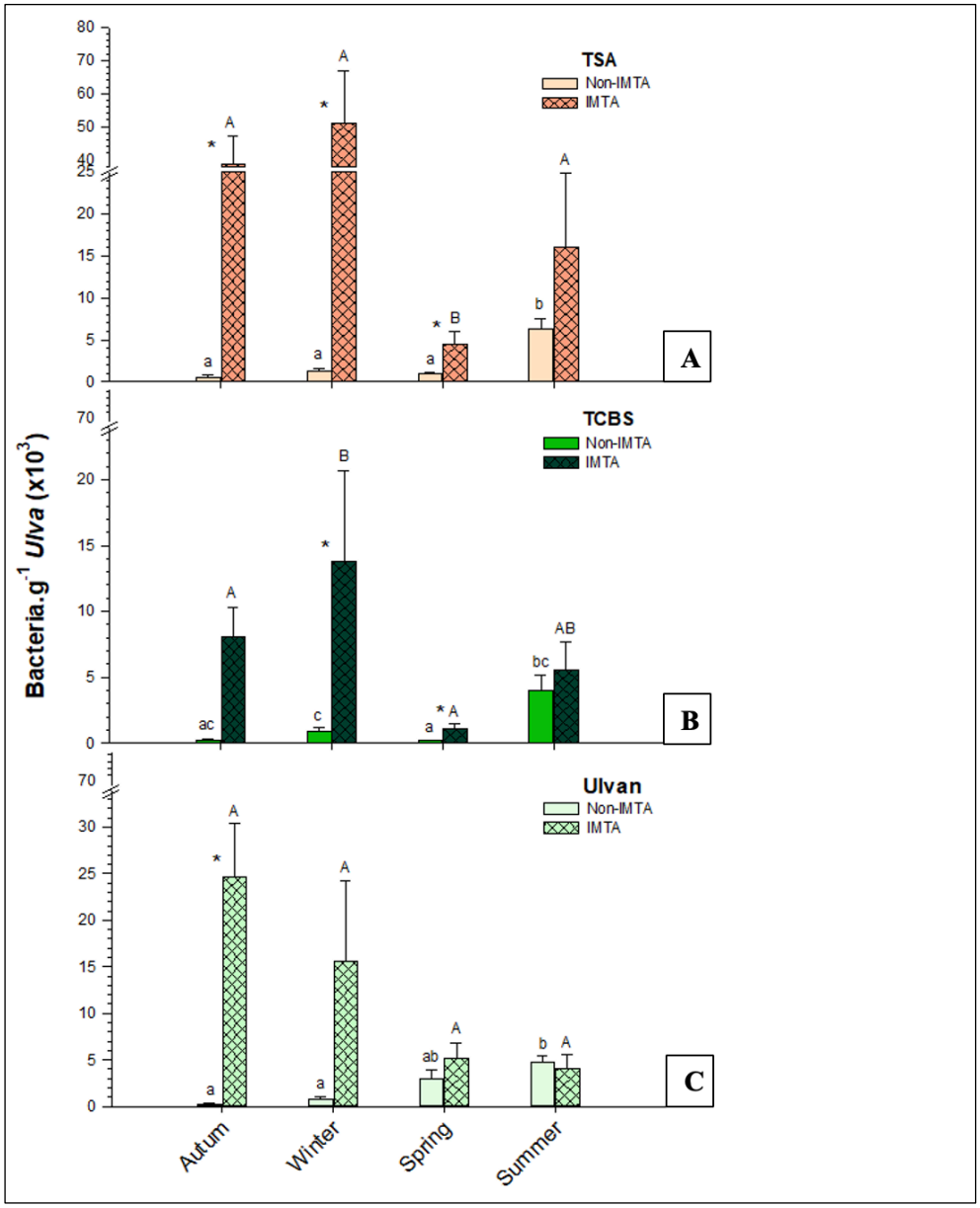

Figure 3.3. Average number of culturable bacteria on *Ulva* sampled across IMTA (N = 16) and non-IMTA (N = 4) systems seasons growing on three selective media; Tryptone Soy Agar (TSA (A)), Thiosulfate Citrate Bile Sucrose Agar (TCBS) (B) and Ulvan (C). Significant difference is marked with an asterisk (Significance was assigned to *p*-values less than 0.05 for one-way ANOVA).

3.3.3 Raw next-generation sequencing data processing

After filtering and quality trimming (Table S3.2), a total of 15 917 078 raw sequence reads were generated across the 60 metagenomic libraries created in this study (Fig. S3.1). The obtained library size was compared at the genus level to check data integrity, where a large difference between the maximum and minimum library size was noted (377 to 683407). The sample with the lowest library size (Ou_H_2; 377 reads) was excluded from downstream analysis, as all other samples had library sizes ranging from 69254 to 683407 reads. After reads were mapped to the SILVA database, a total of 2822 ASVs were detected. Subsequent filtering in MicrobiomeAnalyst resulted in a total of 1154 low abundance features being removed (based on prevalence) prior to conducting alpha and beta diversity analyses, resulting in the total removal of 1154 features. Sequences that could not be assigned to any taxonomic groups were assigned as unclassified.

3.3.3.1 Alpha diversity

Rarefaction curves were constructed across cohorts based on the number of ASVs observed (richness) to assess how exhaustively bacterial communities were sampled. All rarefaction curves approached the saturation plateau, except for one sample from the SW system (U_H_4), indicating that there were sufficient sequence reads (sampling depth) for most samples analysed in this study (Fig. 3.4). The rarefaction curves revealed a large variation in the total number of sequences between samples, which ranged from 300 to 650 ASVs per sample after data filtering.

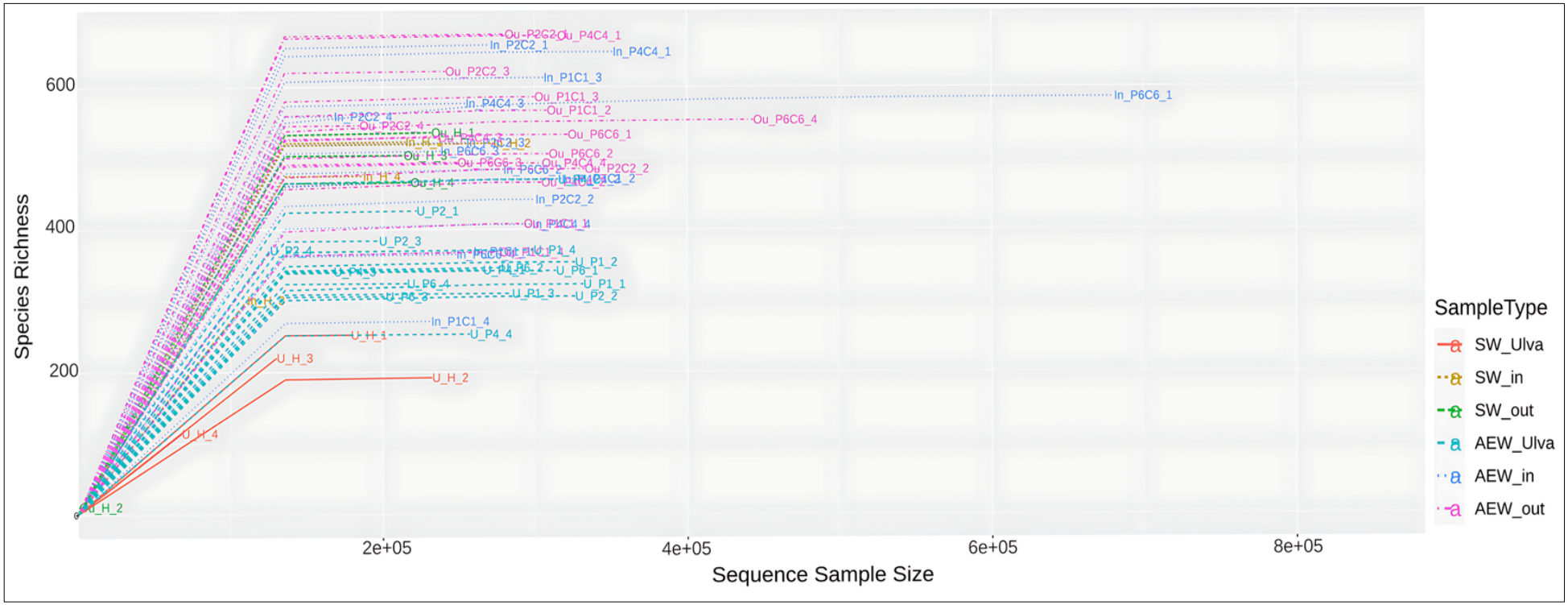

Figure 3.4. Rarefaction curves of filtered ASVs, where samples were grouped into AEW_Out, AEW_In, SW_*Ulva*, AEW_*Ulva*, SW_In, and SW_Out cohorts (N = 60). Samples denoted by "H" are from the SW (non-IMTA), while samples denoted by "P" are from the AEW (IMTA) system. *Ulva* samples are denoted by "U", Inlets are denoted by (In), and outlets are denoted by (Ou).

Within the sample, diversity was assessed across six cohorts within the two-production system types: AEW_In, AEW_Out, AEW_*Ulva* (IMTA) and SW_In, SW_Out, SW_*Ulva* (non-IMTA) (Fig. 3.5). Bacterial diversity at the genus and family level varied across the seawater and *Ulva* samples collected from abalone effluent water (AEW) and seawater (SW) systems. Significant differences were observed between the SW and AEW cohorts for the Simpson diversity index at the genus level (ANOVA; $F = 2.91$; *p*-value = 0.04), indicating a higher degree of uniformity in the bacteria present in the SW_In and SW_Out cohorts compared to the AEW_In and AEW_Out cohorts (Fig. 3.5). Conversely, the Shannon diversity indices obtained from ANOVA showed no statistical difference across the six cohorts ($F = 1.74$; *p*-value = 0.14), indicating similarity in the community richness of the bacterial communities across cohorts. (Fig. 3.5). Nevertheless, the Chao1 richness estimator exhibited a statistically significant increase in the AEW cohorts (In + Out) compared to the SW cohorts (In + Out), as well as a higher richness in the water cohorts (both SW and AEW) compared to the *Ulva* cohorts (AEW_*Ulva* and SW_*Ulva*) ($F = 35.15$, $p < 0.01$). This suggests a greater presence of distinct ASVs in the water columns, particularly in the AEW (Fig. 3.5).

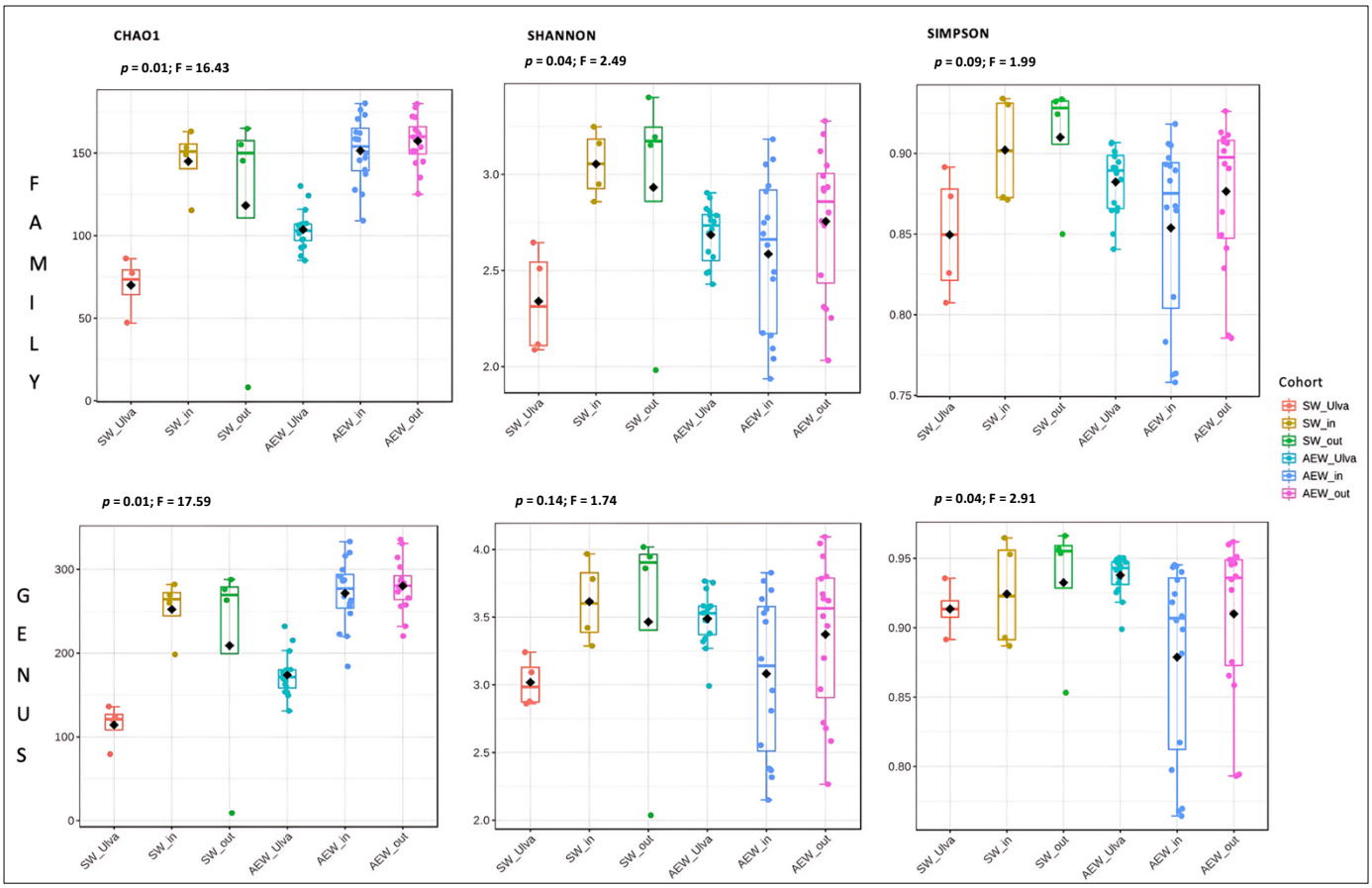

Figure 3.5. Average alpha diversity measures (Chao1, Shannon, and Simpson) for each cohort at family- and genus- level, where the minimum, maximum and mean as well as ANOVA F-values and *p*-values are indicated for each cohort. Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted with "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

3.3.2.2 Seasonal alpha diversity

Bacterial alpha diversity at family- and genus- level was further assessed across seasons and from seawater and *Ulva* samples collected from abalone effluent water (AEW) and seawater (SW) systems. In the AEW, the Chao1 richness estimator (Fig. 3.6) of spring and summer-associated bacterial communities was significantly higher at family and genus level (Fig. 3.6; ANOVA; $p < 0.05$) compared to the other sampling periods, indicating a higher degree of unique ASVs in summer and spring. Among all sampling periods, winter (SW) had the lowest Chao1 richness at family and genus level, while summer (AEW) and spring (AEW) had the highest Chao1 richness. Both autumn (SW) and spring (SW) samples appeared to be similar in the Chao1 richness of microbial communities. Moreover, the Simpson diversity index showed no significant differences across all seasons ($p = 0.19$) at family level, while spring (SW) and

autumn (SW) had the highest Shannon diversity, indicating a high degree of richness during the spring (SW) and autumn (SW) seasons (Fig 3.6). Furthermore, all the *Ulva* samples from AEW and SW systems at family and genus level across seasons had the lowest Chao1 richness, indicating a low degree of unique ASVs. Overall, there was a large amount of temporal variation, i.e., summer and spring from AEW samples had the highest degree of unique ASVs, while spring and autumn from SW samples had the highest microbial diversity. Furthermore, there were also differences observed between the inlet and outlet during autumn in the AEW system, where the outlet had a higher degree of unique ASVs than the inlet (Fig. 3.7; $p = 0.04$). Additionally, the Shannon and Simpson diversity indices were statistically higher in the outlets than in the inlets, indicating a higher microbial diversity in the outlets compared to the inlets across the seasons (Fig. 3.7).

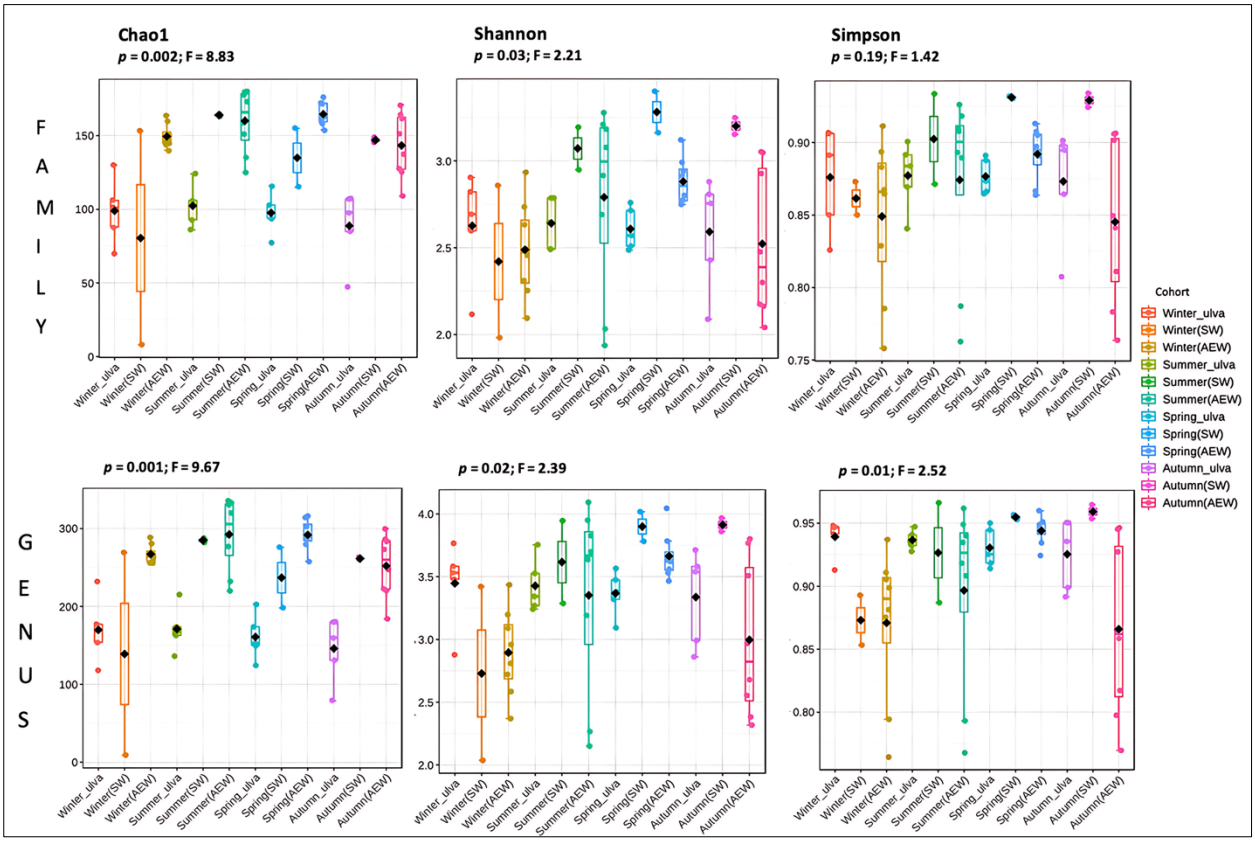

Figure 3.6. Average alpha diversity measures (Chao1, Shannon, and Simpson) for each cohort at family- and genus- level, where the minimum, maximum and mean as well as ANOVA F-values and *p*-values are indicated for each cohort. Samples for each season consisted of grouped inlets and outlet of the respective systems i.e., Winter (SW (In + Out)). *Ulva* samples of the respective systems were grouped together according to sampled season i.e., Winter_*Ulva* consisted of AEW_*Ulva* + SW_*Ulva* samples. Samples for each season AEW system (N=8), samples for each season SW system (N = 2), *Ulva* samples per season (N = 5).

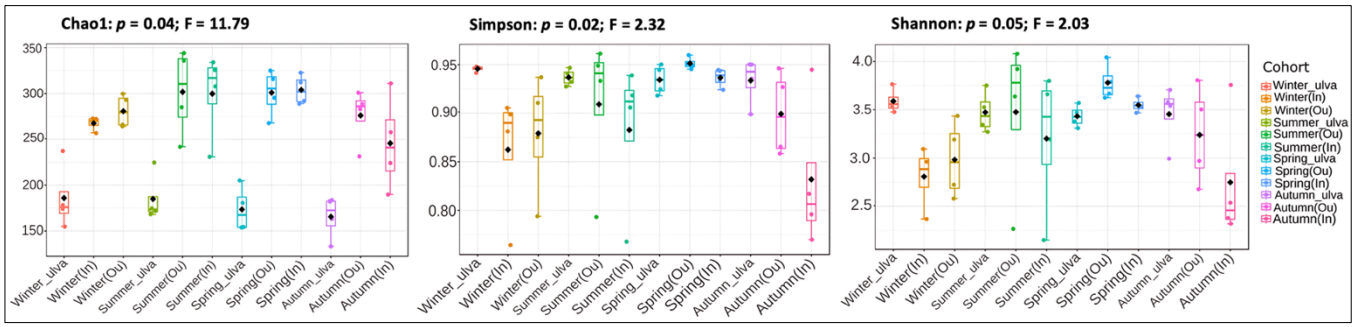

Figure 3.7. Average alpha diversity measures (Chao1, Shannon, and Simpson) for the AEW system at genus- level (excluding all SW samples), where the minimum, maximum and mean as well as ANOVA F-value and *p*-value. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Ou) for respective seasons.

3.3.2.3 Beta diversity

Variation in the composition of the bacterial microbiome observed across all the seawater and *Ulva* samples collected from the AEW and SW systems (beta diversity) was determined using Bray-Curtis dissimilarity indices, where differences were visualised using nonmetric multidimensional scaling (NMDS) plots and dendrograms. The comparison between the six cohorts (AEW_In, AEW_Out, AEW_*Ulva*, SW_In, SW_Out, and SW_*Ulva*) showed a partial degree of overlap between cohorts (Fig. 3.8), indicating similarities in bacterial composition. At the genus and family level, bacterial communities associated with AEW *Ulva* and SW *Ulva* clustered separately, with a small extent of overlap, indicating a high degree of differentiation of the bacterial microbiome associated with *Ulva* grown in the two systems (Fig. 3.8). Similarly, the bacterial communities associated with water collected from the AEW and SW systems clustered separately (Fig. 3.8), with overlap between bacterial communities sampled from the inlet and outlet of each system (AEW or SW). Moreover, a community shift can also be seen in the SW system, with the *Ulva* bacterial community being closer to the outlet than the inlet, indicating that communities share similar features with SW_In. The statistical analysis conducted in this study supports some of the compositional differences observed between cohorts (Table 3.4). The PERMDISP analysis (F-value = 0.40; $p > 0.05$) at genus (Table 3.4) indicated no difference in the homogeneity of variance between groups, and the ANOSIM ($R = 0.43$ to 0.47; $p > 0.01$) and PERMANOVA ($R^2 = 0.46$ to 0.53; $p < 0.01$) results point to moderate compositional differences. Lastly, the permutational multivariate analysis of variance (PERMANOVA) supports the high degree of dissimilarity between samples (F-value = 12,57; $R^2 = 0.54$; $p < 0.01$). The difference between the PERMANOVA and PERMDISP results shows that

groups do not differ in their overall ASV composition, but rather that the various sets of bacterial communities were different between groups, with some overlapping ASVs.

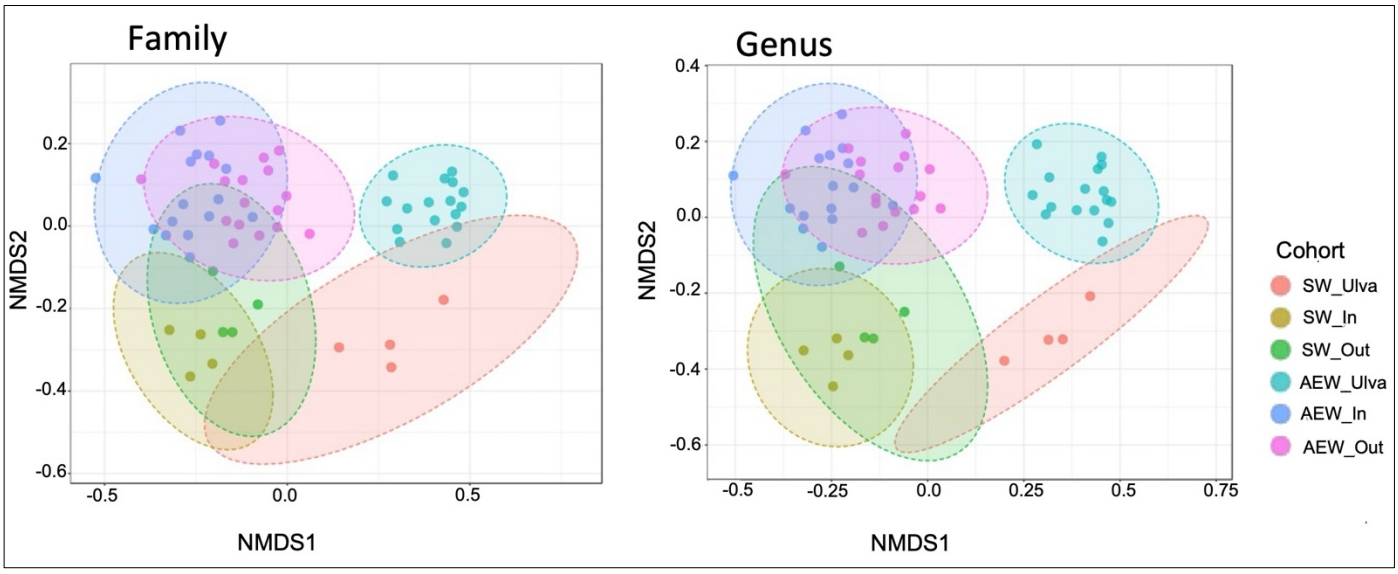

Figure 3.8. Non-metric multidimensional scaling (NMDS) analysis at family (NMDS stress = 0.1296), and genus (NMDS stress = 0.1295) level, indicating compositional differences across the bacterial communities obtained from respective groups. Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

Table 3.4. Statistical assessment of similarity (PERMANOVA, PERMDISP, and ANOSIM) where corresponding *p*-values are indicated in brackets, indicative of moderate differences in bacterial community composition between cohorts.

Analysis	Taxonomic rank	
	Family	Genus
PERMANOVA; R^2	0.53 ($p < 0.001$)	0.54 ($p < 0.001$)
PERMDISP; F	0.83 ($p > 0.78$)	0.40 ($p > 0.94$)
ANOSIM; R	0.47 ($p < 0.001$)	0.45 ($p < 0.001$)

3.3.2.4 Seasonal beta diversity

There also appears to be temporal stability in the community composition. All the AEW seasonal samples were grouped together. Similarly, SW seasonal samples were grouped together, but with some degree of separation from the AEW samples. Interestingly, all the *Ulva* seasonal samples had a high degree of overlap; this is due to the fact that all *Ulva* samples from the two systems were combined for seasonal analysis. As observed from the NMDS analysis, there was a partial overlap of samples between seasons within each system type (AEW or SW) (Fig. 3.9), suggesting bacterial communities are relatively stable over time

or across seasons. The permutational multivariate analysis of variance (PERMANOVA) indicated a degree of dissimilarity between samples (F-value = 12,57; R^2 = 0.54; $p < 0.01$). The statistical analysis conducted in this study, indicates a compositional difference between the respective cohorts (Table 3.5). The PERMDISP analysis (F-value ranging from 0.40 to 0.65; $p > 0.05$; (Table 3.5) indicated limited differences between seasons, whereas the ANOSIM (R = 0.64 to 0.68; $p > 0.01$) and PERMANOVA (R^2 = 0.59 to 0.61; $p < 0.01$) results point to moderate compositional differences.

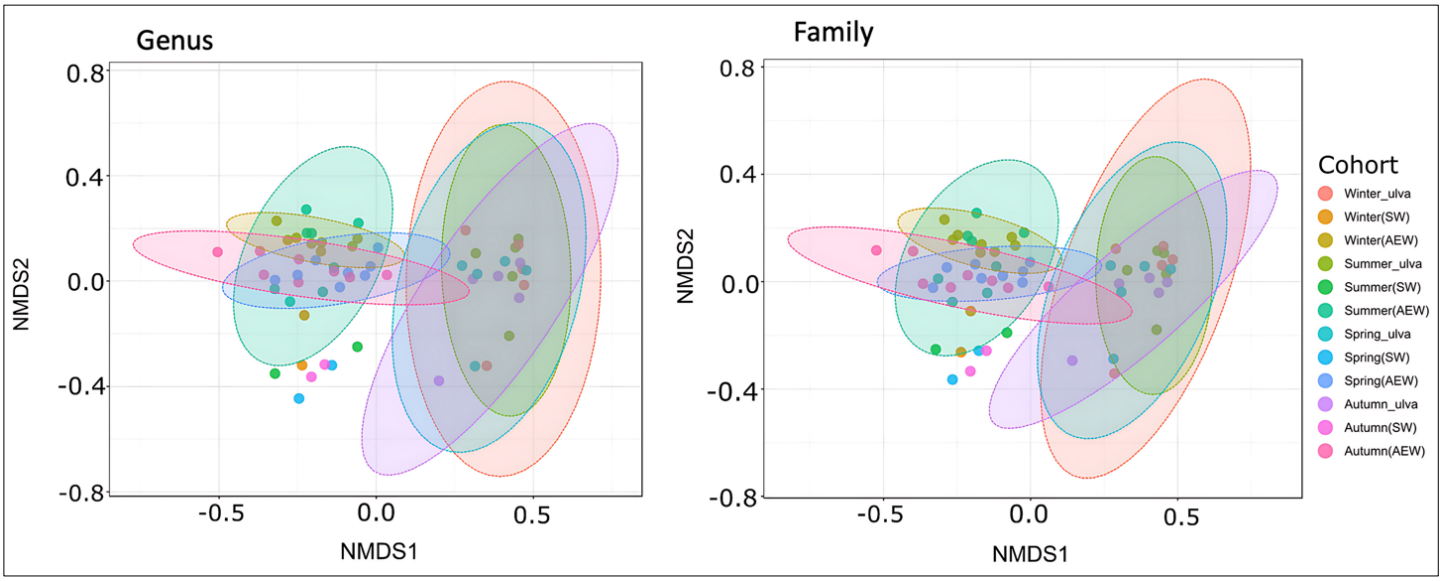

Figure 3.9. Non-metric multidimensional scaling (NMDS) analysis at family-, and genus level, indicating compositional differences across the bacterial communities obtained from respective groups. Samples for each season consisted of grouped inlets and outlet of the respective systems i.e., Winter (SW (In + Out)). *Ulva* samples of the respective systems were grouped together according to sampled season i.e., Winter_*Ulva* consisted of AEW_*Ulva* + SW_*Ulva* samples. Samples for each season AEW system (N = 8), samples for each season SW system (N = 2), *Ulva* samples per season (N = 5).

Table 3.5. Similarity tests (PERMANOVA, PERMDISP, and ANOSIM) where corresponding *p*-values are indicated in brackets, indicative of moderate bacterial community compositional differences across seasons.

Analysis	Taxonomic rank	
	Family	Genus
PERMANOVA; R^2	0.61 ($p < 0.001$)	0.59 ($p < 0.001$)
PERMDISP; F	0.65 ($p > 0.78$)	0.40 ($p > 0.94$)
ANOSIM; R	0.64 ($p < 0.001$)	0.68 ($p < 0.001$)

Further analysis of the seasonal community structure of the AEW system revealed a clustering structure that consists of three separate clusters. The *Ulva* cluster consists of all the *Ulva* samples from different seasons clustering together, indicating a high degree of similarity between the *Ulva* samples (Fig. 3.10). The second cluster was between autumn (inlet and outlet) and spring (inlet and outlet) indicating that spring and autumn samples were more similar to one another, while the third cluster consisted of samples from summer (inlet and outlet) and winter (inlet and outlet), also indicating similarities between the summer and winter samples. However, there was a partial overlap between the latter two clusters, suggesting that bacterial communities are relatively similar with some compositional differences.

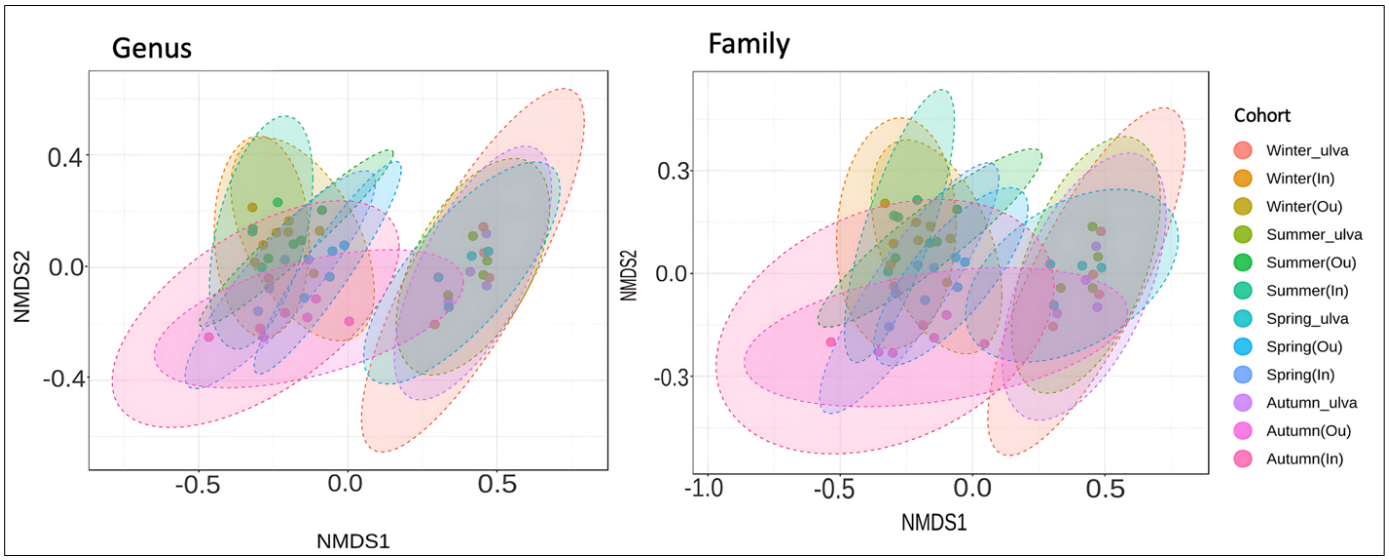

Figure 3.10. Non-metric multidimensional scaling (NMDS) analysis at and genus level (NMDS stress = 0.1295; *p*-value: 0.001) indicating compositional differences across the bacterial communities obtained from abalone effluent water (AEW) seasonal cohorts. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Ou) for respective seasons.

The sample similarities across the six cohorts were also assessed through hierarchical clustering of samples and visualised using dendrograms. The cluster analysis at the genus level supported the NMDS analysis of bacterial communities, with the *Ulva* samples from each system (AEW or SW) clearly clustering with one another and a clear separation observed between the water and *Ulva* samples (Fig. 3.11). Similarly, within the seawater clade, the water samples from each system type clearly clustered with one another, forming separate sub-clades within the main water clade.

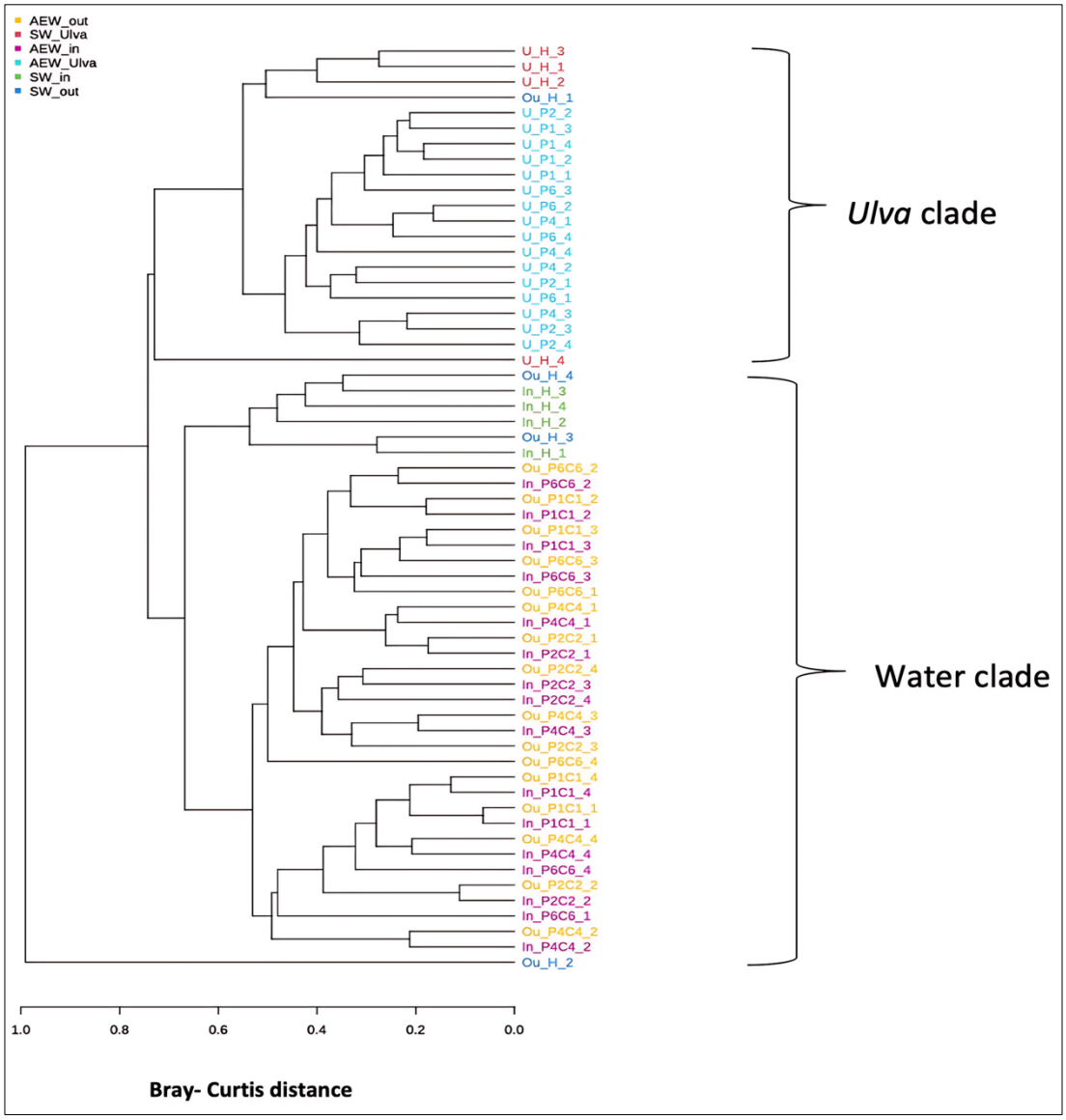

Figure 3.11. Dendrogram illustrating the bacterial community composition relationship among cohorts. The organization of the 60 samples taken from the effluent raceways, seawater tanks and the *Ulva* within each of the systems. The samples broadly fit into two clades which have been denoted as the "Water clade" and the "*Ulva* clade". Samples denoted by "H" are from the SW (non-IMTA), while samples denoted by "P" are from the AEW (IMTA) system. *Ulva* samples are denoted by "U", Inlets are denoted by (In), and outlets are denoted by (Ou).

3.3.2.5 Relative abundance and taxonomic profiling

The presence of diverse bacterial communities was shown by the taxonomic abundance profiling of samples collected across the six cohorts (AEW_In, AEW_Out, AEW_*Ulva*, SW_In, SW_Out, SW_*Ulva*), with 194 ASVs identified at the family level, 315 at the genus level and 320 ASVs identified at the species level after data filtering (Appendix A; Figures S3.1–S3.3). At the genus level, changes in taxonomic abundance were evident when comparing the SW_*Ulva* vs. AEW_*Ulva*, as well as the water samples from the AEW and SW systems when compared to *Ulva*. The four most abundant bacterial orders detected in this study across all cohorts were the Alteromonadales (15%), Flavobacteriales (14%), Vibrionales (11%), and Rhodobacterales (10.5%). The three most abundant genera detected in this study across all cohorts were *Vibrio*, *Pseudoalteromonas*, and genera belonging to the family Rhodobaceraceae. The four most abundant bacterial genera were *Vibrio* (11%), *Pseudoalteromonas* (8.5%), members of Rhodobacterceae (7.4%), and *Leucothri*x (4%) (Fig. 3.12). It is worth noting that from the inlet (incoming effluent from abalone raceways) to the outlet (bioremediated water returning to abalone raceways) of the *Ulva* paddle-raceway system, changes in the bacterial profile were observed for both the effluent and seawater systems. Most notably, the relative abundance of taxa belonging to the *Pseudoalteromonas* and *Vibrio* genera decreased from the inlet to the outlet of *Ulva* paddle-raceways receiving abalone effluent water from 15% to 12% and from 19% to 15%, respectively (Table 3.6). In contrast, the average abundance of *Vibrio* species increased slightly, from 6% to 8%, from the inlet to the outlet of the seawater system (non-IMTA and non-recirculation systems). Interestingly, *Vibrio* only occurred in very low abundance (almost absent) in the *Ulva* cohorts of both systems. At family level, Saprospiraceae had the greatest abundance in SW_*Ulva* (23%) and AEW_*Ulva* (13%), followed by Vibrionaceae in AEW_In (20%) and AEW_Out (15%) (Table 3.6). However, the two families were not differentially abundant across all their respective cohorts.

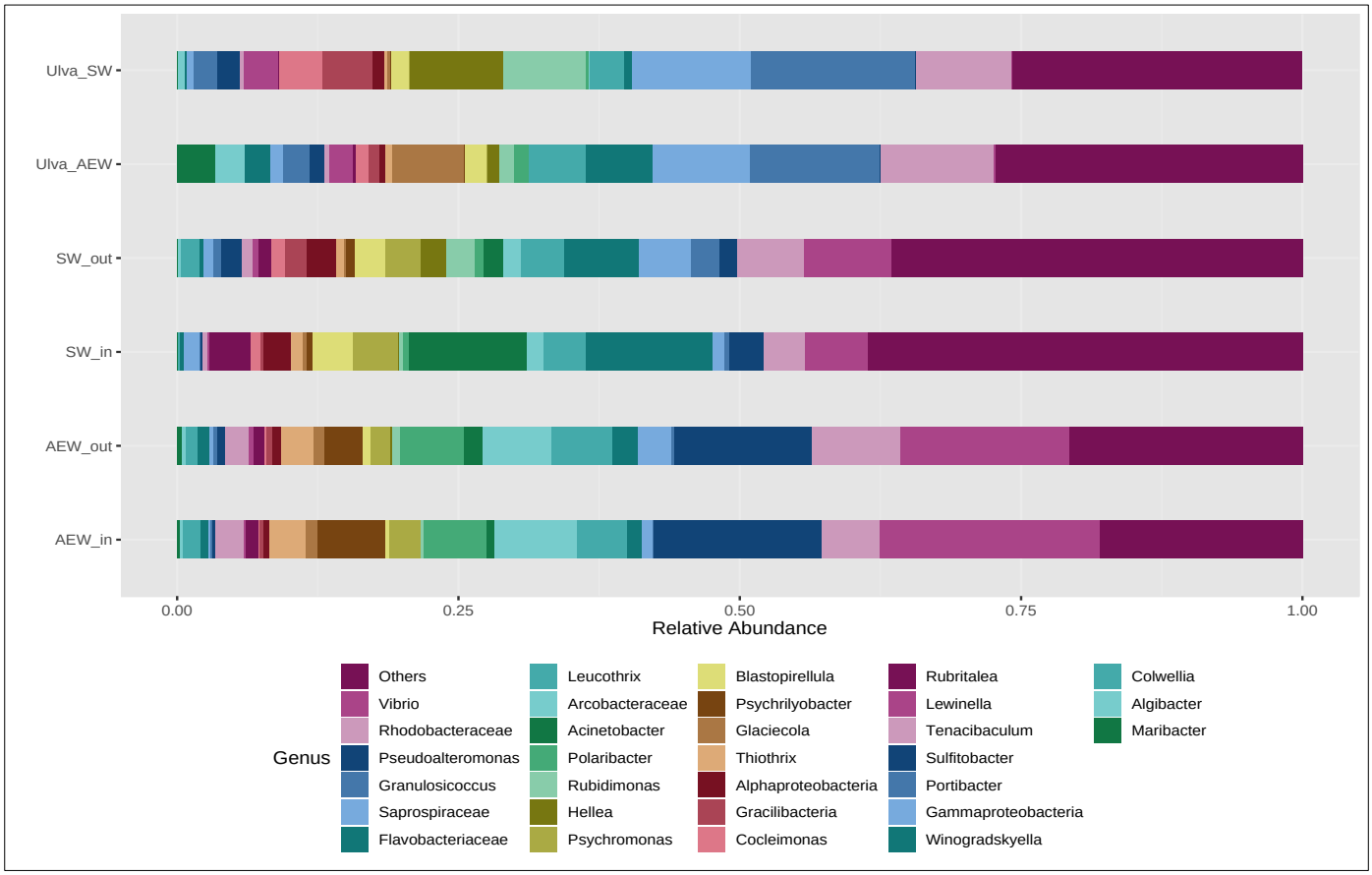

Figure. 3.12. Relative ASV abundance (%) at genus level, across 60 samples where group ASV abundance is indicated for the 30 most abundant ASVs. Less prominent ASVs were merged and denoted by "Others". Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

Table 3.6. Top three most abundant ASVs at family and genus level, with percentage abundance indicated in brackets.

Classification	Cohort	Most prevalent ASVs
Family	SW_In	Flavobacteriaceae (14%) Moraxellaceae (11%) Rhodobacteraceae (8%)
	SW_Out	Flavobacteriaceae (12%) Rhodobacteraceae (14%) Vibrionaceae (9%)
	SW_*Ulva*	Saprospiraceae (23%) Hyphomonadaceae (15%) Granulosicoccaceae (15%)
	AEW_In	Vibrionaceae (20%) Pseudoalteromonadaceae (15) Flavobacteriaceae (12%)
	AEW_Out	Vibrionaceae (15) Flavobacteriaceae (13%) Pseudoalteromonadaceae (12%)
	AEW_*Ulva*	Flavobacteriaceae (21%) Saprospiraceae (14%) Rhodobacteraceae (14%)
Genus	SW_In	*Flavobacteria* (11%) *Acinetobacter (*11%) *Vibrio* (6%)
	SW_Out	*Vibrio* (8%) Flavobacteriaceae *(*14%) Rhodobacteraceae (6%)
	SW_*Ulva*	*Granulosicoccus (15%)* Saprospiraceae (14%) Rhodobacteraceae (8%)
	AEW_In	*Vibrio* (19%) *Pseudoalteromonas* (15%) Arcobacteraceae (7%)
	AEW_Out	*Vibrio* (15%) *Pseudoalteromonas* (12%) Rhodobacteraceae (8%)
	AEW_*Ulva*	*Granulosicoccus* (12%) Rhodobacteraceae (10%) Saprospiraceae (9%)

To determine which taxa are contributing towards the dissimilarity observed in the ordinate analysis, linear discriminant analysis was used to calculate the effect size (LefSe) to test for significant associations between groups and bacterial communities, where the 25 ASVs that most likely explain differences (LDA score > 2; $p < 0.05$) between the cohorts were identified (Fig. 3.13). The results show that ASVs assigned to the genera *Pseudoalteromonas, Psychrilyobacter, Polaribacter, Thiothrix, Rubritalea, Roseimarinus, and Blastopirellula* were more likely to be associated with water samples collected from both the effluent and seawater systems, while ASVs assigned to *Granulosicoccus,* Saprospiraceae*, Glaciecola, Hellea, Rubidimonas, Gracilibacteria,* and *Litorimonas* were more likely to be associated with *Ulva* collected from the two systems. Moreover, ASVs assigned to *Vibrio*, *Pseudoalteromonas, Psychrilyobacter, and Tenacibaculum* are more likely to be associated with samples from effluent water, while ASVs assigned to *Rubritalea, Roseimarinus, and Blastopirellula* are more likely to be associated with the water samples collected from the seawater system.

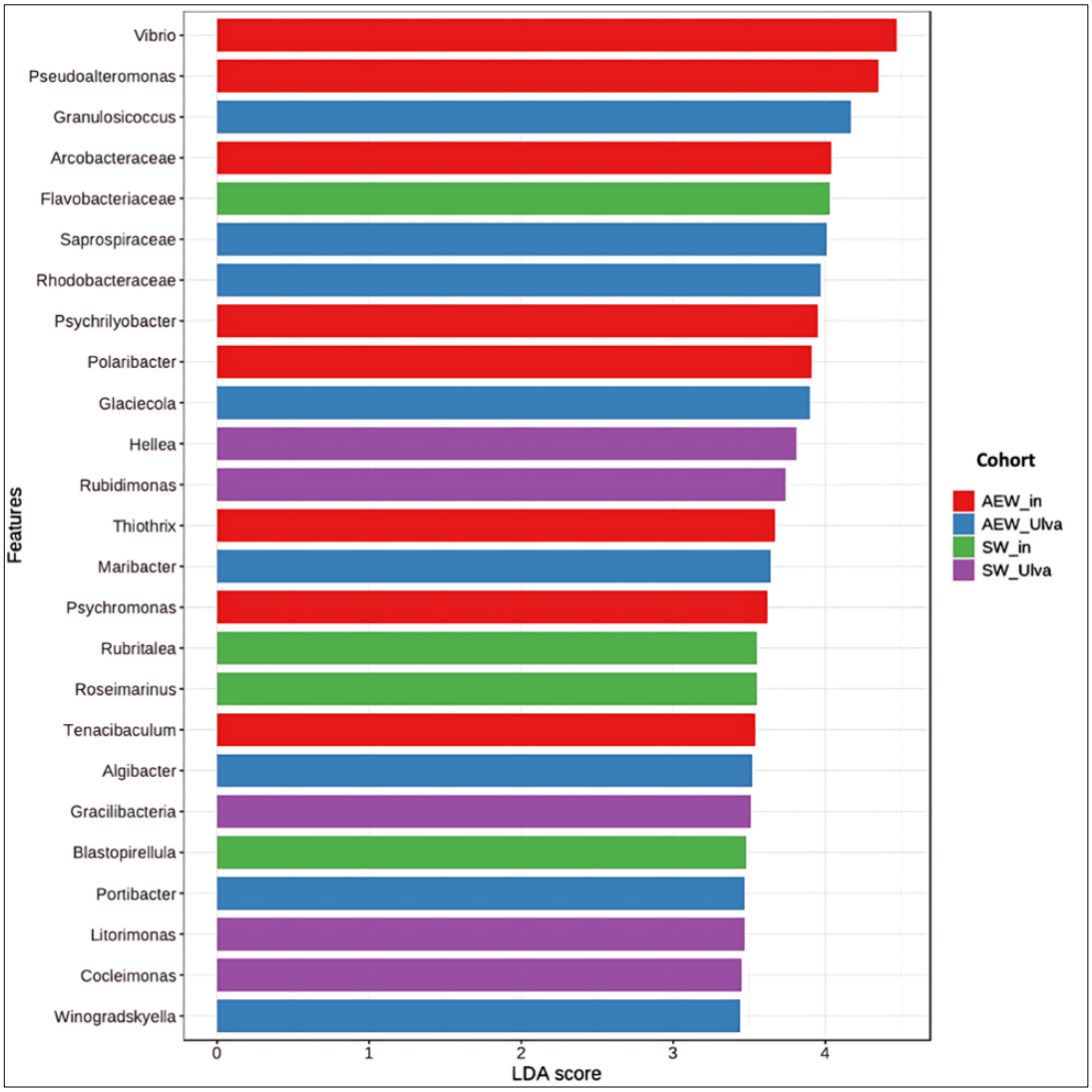

Figure 3.13. Linear discriminant analysis effect size (LEfSe) of bacterial ASVs, with the 25 ASVs most likely to explain differences between cohorts (effluent water inlet (AEW_in), effluent water outlet (AEW_out), effluent water *Ulva* (AEW_*Ulva*), seawater Inlet (SW_in), seawater outlet (SW_out) and seawater *Ulva* (SW_*Ulva*) at genus level at genus level. Different colours represent different classes. The LDA score represents the relevance or effect size of differential abundant feature.

A total of 98 differentially abundant ASVs were identified at the species level, 81 at the genus level, and 31 at the family level. The genus *Vibrio was* differentially abundant and more prevalent in the water cohorts (Fig. 3.14). It was also observed that SW-*Ulva* generally had a lower ASV abundance in most samples. In contrast, various genera of *Vibrio*, *Tateyamaria*, *Nannocystis*, and *Shewanela* were more prevalent in the water of both system types (AEW and SW), whereas *Granulosicoccus* and genera belonging to the family Microtrichaceae were more prevalent on *Ulva* (Fig. 3.14). Genera belonging to *Litorimonas*, *Hellea,* and *Rubidimonas*

were also more prevalent on SW_*Ulva,* whereas taxa belonging to *Granulosicoccus* and *Sphingorhabdus* were more prevalent on *Ulva* cultured in the effluent (AEW) system (3.14). Notably, *Roseobacter, Roseovarius, and Psychrilyobacter* were more prevalent in AEW inlets and less prevalent in seawater outlets (3.14). Similarly, the at species level, taxa belonging to *Thiothrix sp.*, *Tenacibaculum ovolyticum*, *Tenacibaculum todarodis*, *Vibrio tritonius*, *Vibrio hispanicus*, and *Shewanella intestini* were more prevalent in the AEW systems (3.15). Interestingly, *Algitalea ulvae* was more prevalent in the SW_*Ulva.*

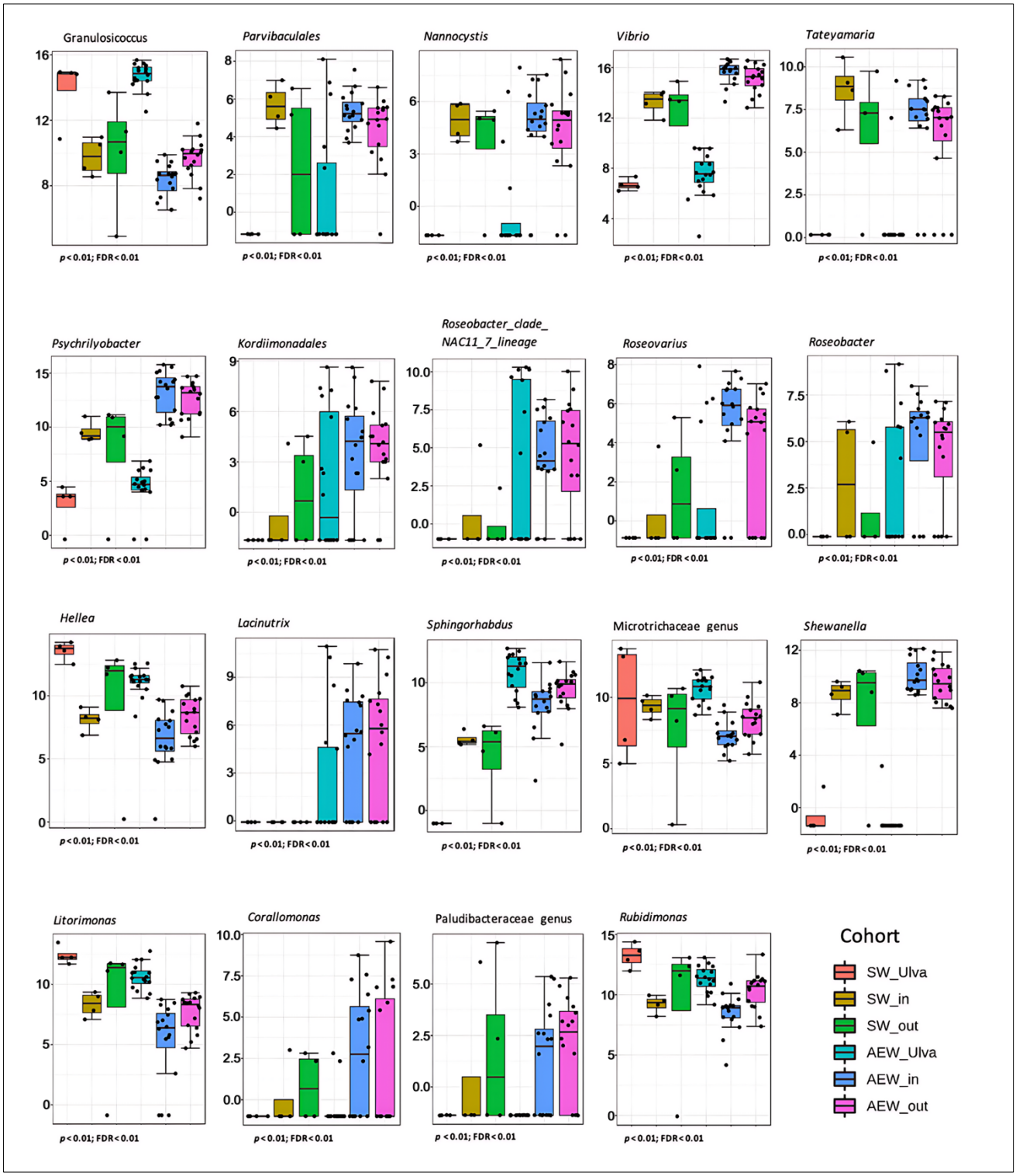

Figure 3.14. Differentially abundant ASVs of AEW, SW and Ulva bacterial communities at genus level, where the log fold change (log2FC), *p*-value and false discovery corrected (FDR is indicated). Samples denoted with "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

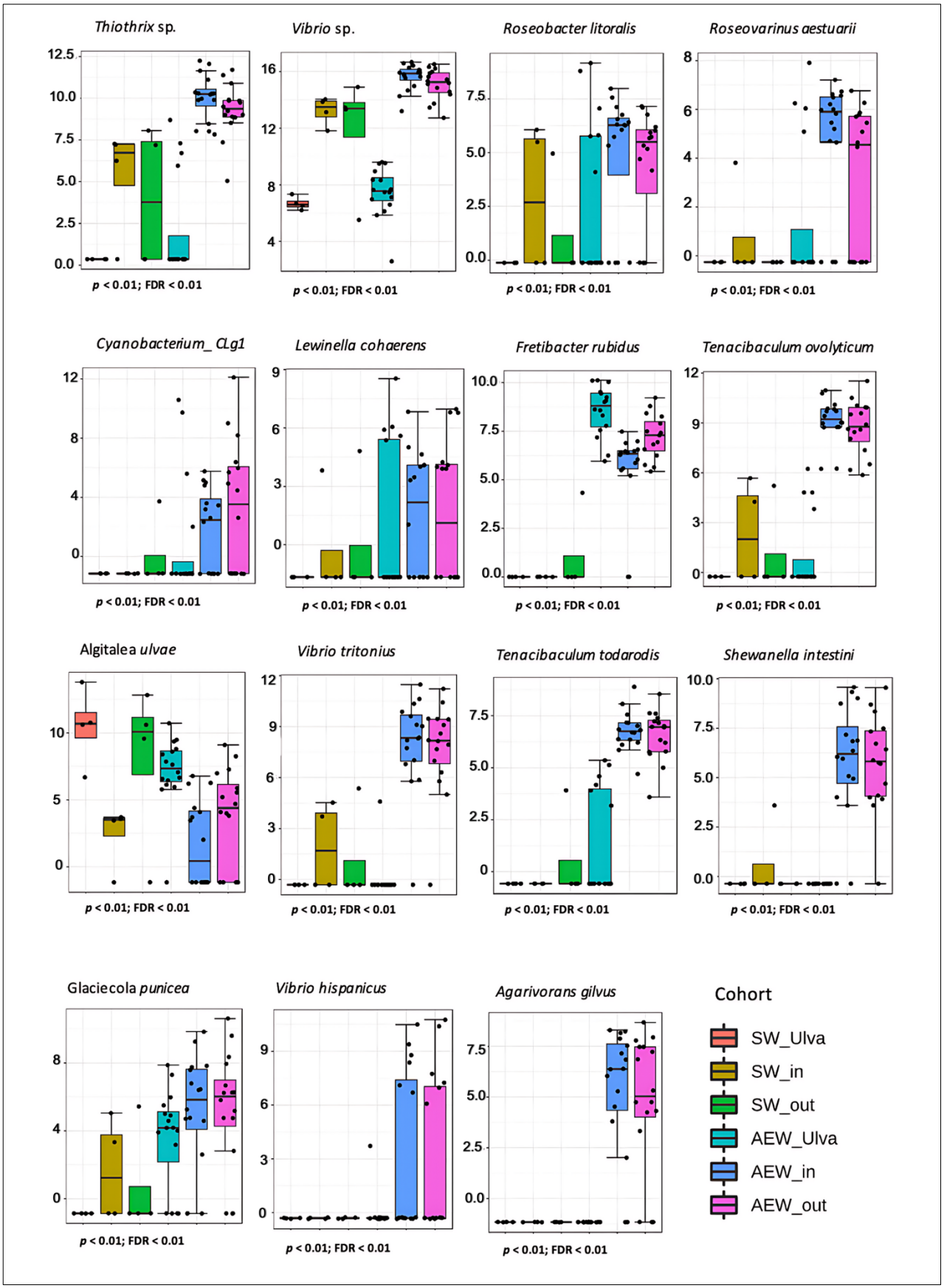

Figure 3.15. Differentially abundant ASVs of AEW, SW and *Ulva* bacterial communities at species level, where the log fold change (log2FC), *p*-value and *p*-value corrected false discovery rate (FDR) is indicated. Samples denoted with "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

Seasonal taxonomic abundance profiling showed the presence of diverse bacterial communities across all seasons (Fig. 3.16). At the family level, the average abundance of Vibrionaceae was greatest in summer (AEW), winter (AEW), and autumn (AEW) at 19%, 15%, and 25%, respectively (Table 3.7). The genus *Acinetobacter* had relatively high abundance in winter (SW), while *Vibrio* was observed to be more abundant in summer when compared to winter and spring. *Pseudoalteromonas* was abundant in the summer (AEW) and winter (AEW). *Psychrilyobacter* was more abundant in AEW than SW throughout all the seasons. *Maribacter* was more abundant on *Ulva* in the summer. *Granulosicoccus* and Saprospiraceae were present on *Ulva* throughout all the seasons. The relative abundance of taxa belonging to various genera across seasons decreased from the inlet to the outlet in the AEW system (Fig. 3.17). During winter, *Vibrio* decreased from 17% to 11% and *Pseudoalteromonas* decreased from 22% to 20%, and in summer, *Vibrio* decreased from 19% to 18%, *Pseudoalteromonas* decreased from 13% to 11%, and *Psychrilyobacter* decreased from 9% to 5%. In spring, *Vibrio* decreased from 13% to 7%, *Psychrilyobacter* decreased from 7% to 5%, and *Thiothrix* decreased from 6% to 4%. Finally, during autumn, *Vibrio* decreased from 30% to 20%, *Pseudoalteromona*s decreased from 21% to 13%, and *Psychrilyobacter* decreased from 3% to 1%.

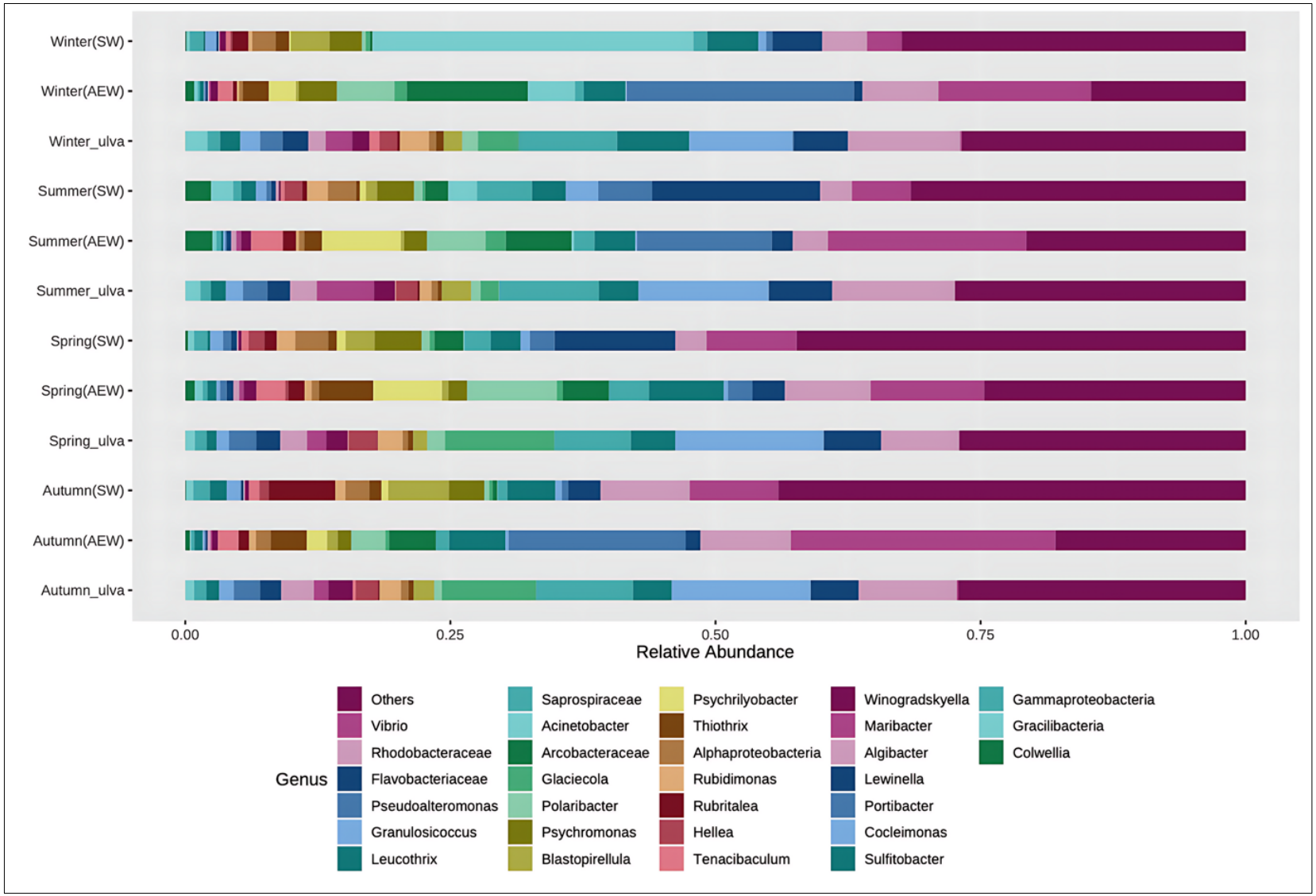

Figure 3.16. Relative ASV abundance (%) at genus level, across seasons where group ASV abundance is indicated for the 30 most abundant ASVs. Less prominent ASVs were merged and denoted by "Others". Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

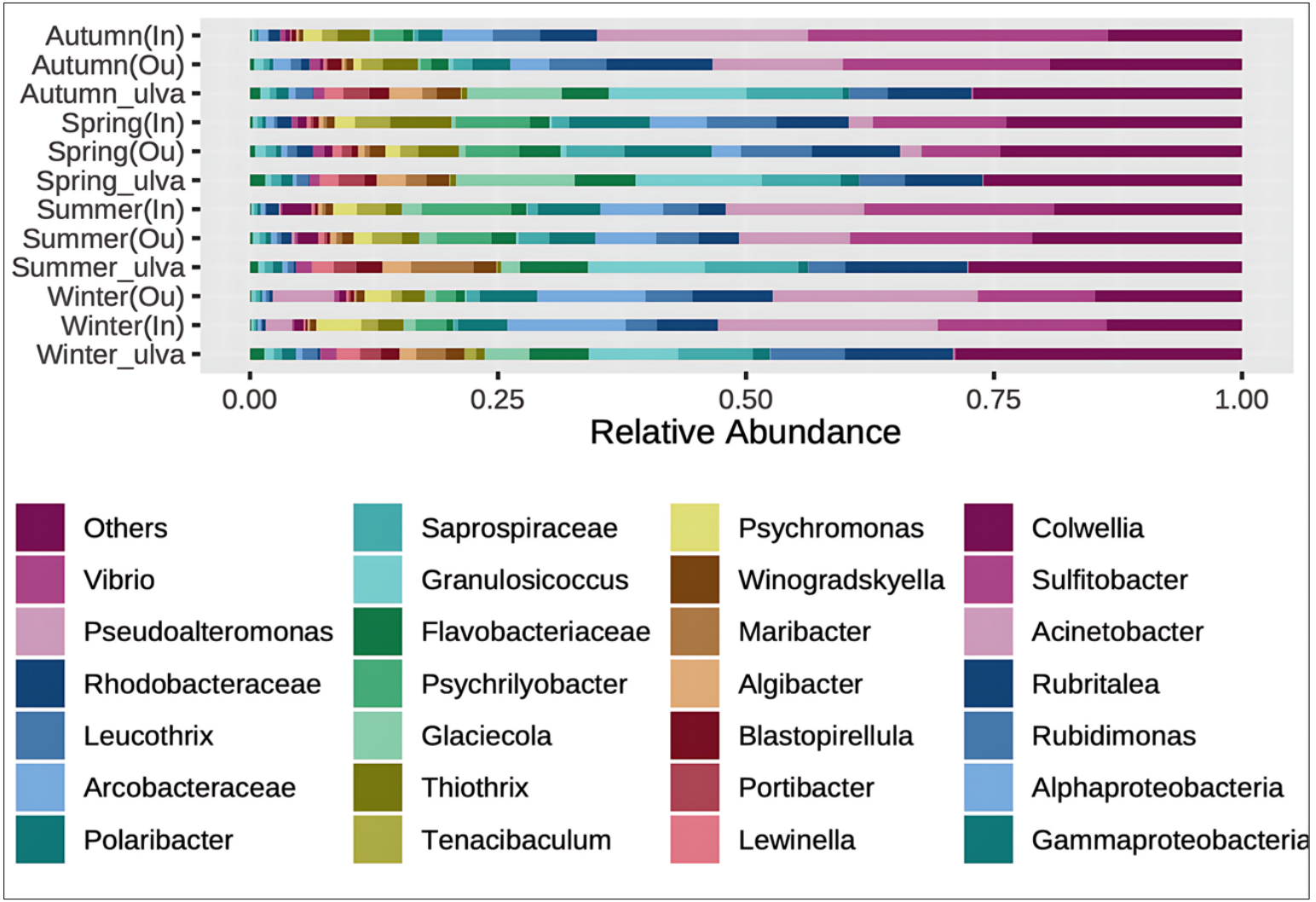

Figure 3.17. Relative ASV abundance (%) from abalone effluent water (AEW) at genus level, across seasons where group ASV abundance is indicated for the 30 most abundant ASVs. Less prominent ASVs were merged and denoted by "Others". Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

Table 3.7. Top three most prevalent ASVs at family and genus level, with percentage abundance indicated in brackets.

Classification	Cohort	Most prevalent ASVs
Family	Winter (AEW)	Pseudoalteromonadaceae (22%) Vibrionaceae (15%) Arcobacteraceae (12%)
	Winter (SW)	Moraxellaceae (30%) Rhodobacteraceae (12%) Flavobacteriaceae (7%)
	Winter (*Ulva*)	Saprospiraceae (17%) Flavobacteriaceae (17%) Rhodobacteraceae (15%)
	Summer (AEW)	Vibrionaceae (19%) Flavobacteriaceae (14%) Pseusoalteromonadacea (13%)
	Summer (SW)	Flavobacteriaceae (19%) Saprospiraceae (8%) Rhodobacteraceae (7%)
	Summer (*Ulva*)	Flavobacteriaceae (21%) Saprospiraceae (15%) Rhodobacteraceae (15%)
	Spring (AEW)	Flavobacteriaceae (19%) Thiothrichaceae (14%) Rhodobacteraceae (12%)
	Spring (SW)	Flavobacteriaceae (15%) Vibrionaceae (10%) Rhodobacteraceae (6%)
	Spring (*Ulva*)	Flavobacteriaceae (16%) Saprospiraceae (14%) Granulosicoccaceae (14)
	Autumn (AEW)	Vibrionaceae (25%) Pseudoalteromonadaeceae (17%) Rhodobacteraceae (11)
	Autumn (SW)	Rhodobacteraceae (13%) Pirellulaceae (10%) Vibrionaceae (9%)
	Autumn (*Ulva*)	Flavobacteriaceae (15%) Saprospiraceae (15%) Granulosicoccaceae (14%)
Genus	Winter (AEW)	*Pseudoalteromonas* (21%) *Vibrio* (14%) Arcobacteraceae (11%)
	Winter (SW)	*Acinetobacter* (30%) *Leucothrix* (5%) Flavobacteriaceae (5%)
	Winter (*Ulva*)	Rhodobacteraceae (11%) *Granulosicoccus* (10%) Saprospiraceae (9%)
	Summer (AEW)	*Vibrio* (19%) *Pseudoalteromonas* (13%) *Psychrilyobacter* (7%)
	Summer (SW)	Flavobacteriaceae (16%) *Vibrio* (6%) Saprospiraceae (6%)
	Summer (*Ulva*)	*Granulosicoccus* (11%) Rhodobacteraceae (11%) Saprospiraceae (10%)
	Spring (AEW)	*Vibrio* (11%) *Poloribacter* (8%) Rhodobacteraceae (8%)
	Spring (SW)	Flavobacteriaceae (11%) *Vibrio* (8%) *Psychromonas* (4%)
	Spring (*Ulva*)	*Granulosicoccus* (14) *Glaciecola* (10%) Rhodobacteraceae (7%)
	Summer (AEW)	*Vibrio* (25%) *Pseudoalteromonas* (17%) Rhodobacteraceae (9%)
	Summer (SW)	Rhodobacteraceae (8%) *Vibrio* (8%) *Rubritalea* (6%)
	Summer (*Ulva*)	*Granulosicoccus* (14%) Rhodobacteraceae (10%) Saprospiraceae (9%)

The seasonal LefSe analysis results show that ASVs assigned to the genera *Vibrio* were significantly associated with autumn (AEW), while *Rubritalea* and *Blastopirellala*, as well as genera belonging to the family Halieaceae, Roseimarinus, and Oceanospirillates, were significantly associated with autumn (SW). Members of Rhodobacteraceae, Rubidimonas, Lewinella, and Portibater were associated with the winter_*Ulva* cohort (Fig. 3.18). Furthermore, genera that commonly occur in the development and morphogenesis of *Ulva*, such as *Granulosicoccus*, *Maribacter*, and Saprospiraceae, were associated with summer_*Ulva* and winter_*Ulva* cohorts (Fig. 18). The univariate method, DESeq2, identified differentially abundant ASVs corresponding to genus and species-level classification. The genus *Granulosicoccus* had a statistically greater abundance ($p < 0.05$) in the *Ulva* across seasons, while *Vibrio* had a statistically greater abundance in the AEW and SW water cohorts across all seasons (Fig. 3.19). Similarly, at the species level, *Tenacibaculum todarodis, Shewanella intestini,* and *Vibrio tritonius* had a statistically greater abundance in the AEW across seasons (Fig. 3.19).

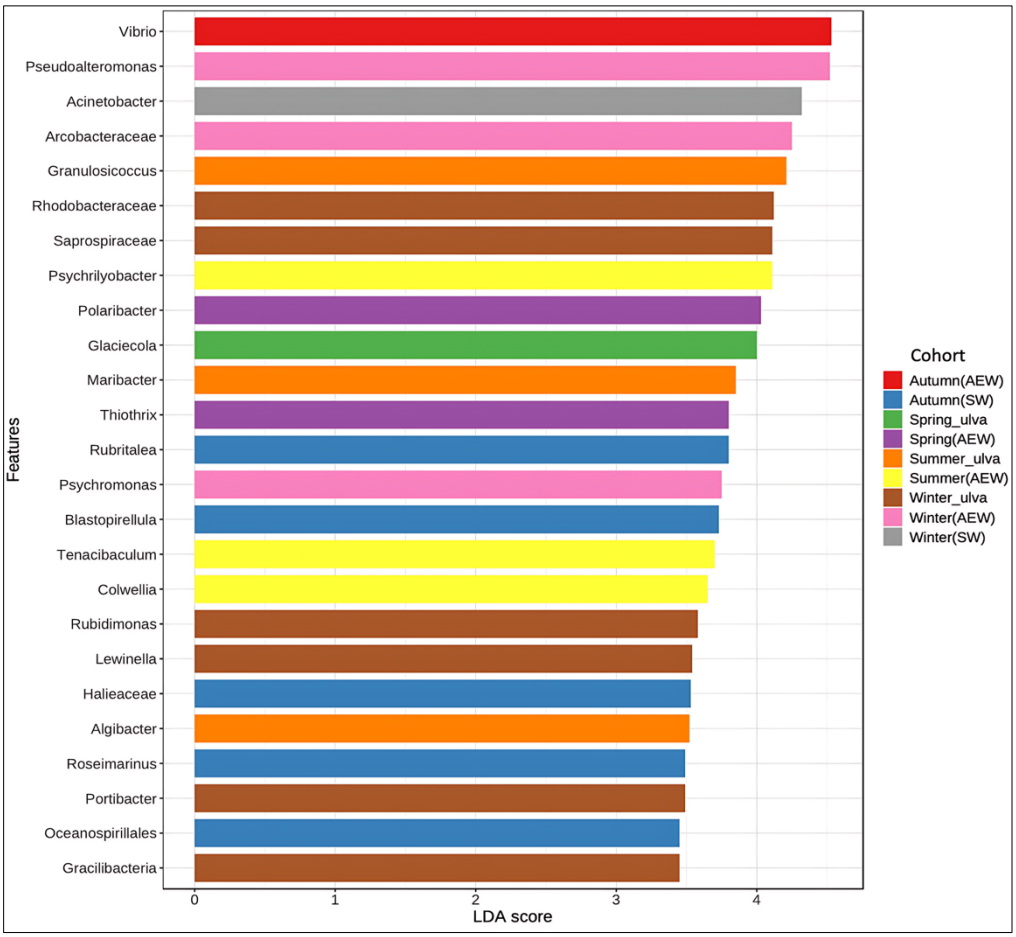

Figure 3.18. Linear discriminant analysis effect size (LEfSe) of bacterial ASVs, with the 25 ASVs most likely to explain differences between seasons (effluent water inlet (AEW_in), effluent water outlet (AEW_out), effluent water *Ulva (*AEW_*Ulva)*, seawater Inlet (SW_in), seawater outlet (SW_out) and seawater *Ulva* (SW_*Ulva*) at genus level. Different colours represent different classes. The LDA score represents the relevance or effect size of differential abundant feature.

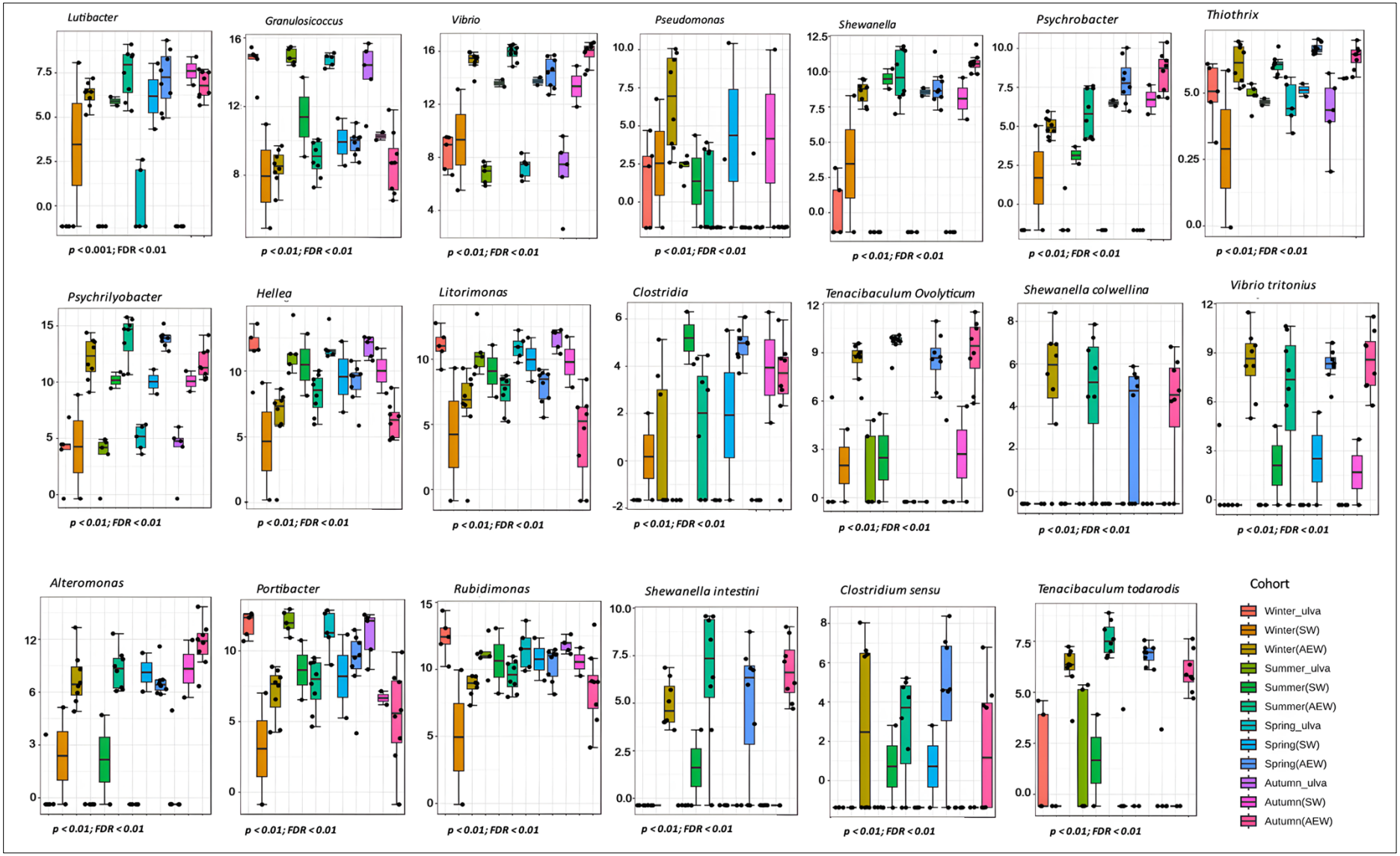

Figure 3.19. Differentially abundant ASVs of AEW, SW and *Ulva* bacterial communities at and genus and species level, where the log fold change (log2FC), *p*-value and *p*-value corrected false discovery rate (FDR) is indicated. seasons (effluent water inlet (AEW_in), effluent water outlet (AEW_out), effluent water *Ulva (*AEW_*Ulva)*, seawater Inlet (SW_in), seawater outlet (SW_out) and seawater *Ulva* (SW_*Ulva*) at genus level.

3.3.2.6 Functional profiling

The putative functional roles of the microorganisms identified from the effluent water and seawater were examined by predicting the functional potential of the bacterial communities using Tax4Fun. Putative functional roles revealed 6250 KOs, which were grouped into 11 KEGG pathways. Figure 3.20 depicts the general metabolic pathways, comparing putative microbiota functions from seawater, effluent water, and *Ulva.* These pathways are mainly associated with one of the six major functional categories, namely metabolism. Nucleotide metabolism, metabolism of cofactors and vitamins, and metabolism of carbohydrates were most represented in the microorganisms of all cohorts studied. In the AEW cohort, there was a pronounced up-regulation of the biosynthesis of other secondary metabolites (Fig. 3.20). Metabolism of other amino acids and amino acid metabolism were more pronounced in the AEW_*Ulva* and SW_*Ulva* cohorts when compared with other cohorts, while amino acid metabolism and xenobiotic biodegradation and metabolism had a higher contribution in the AEW_*Ulva* cohort (Fig. 3.20).

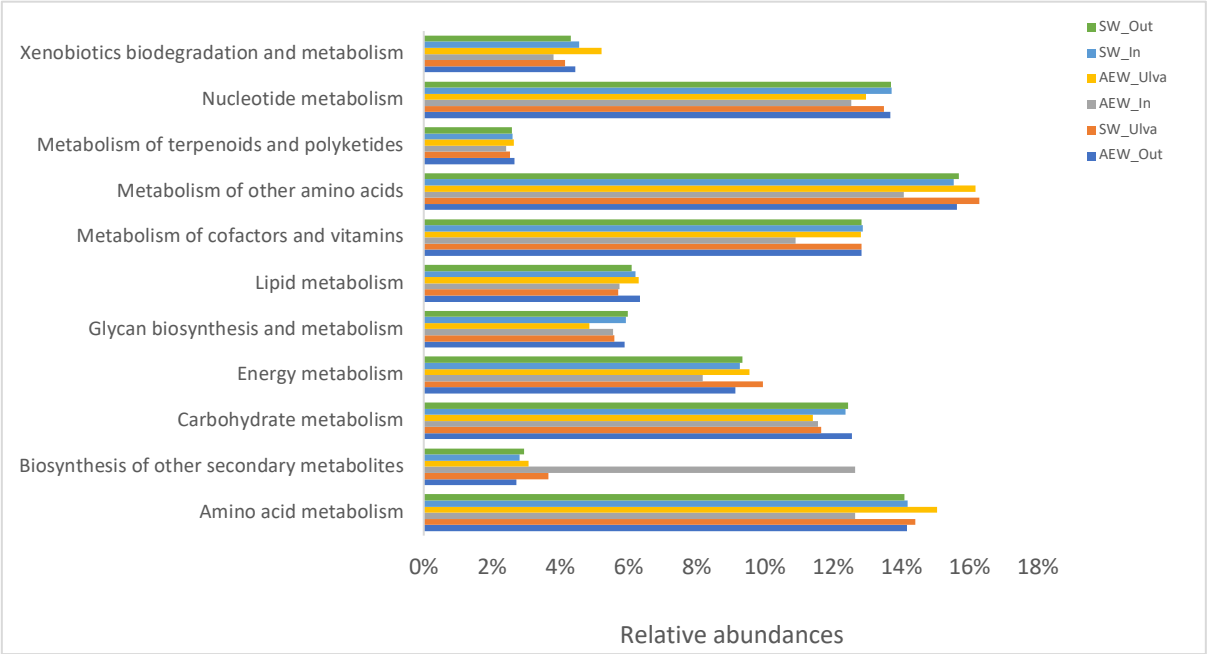

Figure 3.20 Level 2 metabolic pathway abundance as a proportion of parent term "Metabolism" at all 6 at sampling points.

3.4 Discussion

In the current study, the microbial community composition of the effluent water from a production-scale abalone IMTA system was explored, expanding our understanding of the complexity of these systems.

3.4.1 Water parameters

Previous studies have indicated that the release of organic waste, uneaten feed, and fish excrement in aquaculture farming systems increases nutrient loading, particularly for nitrogen and phosphorus (Sarà, 2007; Bouwman et al., 2013; Rubio-Portillo et al., 2019). Given the higher nutrient load within the effluent received from abalone raceways observed when compared to seawater system, free from effluents in the present study (Table 3.3), and in other previous studies in similar systems (Robertson-Andersson, 2003; Probyn et al., 2017) and the fact that bacteria are known to proliferate in nutrient-rich environments (Matsushita et al., 1990; Walker, 2000), it is not surprising that SW system, free from effluents, had a statistically lower species richness than the AEW system. Similarly, a study conducted by Cho & Kim (2000), reported an increase in both bacterial diversity and abundance in subsurface aquifers receiving livestock wastewater input, which corresponds with the observed increase in bacterial diversity inside IMTA raceways. Furthermore, the current study observed increased nitrogen compounds such as nitrate and nitrite in winter and autumn and decreases in summer and spring, which could be attributed to the several factors related to temperature, light availability, biological activity, and feeding practices (Kyritsi et al.,2023; Lin et al., 2023). *Ulva* species readily uptake nutrients from water, including nitrogen and phosphorus, which also facilitate *Ulva*'s growth. This reduces the quantities of nutrients, which could otherwise lead to dangerous overgrowth of certain bacteria (Kang et al., 2011; Hurd et al., 2014). During seasons with high nutrient loads (typically in autumn and winter in the case of this study), *Ulva* and the microorganism associated with *Ulva* play a crucial role in nutrient cycling in marine ecosystem. They help mitigate these impacts of nutrient overload by breaking down nutrients and absorbing excess nutrients, modifying the microbial community structure that would otherwise flourish on these nutrients. This observation is consistent with the current study's findings, which demonstrated lower ASV richness during winter and autumn. These findings indicate a strong relationship between the presence of bacterial communities and the

seasons of the year, suggesting that seasonality and the corresponding environmental conditions, is one of the major drivers shaping bacterial community composition and diversity in aquaculture farms. However, several potential confounding factors may influence the results when samples are aggregated and not analysed separately by season/collection date. Therefore, it is crucial to consider these seasonal differences.

3.4.2 Enumeration of culturable bacteria in effluent water and *Ulva*

Given the higher nutrient load within the effluent received from abalone raceways observed in the present study (Appendix A; Table S3.3) and in other previous studies (Robertson-Andersson, 2003; Probyn et al., 2017) and the fact that bacteria are known to proliferate in nutrient-rich environments (Matsushita et al., 1990; Walker, 2000), it is not surprising that significantly more bacteria were present in the water column of the *Ulva* paddle-raceways receiving effluent water than in the seawater system. Cho & Kim (2000) reported an increase in both bacterial diversity and abundance in subsurface aquifers receiving livestock wastewater input, which corresponds with the observed increase in bacterial abundance inside effluent raceways. In both the AEW (IMTA) and SW (non-IMTA) systems, the macroalgae *Ulva* appears to have a strong inhibitory effect on *Vibrio* species within these systems, as indicated by the significant decrease in bacteria growing on TCBS (*Vibrio* selective media) between the inlets and outlets (Fig. 3.2). With regard to spatial differences, the inhibitory effect of *Ulva* seems to be nutrient-dependent, as a substantial decrease in *Vibrio* and the number of bacteria growing on TSA media was observed in the effluent water system. This could be attributed to the fact that *Ulva* growing in AEW receives more nutrients, is healthier, and is therefore better able to modulate the microbiome. These findings are in line with the results of Lu et al. (2008), who demonstrated a decrease in a strain of *V. anguillarum* in the presence of *U. clathrata*, which was enhanced following the addition of nitrogen and phosphorous.

The abundance of general marine bacteria varied significantly between the inlets and outlets of both water systems, as indicated by the growth on TSA (Fig. 3.2). Abiotic parameters such as oxygen concentration as well as competition among different bacterial species with different metabolic characteristics could contribute to the observed differences in the

microbiome composition of the inlet and outlet within the IMTA and non-IMTA systems. The CFU data exhibited differences across different time points (seasons). There is evidence for seasonal or temporal differences, in which there was a high abundance of Ulvan-specific bacteria and *Vibrio*'s growing on TCBS in both systems in spring and reduced over the course of summer (Fig. 3.2). A shift was also observed from the inlet to the outlet of Ulvan-specific bacteria and *Vibrio* during spring and summer in both systems. On all three types of selective media, the bacterial abundance of *Ulva* cultured in the effluent system was greater than that of *Ulva* cultured in the seawater system (non-IMTA) (Fig. 3.3).

3.4.3 Bacterial alpha diversity

Across all alpha diversity measures, a great amount of genus- and family-level diversity was observed in the SW cohort (SW_In and SW_Out). The high degree of diversity in these cohorts is explained by the interchangeable nature of water with the environment, where fresh seawater is constantly pumped in from the ocean into the *Ulva* systems, which explains the great degree of diversity in these cohorts. However, it is important to note that these observations are based on only eight samples in the SW cohort, and the limited sample size should be taken into account when interpreting the results. When compared with the AEW system, which recirculates 50% of its water, incoming seawater (SW) demonstrated a significantly higher Simpson diversity indices at genus level (p = 0.04), indicating an even distribution of species' abundance in the SW system. These findings appear to be consistent with previous findings (Brailo et al., 2019), which revealed that environmental seawater can be a natural promoter of microbial communities. The Chao1 diversity indices was also significantly higher in the AEW cohorts (in and out) than in the SW cohorts (in and out), indicating the presence of unique ASVs in the nutrient-rich AEW cohorts, while the Simpson diversity index was significantly higher in the non-IMTA system, emphasising even distribution of taxa. However, a similar Shannon diversity index (which accounts for both species richness and evenness) was observed between samples/cohorts across system types at genus level, indicating that despite differences in how Chao1 was significantly higher in the IMTA system (higher richness), these species are not as evenly distributed (lower evenness) as in the non-IMTA and indicating that these differences do not result in a higher overall diversity in one system than the other. The *Ulva* cohort, on the other hand, had the lowest bacterial

abundance and diversity relative to the water systems (SW and AEW). The richness estimator Chao1 was significantly higher for water samples (SW and AEW) ($p < 0.001$) than *Ulva* cohort*s*, indicating a higher prevalence of unique ASVs in the water columns. This could be attributed to the fact that macroalgae can influence the makeup of their associated microbiota by secreting chemical defences (such as proline, dimethylsulphoniopropionate (DMSP), and fucoxanthin; (Paix et al., 2021) and chemoattractants (Kessler et al., 2018; Loos et al., 2019). Hence, the high microbial diversity was observed in the water columns relative to the *Ulva* cohorts. The objective of this study was to capture a holistic view by combining the data, while also accounting for the potential influence of seasons, which necessitated conducting separate analyses for seasons. The increase in the number of unique ASVs and alpha diversity during summer and spring in the AEW system (Fig. 3.7), could be related to the increase in temperature, which was relatively higher than other seasons (Table 3.3). The increased richness during summer and spring could also be attributed to selective nutrient enrichment in the systems, which favours the growth of certain bacterial groups, leading to a less diverse bacterial community.

3.4.4 Bacterial beta diversity

The comparison between water and *Ulva* samples showed that water cohorts (SW and AEW) clustered more closely together at family and genus taxonomic levels in the NMDS plot (Fig. 3.9). Moreover, a partial degree of overlap was observed between SW_*Ulva* and AEW_*Ulva*. Both environmental and host factors influence the difference in the composition of bacterial communities (Vogel et al., 2020), where microbial communities in the surrounding water likely influence bacterial colonisation of the seaweed surface. Seaweeds have a considerable chemical gradient on their surfaces because of the exudation of organic and inorganic substances (collectively called info-chemicals) (Schmidt & Saha, 2021). Excessive microbial colonisation may hinder not only nutrient intake but also reproduction and photosynthesis, among other processes; hence, info-chemicals from seaweed surfaces can operate as a chemical defence against certain microbial colonisers (Gama et al., 2014; Saha et al., 2019). This defence can also be provided by host-associated microbes (Nasrolahi et al., 2012). In the current study, partial overlap between *Ulva* from different systems was observed (Fig. 3.9), indicating that while there were differences, there were also similarities in the bacterial

composition of *Ulva* in the different systems. *Ulva* actively changes its microbiome, favouring microorganisms that promote its growth, health, and ecological success (Ren et al., 2022). The core microbiome is a stable and beneficial community that supports various aspects of *Ulva*'s biology, regardless of environmental variations. This community remains generally stable across different environments and geographical locations, demonstrating a co-evolved and mutually beneficial relationship between *Ulva* and its associated microorganisms. This corresponds with the observed similarities among *Ulva* samples from different systems in the current study. On the other hand, the dissimilarities between AEW_*Ulva* and SW_*Ulva* are consistent with earlier research on marine seaweeds (Michelou et al., 2013; Mancuso et al., 2016; Minich et al., 2018), suggesting that interactions between microbial communities and the surrounding environment might influence the composition of the seaweed microbiome. In one case, high temperatures and high CO_2 partial pressure were found to have the greatest impact on the kelp (*M. pyrifera*) microbiome in a study by Minich et al. (2018). Furthermore, microbial communities have been observed to shift as a result of climate change stressors such as higher mean temperatures and ocean acidification (Minich et al., 2018). Increased temperature may lead to an increased exudation of DMSP on the surface of seaweeds, which may not only attract beneficial bacteria (Kessler et al., 2018), but also pathogenic microbes, thus leading to dysbiosis and ultimately resulting in diseased holobionts. In the current study, *Ulva* samples across all seasons clustered with one another and had the lowest bacterial diversity relative to SW and AEW water samples across all seasons. Furthermore, autumn and spring water cohorts clustered together, indicating similarities in the microbial diversity structure, while summer and winter cohorts also clustered with one another. The NMDS plots show distinct microbial communities on *Ulva* in both systems. This aligns with molecular studies indicating that bacterial communities differ between hosts and environments, with limited core communities specific to the host (Burke et al., 2011a).

3.4.5 Taxonomic profiling of bacterial communities in the IMTA vs. non-IMTA systems

This study was conducted to investigate the bacterial communities in abalone IMTA systems with a focus on the role of *Ulva* in these systems. In this study, 19 phyla were identified, where the bacterial community was mainly composed of Proteobacteria (58%) and Bacteroidota (23%) across all samples (Fig. S3.1). This is consistent with previous findings from other

aquaculture systems (Huang et al., 2016; Rud et al., 2017; Brailo et al., 2019). These phyla, Proteobacteria and Bacteroidota, were present in different relative abundances in the AEW (57%–23%) and SW (54%–24%) cohorts. The identified phyla are common in freshwater systems and seawater, as well as in the gut and skin of fish, and have previously been identified Tilapia and shrimp systems (Giatsis et al., 2015; Zheng et al., 2015). These results are also in line with a previous study that showed the dominance of Proteobacteria in biofilms in an aquaculture system (Schreier et al., 2010). Proteobacteria are characterised as r-strategists with important roles in nutrient recycling (Gonzalez et al., 2000). Their high abundance (64%) in AEW_In might be due to the nutrient conditions in that environment. Actinobacteria have recently received a lot of attention for their potential role as probiotic bacteria in marine and freshwater aquaculture (Das et al., 2008; Jami et al., 2015), emphasising the importance of these beneficial bacteria and their antimicrobial activities on fish and plant health.

Alteromonadales (15%), Flavobacteriales (14%), and Rhodobacterales (11%) were the most abundant orders detected in the study. Previous research suggests that Alteromonadales play an important environmental role in nitrate uptake in marine environments (Wawrik et al., 2012). Taxa belonging to Alteromonadales are widely distributed in marine environments (Bowman et al., 1997). In the current study, Alteromonadales exhibited a higher abundance in the AEW cohort than SW. It is possible that members of this order were enriched in water samples, especially the AEW_In, due to high nutrient inputs from abalone feed during intensive abalone production. Most of the ASVs assigned to Alteromonadales belonged to the *Pseudoalteromonas* genus (Table 3.2). A strain of *Pseudoalteromonas* has previously been tested with promising results as a protective agent against *Vibrio* infections in European abalone (*Haliotis tuberculate*) (Offret et al., 2019). This genus is composed of ubiquitous bacterial species in marine environments. Interestingly, *Vibrio* was observed to have an increased relative abundance in the AEW_In cohort despite the abundance of *Pseudoalteromonas* and its inhibitory effect on various *Vibrio* species. Some species of the genus *Pseudoalteromonas* are known as probiotics with the effects of reducing the larval mortality of fish and shrimp (Hjelm et al., 2004; Pham et al., 2014), inhibiting *Vibrio* (Del Castillo et al., 2008; Fjellheim et al., 2010), and promoting digestion (Tzuc et al., 2014; Leyton et al., 2017). Previous research suggests that members of this genus may contain valuable

biocontrol strains for application in aquaculture (Holmström & Kjelleberg, 1999; Wesseling et al., 2015).

A high abundance of Rhodobacterales was also observed in all cohorts. Members of this order are well known for their metabolic versatility (e.g., photosynthesis, CO_2, nitrogen fixation, and sulfur oxidation), which can significantly contribute to nutrient cycling and improve water quality (Gupta & Mok, 2007; Voget et al., 2015). Previous research suggests that the Roseobacter clade (Rhodobacterales) may play an important role in the development of fish pathogens in aquaculture systems (Hjelm et al., 2004; Martins et al., 2018). D'Alvise et al. (2010), for example, demonstrated that *Vibrio*-antagonistic *Roseobacter* (a producer of tropodithietic acid, TDA) was capable of suppressing the development of the fish pathogen *Vibrio anguillarum* in model systems simulating a fish larval aquaculture environment. The ASVs assigned to Rhodobacterales were present in all cohorts, including *Ulva. The* Flavobacteriales order was also abundant across all cohorts. Members of this order are well-known for their ability to form biofilms on surfaces in marine environments (Nocker et al., 2004; Webster & Negri, 2006). Flavobacteriales are represented by ASVs assigned to the genus *Polaribacter.* This genus was present in the water cohorts but showed a much higher abundance in the AEW (inlet and outlet) cohorts. Members of this genus have been found in RAS compartments in different geographic locations (Rud et al. 2016). In contrast, Rud et al. (2016) discovered a higher abundance of *Polaribacter* spp. in tank biofilms than in water in a RAS system.

The genus *Vibrio* is one of the most important gram-negative pathogenic bacteria in abalone mariculture (Austin & Austin, 1993). Several *Vibrio* spp. were observed in the current study (Fig. 3.12). Although they did not occur in high abundance throughout most cohorts, their abundance was much lower in the SW_*Ulva* and AEW_*Ulva* cohorts, suggesting that *Ulva* has inhibitory effects on the growth and motility of *Vibrio* spp. According to a study conducted by Qiao et al. (2021), *U. fasciata* significantly inhibited the motility and biofilm inhibition of *V. parahaemolyticus,* proving that seaweed *U. fasciata* has promising prospects as an environmentally friendly preventative measure to limit the occurrence of vibriosis in mariculture. Moreover, the current study found that the relative abundance of taxa belonging

to *Pseudoalteromonas*, *Psychrilyobacter*, and *Vibrio* genera reduced from the inlet to the outlet of *Ulva* paddle-raceways that received abalone effluent water, owing to *Ulva* spp.'s antimicrobial capabilities or that of the microorganisms associated with it. Thus, the data in the current study support the hypothesis that *Ulva* spp. positively regulates microbial dynamics in aquaculture systems, reducing potential harmful bacteria and improving the overall microbial health of the system. The species *Vibrio campbellii, V. alginolyticus* and *V. parahaemolyticus*, *V. harveyi* and *V. splendidus* I, *V. carchariae*, and *V. anguillarum* have previously been found to be associated with abalone diseases. However, the *Vibrio* spp. listed in the current study had low prevalence across all cohorts. The species *V. tritonius*, which was observed to be higher in AEW_In, plays an important role in the production of hydrogen in mesophilic conditions, not only from mannitol, which is the major carbohydrate found in brown seaweed, but also in powdered seaweed. The species has an advantage over other genera in that it can produce significant amounts of H_2 under more saline conditions (Matsumura et al., 2014). It should be noted, however, that while the 16S rRNA gene can be used to classify *Vibrio* at the genus level, it may not have enough resolution for *Vibrio* at the species level (Thompson et al., 2005) and should be used with caution when interpreting the diversity of *Vibrio* communities.

In the AEW_In cohort, *Agarivorans gilvus* was identified as being differentially abundant compared with other cohorts. Members of the aerobic genus *Agarivorans* can produce agarose and catalyse the hydrolysis of agar. Several fish and shellfish pathogens, such as *Tenacibaculum* spp. and *Psychrobacter* spp., were detected in this study. Both genera were observed to have increased relative abundance in the AEW_In cohort and have been previously reported to have several species that are fish pathogens (Piñeiro-Vidal et al., 2008; Olsen et al., 2017). Members of the Rickettsial group, although less prominent, were also present in this study. Although not detected in the current study, a member of the Rickettsia-like prokaryote (RLP), *Xenohaliotis californiensis*, is a well-known pathogen that causes withering syndrome in abalone. The organism invades the digestive gland, and the animal exhibits a loss of condition and atrophy of the foot muscle. Moreover, actinomycetes were also present in this study. These gram-positive bacteria can produce secondary metabolites that are commercially and biotechnologically important. Antibiotics, anticancer agents,

immunosuppressive agents, and enzymes are examples of secondary metabolites commonly used in medicine. In addition, these metabolites are known to possess antibacterial, antifungal, neuritogenic, anticancer, antialgal, antimalarial, and anti-inflammatory properties (Das et al., 2006; Valli et al., 2012; Singh et al., 2014). These actinomycetes may aid abalone defence mechanisms. Abalones are herbivores that primarily consume algae, which are composed of carbohydrate molecules such as alginate and cellulose (Erasmus et al., 1997). Enteric bacteria play a critical role in abalone digestion, particularly in the breakdown of cellulose (Daume, 2006). Large numbers of bacteria from the genera *Vibrio, Pseudomonas, Enterobacteria*, *Flavobacteria*, and *Moraxella* populate the digestive system of abalone, assisting in the digestion of seaweed (Erasmus et al., 1997). Bioremediation of nitrogenous compounds relies on maximising chemolithotrophic processes that remove potentially toxic compounds (ammonia and nitrite) via nitrification, which is mediated in a stepwise process by ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB). Taxa known to be involved in NOB, such as Nitrobacter, Nitrobacter, and Nitrococcus, were not identified; however, Nitrosomanadaceae were present in the AEW cohort, and although in low abundance (counts < 10), it does indicate that nitrification was occurring in the AEW system. Denitrifying bacteria are also considered important, particularly in recirculating aquaculture systems where nitrate accumulates because of the high nitrification capacity of biofilters. Taxa known to be involved in denitrification, such as *Pseudomonas*, were identified in the AEW system (inlet, outlet, and *Ulva*). *Pseudomonas* is a very diverse group, showing adaptability to a range of environmental niches and a broad ecological distribution, Furthermore, ASVs assigned to the family Pseudomonadaceae, which contains denitrifying and N_2-fixing bacteria, were present in the AEW system.

3.4.6 Putative metabolic functions

To examine the role of microorganisms in the IMTA and non-IMTA systems, Tax4fun was used to analyse the 16S rRNA sequencing data to identify putative functional roles. One of the major functional categories, metabolism, was significantly associated with all cohorts. The upregulated function of metabolism suggested the active involvement of microorganisms in the system. These results are consistent with those of previous studies, which reported that metabolism was the predominant pathway among the six KEGG pathways (Jeong et al., 2018;

Xie et al., 2020). Among the subcategories of these major pathways, carbohydrate, amino acid, cofactor, and vitamin metabolic pathways were the predominant pathways across cohorts. Metabolism of cofactors and vitamins pathway includes ubiquinone biosynthesis, which has been described as having bioactive compounds related to energy metabolism, immunological competence, and antioxidation (Pravst et al., 2010). Similarly, KEGG pathways related to amino acid metabolism were highly abundant in all cohorts, more so in the AEW_*Ulva* and SW_*Ulva* cohorts. Amino acids such as tryptophan, phenylalanine, and lysin are commonly mentioned as essential nutritional requirements in several fish (Wilson & Halver, 1986). Another important KEGG pathway present in all cohorts was lipid metabolism, which includes the biosynthesis of unsaturated fatty acids and omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid). This pathway was observed to be higher in the AEW-*Ulva* cohort. Moreover, the predicted functionality profile demonstrated the potential for enrichment of xenobiotic biodegradation and metabolism in the AEW_*Ulva* cohort. This could be attributed to the enhanced capacity and the metabolic versatility of microorganisms in the AEW_*Ulva* cohort to break down and metabolize xenobiotic compounds (i.e. from food additives, pollutants or drugs) introduced into the system (Hausner & Bathe, 2011). The presences of bacteria with xenobiotic degradative potential such as *pseudomonas* (Tirkey et al., 2022) and vital oil-degrading bacteria such as *Oleispira* (Tremblay et al., 2019) in the AEW_*Ulva* cohort supports with this observation. However, it is important to note that while the interpretation in the current study might be limited, shotgun sequencing should be employed in future studies to improve our understanding of the metabolic pathways and the functional capabilities of microorganisms in these systems.

3.5 Conclusions and recommendations

Land-based abalone production has been increasing dramatically over recent years, leading to new challenges in fish health, water chemistry, and potential impacts on later life performance. This study utilised metagenomics to provide insight into the function of IMTA abalone effluent, seawater and *Ulva* microbiota. The 16S rRNA gene sequencing showed that the microbiota was dominated by Proteobacteria and Bacteroidota phyla in both AEW, SW, and *Ulva*, whereas genera such as *Vibrio*, *Granulosicoccus*, and *Pseudoalteromonas* dominated both AEW and SW systems. Comparative metagenomic analysis showed that all

metabolism categories were enriched in the systems. Most importantly, taxa that have previously been recognised for their role in *Ulva* morphogenesis, defence mechanisms against harmful bacteria, and assisting in the digestion of *Ulva* in abalone were identified in the IMTA system. Taxonomic profiling suggested that the bacteria inhabiting the *Ulva*, or the *Ulva* itself, can release antimicrobial compounds capable of inhibiting *Vibrio* spp. and other bacteria. As such, *Ulva* has an indirect impact on the microbiome composition of the system. There were some seasonal variations in the community structure of bacteria, such as a high diversity of bacterial communities in the summer and spring (AEW) cohorts. This could be because increased environmental temperatures can influence the temperature of the system in which animals are being cultured, and this, together with organic nutrients, can create an environment that is perfect for the proliferation of other bacterial species. To understand why certain groups of microbes are abundant in AEW or SW systems and across seasons, future research should focus on increasing sample size and using shotgun metagenomic approaches to identify the dominant microbiome functions and determine their importance for host seaweed-abalone growth and adaptation in IMTA systems. The implementation of effective aquaculture practises, in conjunction with the deployment of biomonitoring techniques, is essential for achieving sustainable and productive aquaculture. Monitoring water quality indicators such as temperature, salinity, dissolved oxygen, pH, and nutrient levels is crucial for cultivated species, and automated sensors and real-time monitoring systems improve data collection efficiency. For future studies, biosensors should be considered for the identification and quantification of targeted waterborne diseases or contaminants. Monitoring can be prioritised by season, as demonstrated in this study, where high microbial diversity was observed during summer and spring seasons. Although biosensors exist for the detection of *Vibrio*, additional development may be necessary to integrate them into IMTA systems (Stocker et al., 2003). Moreover, during periods of higher *Vibrio* numbers, it is advisable to implement more stringent biosecurity measures aimed at preventing the introduction of *Vibrio*.

Chapter 4: Fungal communities associated with recirculating abalone-*Ulva* integrated aquaculture systems

4.1 Introduction

More than half of the fish used for human consumption is produced from aquaculture, making it the world's fastest-growing food-producing industry (Nielsen et al., 2016; Fečkaninová et al., 2017; Liu et al., 2017; O'Neill et al., 2019; O'Neill et al., 2020; O'Neill & Rowan, 2022). Aquaculture has consistently expanded at a rate of 5.8% annually (2001–2016), reaching 46% in 2016 from 25.7% in 2001 (FAO, 2018). This is mostly attributable to the commercialisation of farmed aquatic animals such as shrimp, salmon, bivalves, tilapia, and catfish (Sukhdhane et al., 2018). There has also been a growing interest in the exploitation of low-trophic species and low-cost environmentally friendly "natural" aquaculture processes, which has led to the adoption of integrated multi-trophic aquaculture systems (IMTA) and accelerated efforts to adopt eco-innovation and improve the monitoring of traditional processes (Granada et al., 2016; Tahar et al., 2018a, 2018b; Naughton et al., 2020). This practice minimises or eliminates waste and boosts the overall productivity of the food system (Troell et al., 2003; Neori et al., 2004; Chopin, 2006). South Africa has been successfully practicing IMTA for over two decades by integrating the production of a local abalone species *Haliotis midae,* as the primary cultured species with the seaweed *Ulva lacinulata* as a biofilter and a source of supplementary feed for the abalone. The conversion of waste from abalone into a resource helps mitigate the potentially negative environmental effects of the commercial process (Biswas et al., 2020; Sickander & Filgueira, 2022). However, there are potential biosecurity risks associated with water recirculation and the utilisation of extractive species reared in effluent water in the IMTA systems as supplementary feed because extractive organisms can accumulate pollutants, such as heavy metals, and harbour potentially harmful or opportunistic microorganisms that could be transferred between systems on the farm and compromise healthy animals.

There has been limited research on the possible human and animal health benefits or risks of growing organisms, such as abalone and seaweed, in integrated aquaculture systems and the

effects of this integration on the microbiota, particularly with respect to fungi. Fungal infections are the second most prevalent cause of disease after bacterial infections, causing substantial losses in the aquaculture sector (Ramaiah, 2006; Van West, 2006; Gonçalves & Gagnon, 2011). These organisms present a significant risk to global health, biodiversity, and conservation because of their ability to infect and adapt to various hosts and environments (Andreou et al., 2009; Al-Shorbaji et al., 2015). In recent years, there has been a significant increase in the frequency and prevalence of fungal pathogens and fungal-like infections in cultured fisheries (Yanong, 2003; Holdich et al., 2009; Gozlan et al., 2014; Cutuli et al., 2015; Magwaza et al., 2017). The kingdom of fungi encompasses a broad range of fungal species, mostly from the Ascomycetes and Oomycetes, with morphological structures ranging from unicellular to multicellular. These microorganisms are highly diversified ecologically, phylogenetically, and morphologically (Alexopoulos et al., 1996; Seyedmousavi et al., 2013; Gozlan et al., 2014; Siriyappagouder et al., 2018). The majority of these organisms are widespread within aquatic environments, thereby posing a significant risk to aquaculture, and some have been shown to cause mass mortality (Kohlmeyer & Kohlmeyer, 1979; Hyde et al., 1998; Magwaza et al., 2017;). For example, aquaculture mortality caused by members of Ascomycetes, including *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., have been previously documented (Blaylock et al., 2001; Zhang et al., 2021). Previous case studies from India revealed concurrent infection of *Labeo calbasu* with *Aspergillus flavus* and *Aspergillus terrerus* (Chauhan et al., 2014).

Members of the Oomycetes, also known as 'water molds', are among the most significant emerging threats to the sustainability of aquaculture (Van West, 2006; Jiang et al., 2013b). They are increasingly being recognised as emerging pathogens of commercially important fish, molluscs, and shellfish and represent a recurring threat to global food security (Hatai et al., 2000; Leano et al., 2002; Muraosa et al., 2009; Macey et al., 2011, 2012; Muraosa et al., 2012). One of the most devastating diseases in the fish industry is caused by the oomycete *Aphanomyces invadans*, which is the causative agent of epizootic ulcerative syndrome (EUS), which has led to mass mortality in many countries of a wide variety of both wild and cultivated freshwater and brackish water fish species (Kumaresan et al., 2015). In Japan, five oomycete

genera have been identified as pathogenic oomycetes of marine shellfish, including abalone (Hatai et al., 2012). Members of the genera *Lagenidium, Haliphthoros*, *Halocrusticida, Halioticida*, and *Atkinsiella* were among these pathogens. *Halioticida* infections have been documented in abalone species, including *Haliotis midae, Haliotis rufescens,* and *Haliotis sieboldii* (Muraosa et al., 2009), where the animals exhibited typical clinical signs of tubercle mycosis. Members of the class Peronosporomycetes (formerly Oomycetes) are known to cause infections in abalone as well as in other commercially important marine species, including shrimp, crabs, and fish, and have severe consequences for commercial aquaculture operations (Willoughby, 1985; Kitancharoen et al., 1994; Roza & Hatai, 1999; Leao, 2002; Chukanhom et al., 2003; Atami et al., 2009; Muraosa et al., 2009). Other oomycetes, such as *Haliphthoros milfordens, Halocrusticida awabi*, *Atkinsiella dubia*, *Fusarium, Achlya*, and *Saprolegnia*, have previously been demonstrated to be major fish pathogens that affect marine fish and shellfish (Hatai, 1982; Noga, 1993; Kitancharoen et al., 1994; Nakamura & Hatai, 1995; Hatai, 2012; Oidtmann, 2012).

Oomycetes not only cause severe disease in shellfish but also in commercially cultivated seaweed. One of the most devastating diseases recorded in the seaweed industry is caused by the oomycetes *Olpidiopsis porphyrae* and *Pythium porphyrae* (Kim et al., 2014). As the industry develops and intensifies rapidly, disease outbreaks are an increasing source of concern. In addition, oomycete infections have been found in commercially important North Sea seaweeds such as *Chondrus crispus*, *Palmaria palmata*, and other kelp species (Bernard, 2018). Various seaweed-fungal associations have been studied, and it has been shown that red seaweeds (*Gelidiella, Gracilaria, Grateloupia, Halymenia, Palmaria, Plocamium, Portieria, Pyropia)* and brown seaweeds (*Adenocystis, Ascophyllum, Desmarestia, Dictyota, Fucus, Lobophora, Padina, Phaeurus, Sargassum, Stocheospermum*, and *Turbinaria*) harbour greater fungal species diversity than green seaweeds (*Acrosiphonia, Caulerpa, Halimeda, Monostroma,* and *Ulva*) (Suryanarayanan et al., 2010). Green seaweeds' short life cycle, slow growth of endosymbionts, and inhibitory function may contribute to low fungal diversity in association with these plants (Zuccaro & Mitchell, 2005). Overall, these pathogens reduce

seaweed biomass and production quality (Bernard, 2018; Ward et al., 2019), resulting in significant economic losses.

Not all fungi are detrimental, some fungi provide advantages in marine ecosystems. In most situations, fungi and oomycetes are saprophytic and perform an important ecological service by decomposing dead organic matter. Fungi isolated from marine or marine-related habitats are producers of several bioactive compounds, that have demonstrated antimicrobial activity against human and fish pathogenic microorganisms (Debbab et al., 2011; Tarman et al., 2011). For example, fungal strains such as *Penicillium spp*., *Fusarium spp*., and *Aspergillus* spp. isolated from marine plants and invertebrates have demonstrated a broad spectrum of antibiotic activity (Zhang et al., 2009; Zhou et al., 2014). The halotolerant yeast *Debaryomyces hanseniii*, capable of growing in a variety of environments, including the gut of fish (Andlid et al., 1995; Raggi et al., 2014), has been successfully used as a probiotic for cultivated species such as *Mycteroperca rosacea* (leopard grouper), *Sparus aurata* (gilthead seabream), and *Dicentrarchus labrax* (sea bass) and has been demonstrated to have positive effects on their survival, digestive functions, and immune function (Tovar-Ramírez et al., 2004, 2010; Reyes-Becerril et al., 2008; Reyes-Becerril et al., 2011). The study conducted by Macey and Coyne (2005) further demonstrated that feeding abalone a diet supplemented with a mixture of three probiotic, which included *D. hansenii* AY1, increased the protease activity in the intestinal region of the abalone gut, which improved protein digestion and absorption in the gastrointestinal tract of *H. midae* – in addition to stimulating the immune system and improving growth of the cultured abalone.

While progress has been made in documenting the abundance and diversity of fungal and oomycete (hereafter collectively referred to as fungi/fungal) communities in marine habitats, the diversity reported is still substantially lower than that in terrestrial environments and accounts for less than 1% of all known species (Jones, 2011; Serma, 2019), making them a poorly studied group. Recent advances in next-generation sequencing (NGS) have contributed to a significant increase in the number of fungi identified in the marine environment (Richards et al., 2015; Picard, 2017; Xu et al., 2017). The use of the fungal marker gene, the internal

transcribed spacer (ITS), offers a higher species resolution for identifying fungi (Schoch et al., 2012; Banos et al., 2018). As a result, fungal diversity is mostly studied using the ITS region of rDNA for barcoding (Roe et al., 2010; Dentinger et al., 2011; Schoch et al., 2012). However, marine fungi are underrepresented in marine metagenomic sequence data because amplicon sequencing based on the fungal ITS rDNA region also coamplifies other eukaryotes (invertebrates, plant hosts, or gelatinous zooplankton) (Amend et al., 2019; Gladfelter et al., 2019). Moreover, due to the lack of reference sequences in databases, many of the fungal taxa recovered by environmental ITS sequencing can merely be identified at the phylum or kingdom level (Banos et al., 2018). However, as recommended by the international fungal barcoding consortium, the ITS region of the nuclear ribosomal RNA gene is the primary fungal barcoding gene because it has the highest probability of identifying the widest range of fungi (Schoch et al., 2012; Fajarningsih, 2016). Hence, in the current study, the ITS2 region was selected to capture the diversity of the fungal community within the integrated abalone-*Ulva* IMTA systems, with 50% water recirculation, at Bufflejags Abalone farm in the Western Cape province of South Africa. The ITS2 region was targeted for NGS using primers ITS3_KYO2 and ITS4_KYO3 (Toju et al., 2012) (as demonstrated in Chapter 2). Therefore, the primary aim of this chapter is to explore the benefits and potential disease risks associated with fungal communities in such IMTA systems. Additionally, it aims compare the microbial communities of seawater from two different systems: an *Ulva* paddle raceway system that does not receive effluent, and an integrated multi-trophic aquaculture (IMTA) system that receives abalone effluents and recirculates 50% of its effluent. The study hypothesises that the IMTA system will have a more diverse fungal microbiome than the non-IMTA system and that the presence of *Ulva* spp. will favourably influence microbial dynamics, reducing potentially harmful fungi and oomycete and improving the system microbiome overall.

4.2 Materials and Methods

4.2.1 Sampling and DNA Isolation

Water (N = 40) and *Ulva* (N = 20) samples were collected from Buffeljags abalone farm, which is situated in a coastal area near a remote settlement of Buffeljags on the Cape South Coast of South Africa (34.7550° S, 19.6154° E). Both *Ulva* and water samples were collected from two separate *Ulva* production systems on the farm across four seasons: autumn (March 2020), winter (August 2020), spring (October 2020), and summer (January 2021). One system consisted of tanks that received seawater directly from the adjacent coastline (SW/non-IMTA), whereas the other system was comprised of raceways receiving abalone effluent water (AEW/IMTA). The *Ulva* samples were collected from within each *Ulva* raceway system, whereas the water samples were collected from the effluent seawater flowing from the abalone tanks into the *Ulva* raceway, henceforth referred to as the inlet, and the 'bioremediated' water flowing out of the *Ulva* raceways into the abalone tanks, henceforth referred to as the outlet of each system. Across the two systems, samples were grouped into six cohorts, namely AEW_in, AEW_out, AEW_*Ulva*, SW_in, SW_out, and SW_*Ulva*. A comprehensive description of the systems can be found in Chapter 3 and a comprehensive description of the samples can be found in Table S4.1. For the isolation of fungi and oomycetes from seawater and *Ulva*, samples were collected from the SW and AEW systems. Genomic DNA was isolated from the frozen microbial pellets, obtained from the seawater samples and *Ulva* using the QIAamp® DNA Micro kit (Qiagen, Cat. No. 56304) following the manufacturer's instructions. Details for the collection and preparation of DNA samples from seawater and *Ulva* samples are as described in Chapter 3, Section 3.2.

4.2.2 PCR amplification of ITS2 region

Fungal community profiling was performed by amplifying the nuclear ribosomal internal transcribed spacer 2 (ITS2) region with locus-specific forward (ITS3_KYO2) and reverse (ITS4_KYO3) (Toju et al., 2012) primers (Table 4.1). Each polymerase chain reaction (PCR) was carried out in a total reaction volume of 25 µL and consisted of 2.5 µL of 5 ng/µL DNA, 12.5 µL 2X KAPA HiFi HotStart Readymix (Roche; Cat # 07958935001; Lot: 0000105448), 0.5 µL of each primer, and 9 µL ddH_2O. The ITS2 amplicons were eluted in a volume directly

proportional to their concentration to normalise the amplicon concentration for indexing. The elution volumes ranged from 5 to 52.5 µL of 10 mM Tris, pH 8.0. A positive control consisting of equimolar amounts of DNA from 8 fungal species (*Fusarium* spp., *Halioticida noduliformans*, *Pythium* spp., *Saprolegnia ferax*, *Saprolegnia parasitica*, *Saprolegnia australis*, *Aphanomyces astaci*, and *Achyla bisexual*) previously isolated from freshwater and marine environments (Mariska Greeff-Laubscher, pers. Comm.), were included at the same concentration as described above. The PCR amplification cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 25 cycles at 94°C for 20 s, 47°C for 30 s, and 72°C for 20 s, followed by 72°C for 7 min. Negative controls, containing all components other than the DNA templates, were run in parallel. Amplified PCR products, together with the controls (negative and positive), were electrophoresed on a 1% agarose gel at 100V to confirm successful amplification, reaction specificity, and fragment size (540-660 bp fragment expected). PCR products (1 µL of each) were analysed on an Agilent 4200 TapeStation system using a D1000 ScreenTape Assay (Agilent, Cat # 5067-5582) to determine amplicon sizes. All PCR reactions and subsequent quality control steps were performed at the Centre of Proteomics and Genomics Research (CPGR; https://www.cpgr.org.za/).

Table 4.1. Primers used in the study to amplify the nuclear ribosomal internal transcribed spacer 2 (ITS2) region containing Illumina adapter overhang sequences underlined.

Primer	Oligonucleotide sequence (5'- 3')	Targeted region (size in bp)	Annealing temperature	Source
ITS3_KYO2F	GATGAAGAACGYAGYRAA TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CTBTTVCCKCTTCACTCG	ITS2 (540-660)	47 °C	Toju et al., 2012
ITS4_KYO3R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG			

4.2.3 Library preparation and next-generation sequencing

PCR purification, indexing, and library preparation were performed at CPGR, where the PCR products from each sample were used to create libraries with unique barcodes (N = 64 libraries). Briefly, PCR products were purified using AMPure XP beads (Beckman Coulter; Cat # A63881; Lot 17970000) as per the manufacturer's instructions. Thereafter, dual indices and Illumina sequencing adaptors were attached to the purified PCR products using the Nextera XT index Kit v2 (Illumina; Cat # 15052163; Lot 20400443). KAPA HiFi HotStart ReadyMix was

used for the latter PCR reactions, whereafter a second PCR purification step was carried out to purify the indexed sequencing libraries using AMPure XP beads. After purification of the individual libraries, the concentration of dsDNA in each library was quantified using the fluorometric Qubit 1 × dsDNA HS Assay (Thermo Fisher Scientific; Cat# Q33231). Library sizes were verified using the D1000 assay on the Agilent 4200 TapeStation.

The DNA concentration in each of the ITS2 libraries was normalised, and 50 ng of each ITS2 library was pooled for sequencing in separate sequencing runs. The concentration of amplifiable Illumina adapter-ligated molecules in the final library pools was confirmed by qPCR using the KAPA Illumina Library Quantification Kit (Roche; Cat # 07960204001; Lot: 0000090011). A library dilution series of 1:10 000, 1:100 000, 1:1000 000, and 1:10 000 000 of each library pool was quantified. The overall fragment sizes of the pooled ITS2 libraries were determined using the D1000 assay on the Agilent 4200 TapeStation. The indexed ITS2 libraries ranged from 528 to 701 bp for the ITS2 gene region. The larger fragments are likely to be underrepresented in the sequencing as they are likely to cluster less efficiently than the shorter fragments. The size - adjusted library concentrations were calculated, and the libraries were diluted to 4 nM. The 4 nM pooled sequencing libraries were denatured using 0.2 N NaOH and diluted to 5 nM. The libraries were combined with the denatured PhiX control [Illumina, Cat # 15017666; Lot: 20379193 (batch 1) and Lot 20472954 (batch 2)] at a spike-in concentration of 10% v/v, as advised by the Illumina 16S Metagenomic Sequencing Library Preparation guide. Additionally, the sequencing libraries were heat denatured for 2 mins and placed on ice for 5 mins. The denatured libraries were loaded on the Illumina MiSeq instrument and sequenced using the Illumina MiSeq Reagent Kit v2 (500 cycles), consisting of the buffer cartridge, the flow cell, and incorporation buffer. The sequencer was programmed to perform a paired-end, dual - indexed 2 × 250 - cycle sequencing run for each library pool. FASTQ files were automatically generated at the end of each run and saved on the MiSeq onboard computer. The quality of the sequence run was assessed using Illumina Sequence Analysis.

4.2.4 Raw data processing, data filtering and normalisation

Illumina MiSeq software was used to assess the raw data through the 16S Metagenomic workflow. After sequencing, the primary analysis of the raw FASTQ data was processed using QIIME2 (version 2022.2; *http://qiime2.org/*) (Caporaso et al., 2020). Computations were performed using facilities provided by the University of Cape Town ICTS High Performance Computing team: hpc.uct.ac.za. The qiime2-dada plugin was used for filtering, dereplication, and chimera removal. Given the improved resolution observed in Chapter 2 using only the forward reads for subsequent analyses, reverse reads were discarded, and forward reads were used to assess the fungal and oomycete communities in the IMTA vs. non-IMTA systems in this chapter. Forward reads were trimmed to a maximum read length of 228 bp to remove the ends of reads that contain higher expected error rates. The QIIME2 plugin, ITSxpress (Rivers et al., 2018), was used for the identification of exact sequence variants in the region of interest (ITS2). Taxonomic assignments were carried out for the amplicon sequence variants (ASVs) using the Naïve Bayes classifier q2-feature-classifier in QIIME2 (https://github.com/qiime2/q2-feature-classifier) (Bokulich et al., 2018), with a confidence threshold of 0.97. The UNITE database (Nilsson et al., 2018) was combined with a custom oomycete database that was curated using a *H. noduliformans* sequence that was amplified using PCR, sequenced using sanger sequencing, and analysed to get a consensus sequence that was added to the custom reference database.

4.2.5 Data analysis

Before conducting any statistical analysis, the ASVs were filtered using the online webtool MicrobiomeAnalyst (Dhariwal et l., 2017; Chong et al., 2020) to retain those that had a count of at least two in two samples at 10% prevalence. Features (ASVs) containing only or mostly zeros were removed to account for possible sequencing errors. Subsequently, this marginally filtered dataset was used to calculate alpha (within sample) diversity statistics and to quantify overall ASV abundance. Alpha diversity measures were assessed using the R *phyloseq* (McMurdie & Holmes, 2013) and *vegan* (Lixon, 2003) packages implemented in MicrobiomeAnalyst. Various alpha diversity measures were calculated, including the Chao1 (Chao, 1984), Shannon (Shannon, 1948), and Simpson (Simpson, 1949) indices to assess

species uniqueness, richness, and evenness, respectively. The statistical significance of differences between cohort-wise (AEW_In, AEW_Out, AEW_*Ulva*, SW_In, SW_Out, and SW_*Ulva*) alpha (α) diversity was assessed using an analysis of variance (ANOVA; statistical significance at $p < 0.05$) for each alpha diversity measure. Relative log expression (RLE) transformation (Hawinkel 2015) was performed to account for uneven sequencing depth, under-sampling, and data sparsity. The normalised dataset was used for multivariate beta (β) diversity (between samples) tests and univariate differential abundance analysis. The β-diversity analyses were also carried out in MicrobiomeAnalyst using the same R packages, whereby two multivariate ordination methods, a between-sample distance based on principal co-ordinate (PCoA) and a between-sample similarity based on non-metric multidimensional scaling (NMDS) analyses, were conducted at phylum and genus levels to provide an overview of distance and similarity present in IMTA (AEW) and non-IMTA (SW) systems, as well as the mycobiome on the *Ulva* within each system. Both analyses were based on a Bray - Curtis distance matrix. The PCoA analysis evaluates the between-sample distances based on a Bray-Curtis dissimilarity matrix in a linear fashion, whereas the NMDS analysis scales the between-sample distances according to rank to assess cohort variation, which is particularly useful for counts of abundance. Overall, NMDS has been found to possess greater power when assessing non-linear relationships between cohorts (Ramette, 2007). Corresponding statistical significance ($p < 0.05$) was evaluated using permutational multivariant analysis of variance (PERMANOVA; Anderson, 2001), permutational analysis of multivariate dispersions (PERMDISP; Anderson, 2006), and lastly, through an analysis of similarities (ANOSIM; Clarke, 1993). Hierarchical dendrogram relationships between cohorts were assessed at the genus level through a sample-based clustering analysis, based on the Bray-Curtis distance matrix and Ward clustering algorithm, and visualised as a cluster analysis. The univariate method, DESeq2 (Love et al., 2014), at an adjusted *p*-value cut-off of 0.05 was used to assess the differential abundance of ASVs at the family and genus levels across cohorts. The false discovery rate (FDR) was calculated to adjust *p*-values for multiple comparisons to reduce the possibility of type I errors (false positives) (Benjamini and Hochberg 1995). Boxplots were constructed for the top 20 differentially abundant ASVs at the family and genus levels. Using the normalised dataset, the mean, variance, and mean dispersion estimates for each ASV

were calculated to identify ASVs with means that exceeded the threshold calculated for that ASV (statistical significance: $p < 0.05$) (Hawinkel 2015).

Overall ASV abundance was assessed by plotting bar graphs at corresponding levels of taxonomic classification based on relative (%) abundances (n < 10 counts are merged and denoted as “others”). The linear discriminant analysis effect size (LEfSe) was calculated using linear discriminant analysis to test for significant associations between cohorts and bacterial communities, and the 25 ASVs that most likely (Kruskal-Walls rank sum test; significance: $p < 0.05$; LDA score > 2) explain differences between the cohorts were identified. Following that, the significant ASVs ($p < 0.05$) were retained, and a pairwise Wilcoxon test was used to assess the effect size of the significant ASVs in the linear discriminant analysis. The samples were collapsed into their respective cohorts based on the systems (AEW and SW) they were collected from and the components of the systems (inlet, outlet, and *Ulva*) across different seasons. However, as a result of the absence of duplicate samples, the SW cohorts were excluded for the purpose of seasonal analysis to avoid collapsing *Ulva* from different systems (SW_*Ulva* and AEW_*Ulva*) into one season, i.e., Winter_*Ulva* (SW_*Ulva* and AEW_*Ulva*).

4.3 Results

4.3.1 Fungal raw data processing

In total, 19 919 573 raw sequence reads were obtained from all samples (Table S4.1) included in this study, with a minimum and maximum read count within samples ranging from 42 121 to 123 0705 and an average of 321 288 reads per sample. After quality filtering and exclusion of unidentified sequences (18 261 919) (Table S4.1), a total of 1 657 658 sequence reads were retained across the 60 metagenomic libraries created in this study, averaging 27 627 reads per sample. The obtained library size was assessed to check data integrity, and a large difference between the minimum and maximum library sizes was noted between samples (15 to 521 190). Three samples (U_H_4, U_H_1, and U_H_2) were excluded for downstream analysis due to their low read count, resulting in 27 621 average reads per sample. After reads were mapped to the custom reference database, a total of 169 individual ASVs were detected. Subsequent filtering in MicrobiomeAnalyst resulted in the removal of 40 low abundance features, and 4 low variance features were removed based on the inter-quartile range (iqr). The number of features remaining after the filtering steps was 125 ASVs prior to conducting the beta diversity analyses. Sequences that could not be assigned to any taxonomic groups were assigned as unclassified.

4.3.2 Alpha diversity

Subsequently, two ecological diversity metrics (α- and β-diversity) were assessed to evaluate the microbial community compositional differences across the six cohorts and contribute to our knowledge of fungal communities associated with recirculating aquaculture systems, particularly an abalone-*Ulva* IMTA system, across different seasons. Rarefaction curves were plotted to account for sampling depth. The rarefaction curves reached a plateau, except for two samples (U_H_2 and U_H_3) (Fig. 4.1), indicating that there was sufficient sequence depth for the majority of the reads in those samples.

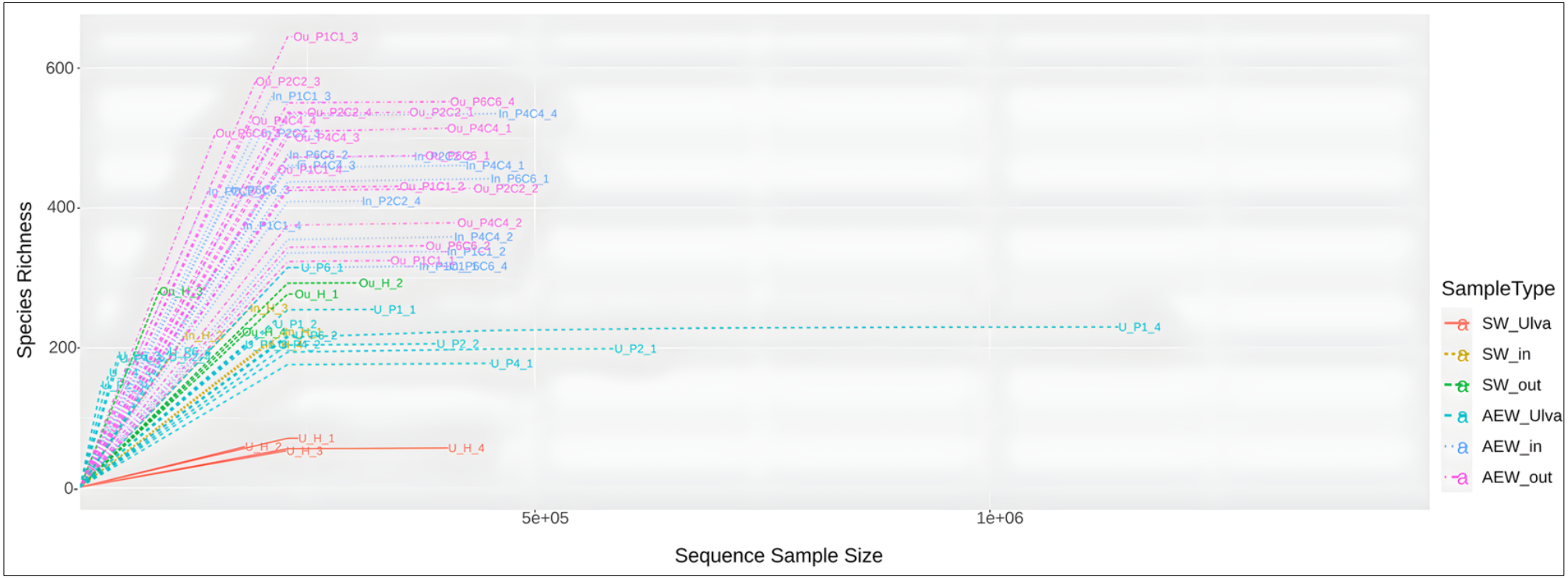

Figure 4.1. Rarefaction curves of unfiltered ASVs, where samples were grouped into AEW_Out, AEW_In, SW_*Ulva*, AEW_*Ulva*, SW_In, and SW_Out cohorts (N = 60). Samples denoted by "H" are from the SW (non-IMTA), while samples denoted by "P" are from the AEW (IMTA) system. *Ulva* samples are denoted by "U", Inlets are denoted by (In), and outlets are denoted by (Ou).

All the diversity measures (Chao1, Simpson, and Shannon indices) of taxa that were assigned to taxonomy showed that the mycobiome associated with the effluent water (AEW) samples was significantly less diverse than that of seawater SW samples (ANOVA; $p < 0.001$) (Fig. 4.2). Specifically, the Chao1 index, which represents the observed richness in terms of the number of genera identified, showed that the abalone effluent water had a significantly lower richness than the non-effluent/seawater samples, indicating that there are a higher number of unique ASVs in the SW tanks. The mycobiome of *Ulva* from both the seawater and effluent systems was significantly less diverse compared to the mycobiome associated with water samples (ANOVA; $p < 0.001$) in both systems. Interestingly, *Ulva* samples from both the SW and AEW systems had a similar number of unique ASVs (Fig. 4.2), indicating the extent of diversity associated with *Ulva* is similar regardless of the system type. The water samples in the SW cohort (SW_In and SW_Out) had a higher evenness (Shannon index) compared to the AEW_In, AEW_Out, and AEW_*Ulva* cohorts, which indicates a more even distribution (uniformity of the abundance of each species present) of taxa in the SW cohort. It is important to note that similar patterns were observed at the family and genus levels. However, these observations are based on only eight samples in the SW cohort, and the limited sample size should be taken into account when interpreting the results.

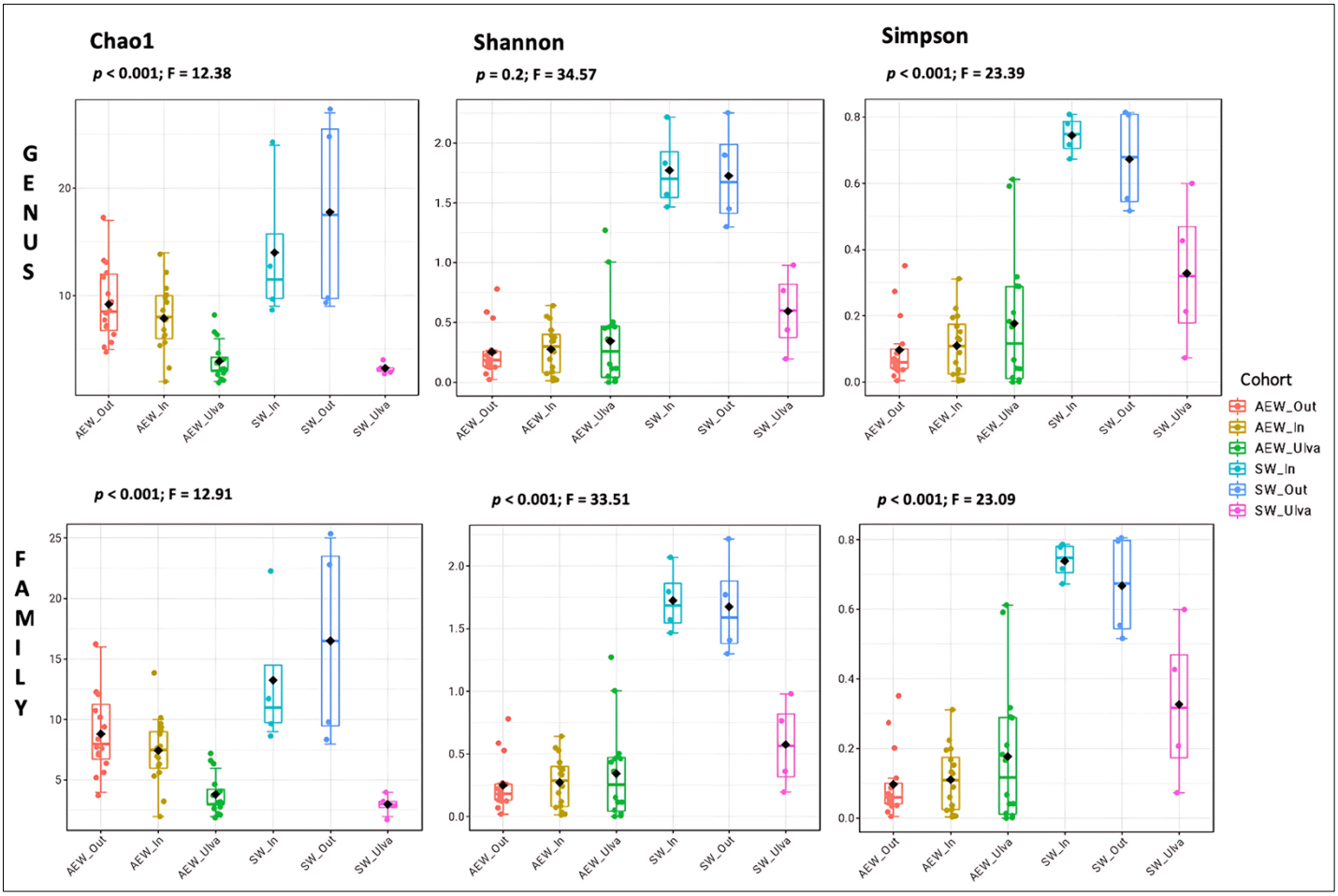

Figure 4.2. Average alpha diversity measures (Chao1, Shannon, and Simpson) for each cohort (AEW_Out, AEW_IN, AEW_Ulva, SW_Out, SW, IN and SW_Ulva) at family- and genus- level, where the minimum, maximum and mean as well as ANOVA F-values and *p*-values are indicated for each cohort. Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

The fungal diversity across cohorts were further analysed by including ASVs that were not assigned to taxonomy (Fig. 4.3), hence providing a truer representation of the data. The ASV richness varied significantly across six cohorts from the two systems (SW and AEW). Significant differences were observed between the *Ulva* cohorts and water cohorts (SW and AEW), where Chao1 was significantly higher ($p < 0.001$) for water cohorts when compared to *Ulva* cohorts (AEW_*Ulva* and SW_*Ulva*), indicating a higher degree of unique ASVs in the water columns. Significant differences were also observed between the SW and AEW cohorts for the Shannon diversity index (ANOVA; $F = 4.97$; $p < 0.001$), indicating a significantly higher degree of richness of the fungi in the SW_In and SW_Out cohorts compared to the AEW_In and AEW_Out cohorts

(Fig. 4.3). Similarly, the Simpson diversity index was also significantly higher (ANOVA; F = 5.84; p < = 0.001), indicating a high degree of uniformity in the fungi present in the SW_In and SW_Out cohorts compared to the AEW_In and AEW_Out. However, the richness estimator Chao1 was significantly higher for the AEW cohorts when compared to the *Ulva* SW cohorts (F = 55.076, p < 0.001), indicating a higher degree of unique ASVs in the AEW cohorts (Fig. 4.3).

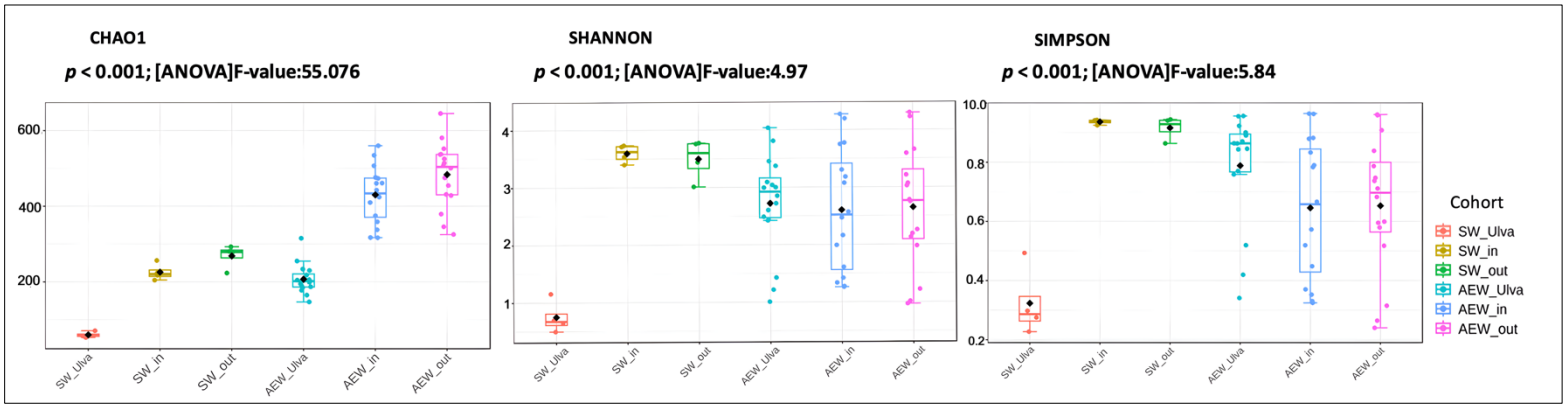

Figure 4.3. Average alpha diversity measures (Chao1, Shannon, and Simpson) for the observed fungal amplicon sequence variant (ASVs) for each cohort (AEW_Out, AEW_IN, AEW_*Ulva*, SW_Out, SW, IN and SW_*Ulva*) where the minimum, maximum and mean as well as ANOVA F-values and *p*-values are indicated for each cohort.

4.3.3 Seasonal alpha diversity

The alpha diversity was evaluated at genus level to assess seasonal fungal diversity. Fungal diversity varied significantly among sampling periods (different seasons) from the respective cohorts (AEW, SW, and *Ulva* from either system type) (Fig. 4.4). Significant differences were observed for the Chao 1 (F = 11.2, p < 0.001), Shannon (F = 15.7; p < 0.001) and Simpson (F = 9.7; p < 0.001), diversity indices (Fig. 4.4). The richness estimator Chao1 was significantly higher in summer and winter samples collected from the SW system (F = 11.2; p < 0.001) compared with all the other samples, indicating a higher degree of unique ASVs in these samples. Moreover, the Shannon diversity exhibited a high degree of richness during the winter (SW) and summer (SW) cohorts (p < 0.001), while the Simpson diversity exhibited a high degree of diversity for all the water cohorts across seasons (p < 0.001), indicating a high degree of uniformity in the fungi present. The *Ulva* cohorts displayed the lowest level of alpha

diversity and unique ASVs ($p < 0.001$) across all seasons. Nevertheless, as a result of the absence of duplicate samples, the SW cohorts were excluded for the purpose of seasonal analysis, and the AEW cohort was evaluated independently (Figure 4.5). Upon conducting a more comprehensive assessment, there was a difference observed between the inlet and outlet of the AEW summer season. Specifically, the outlet exhibited a higher degree of unique ASVs compared to the inlet (Figure 4.5; $p = 0.003$). No significant statistical differences were observed for the Shannon and Simpson diversity indices of the AEW system (Figure 4.5).

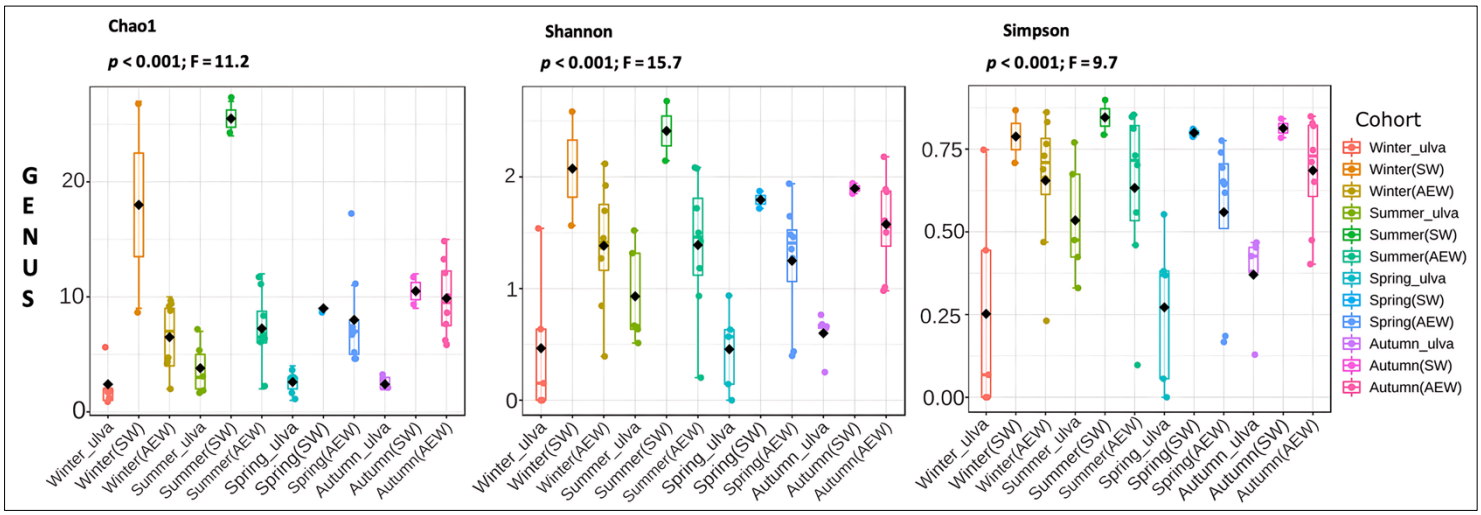

Figure 4.4. Average alpha diversity measures (Chao1, Shannon, and Simpson) for each cohort at genus-level across seasons where the minimum, maximum and mean as well as ANOVA F-values and *p*-values are indicated for each cohort. Samples for each season consisted of grouped inlets and outlet of the respective systems i.e., Winter (SW (In + Out)). *Ulva* samples of the respective systems were grouped together according to sampled season i.e., Winter_*Ulva* consisted of AEW_*Ulva* + SW_*Ulva* samples. Samples for each season AEW system (N=8), samples for each season SW system (N=2), *Ulva* samples per season (N= 5).

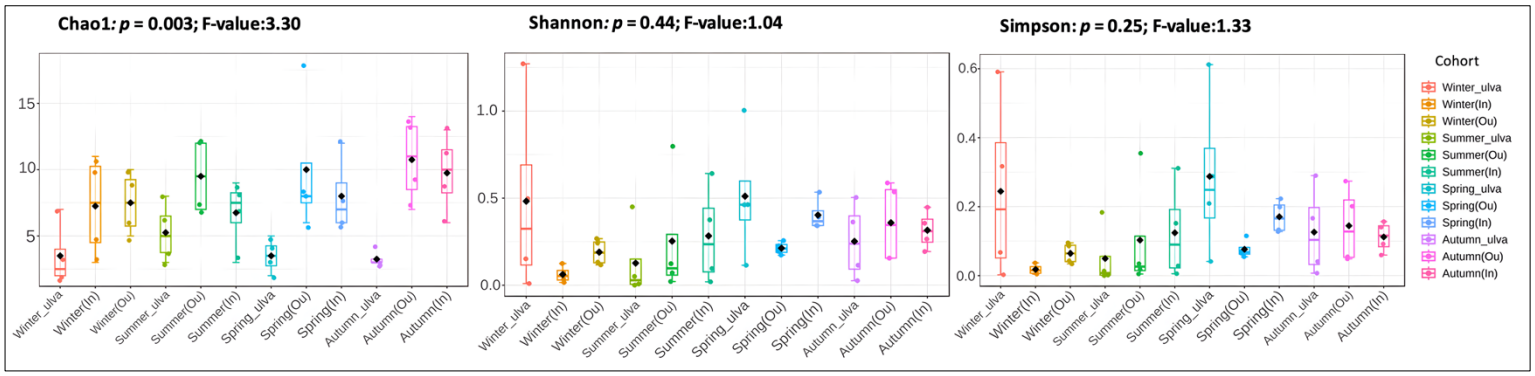

Figure 4.5. Average alpha diversity measures (Chao1, Shannon, and Simpson) for each AEW cohort at genus-level across seasons where the minimum, maximum and mean as well as ANOVA F-values and *p*-values are indicated for each cohort. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Ou) for respective seasons.

4.3.4 Beta diversity

A beta analysis was conducted for principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) to elucidate the diversity between the SW (non-IMTA) and AEW (IMTA) cohorts. The comparison between the six cohorts (AEW_In, AEW_Out, AEW_*Ulva*, SW_In, SW_Out, and SW_*Ulva*) showed a partial degree of overlap between cohorts (Fig. 4.6), indicating differences in fungal composition. The PCoA analysis exhibited a degree of separation between the AEW and SW water cohorts. The *Ulva* cohorts from both systems clustered more closely to the SW cohorts, indicating that the *Ulva* mycobiome was more similar to the SW mycobiome than the AEW mycobiome. Moreover, the AEW_*Ulva* cohort clustered closely with the SW_*Ulva* cohort, indicating similar fungal diversity between the *Ulva* cohorts. Notably, the ANOISM analysis for the PCoA ordination plot showed significant differences between cohorts (ANOISM; $p < 0.01$), and significant differences in the dispersion were detected between the fungal communities of the AEW and SW cohorts (PERMDISP; $p < 0.01$) (Table 4.2). Moreover, the NMDS analysis demonstrated that fungal communities associated with AEW_*Ulva* and SW_*Ulva* clustered separately, with a small extent of overlap, indicating a high degree of differentiation of the fungal mycobiome associated with *Ulva* grown in the two systems, with a moderate sharing of fungal communities. Similarly, the fungal communities associated with water collected from the AEW and SW systems clustered separately (Fig. 4.6), with overlap between fungal communities

sampled from the inlet and outlet of each system (AEW or SW). This was further supported by the statistical analyses (Table 4.2). The permutational multivariate analysis of variance (PERMANOVA), which tests for homogeneity across data points, further supports the high degree of dissimilarity with a significant difference (PERMANOVA; R^2 = 0.51, p < 0.001) between the cohorts (Table 4.2). The ANOISM (R = 0.38, p < 0.001) and PERMDISP (F = 17.6, p < 0.001), further supported compositional differences between cohorts. These results indicate that the diversity of fungal communities between the systems (AEW and SW) was significantly different.

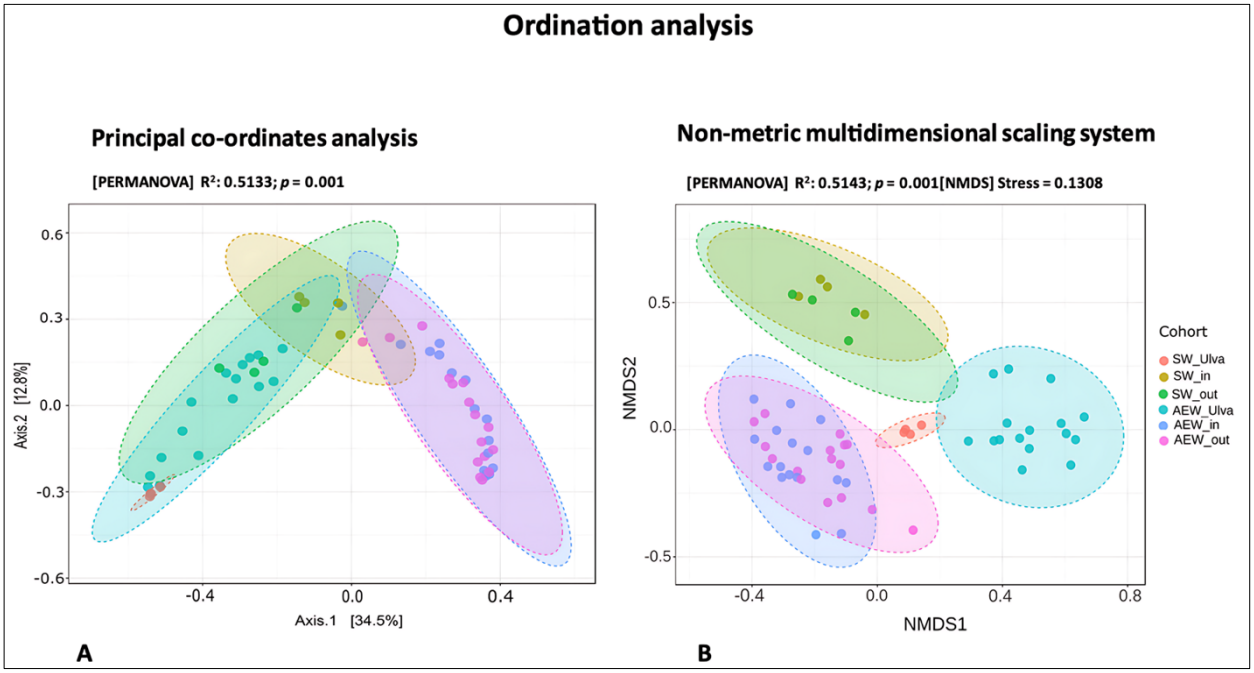

Figure 4.6. Principal co-ordinate analysis (PCoA; A) and Non-metric multi- dimensional scaling analysis (NMDS; B) for the observed fungal amplicon sequence variant (ASVs) for each cohort (AEW_Out, AEW_IN, AEW_*Ulva*, SW_Out, SW, IN and SW_*Ulva*) where the minimum, maximum and mean as well as ANOVA F-values and *p*-values are indicated for each cohort.

Table 4.2. Statistical assessment of similarity (PERMANOVA, PERMDISP, and ANOSIM) where corresponding *p*-values are indicated in brackets, indicative of moderate differences in bacterial community composition between cohorts.

Analysis		
Analysis	PCoA	NMDS
PERMANOVA; R^2	0.51($p < 0.01$)	0.51 ($p < 0.001$)
PERMDISP; F	8.31 ($p < 0.001$)	17.6 ($p < 0.001$)
ANOSIM; R	0.64 ($p < 0.01$)	0.38 ($p < 0.001$)

4.3.5 Seasonal beta diversity

A beta analysis was also conducted for principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) for seasonal diversity at the genus level. A high degree of overlap between samples was exhibited from each cohort collected from spring, summer, autumn, and winter (Fig. 4.7). No distinct seasonal patterns were observed based on the NMDS and PCoA, indicating a degree of similarity in the diversity of mycobiome across seasons. However, the statistical analysis on PCoA (PERMANOVA; R^2 = 0.56, p = 0.001, PERMDISP; F = 17.6, $p < 0.001$, ANOISM; R = 0.33, $p < 0.001$) indicated a significant difference between the cohorts. This was further supported by the NMDS analysis (PERMANOVA; R^2 = 0.56, p = 0.001, PERMDISP; F = 2.28, p = 0.02, ANOISM; R = 0.33, $p < 0.001$) (Table 4.3). The substantial seasonal overlap observed between samples from both PCoA and NMDS analyses may potentially be attributed to the limited sample size, which could be improved with a larger sample size. Moreover, in order to conduct a more comprehensive seasonal analysis, the samples collected from the SW cohort were excluded from the analysis due to the lack of replicates. In this case, samples clustered according to water samples (AEW_In and AEW_Out) vs. *Ulva* samples, with no seasonal patterns observed (Fig. 4.8).

Table 4.3. Statistical assessment of distance (PCoA) and similarity (NMDS) (PERMANOVA, PERMDISP, and ANOSIM) where corresponding *p*-values are indicated in brackets, indicative of moderate differences in bacterial community composition across seasons at genus level.

Analysis	PCoA	NMDS
PERMANOVA; R^2	0.56 (p = 0.001)	0.56 (p = 0.001)
PERMDISP; F	17.6 (p < 0.001)	2.28 (p = 0.02)
ANOSIM; R	0.33 (p < 0.001)	0.33 (p < 0.001)

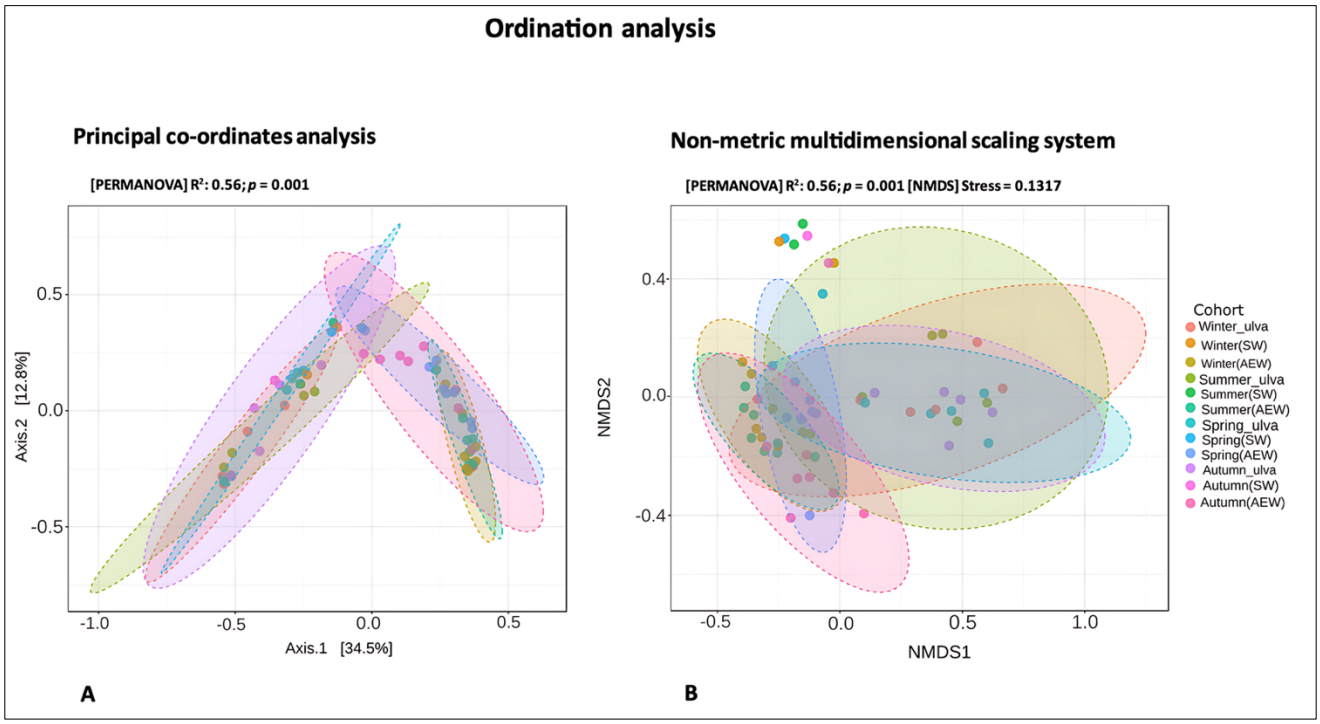

Figure 4.7. Principal co-ordinate analysis (PCoA; A) and Non-metric multi- dimensional scaling analysis (NMDS; B) at genus level showing the difference in fungal communities of non-IMTA and IMTA samples based on Bray–Curtis dissimilarity metrics shows the β-diversity of the fungal communities across seasons. Samples for each season consisted of grouped inlets and outlet of the respective systems i.e., Winter (SW (In + Out)). *Ulva* samples of the respective systems were grouped together according to sampled season i.e., Winter_*Ulva* consisted of AEW_*Ulva* + SW_*Ulva* samples. Samples for each season AEW system (N=8), samples for each season SW system (N=2), *Ulva* samples per season (N= 5).

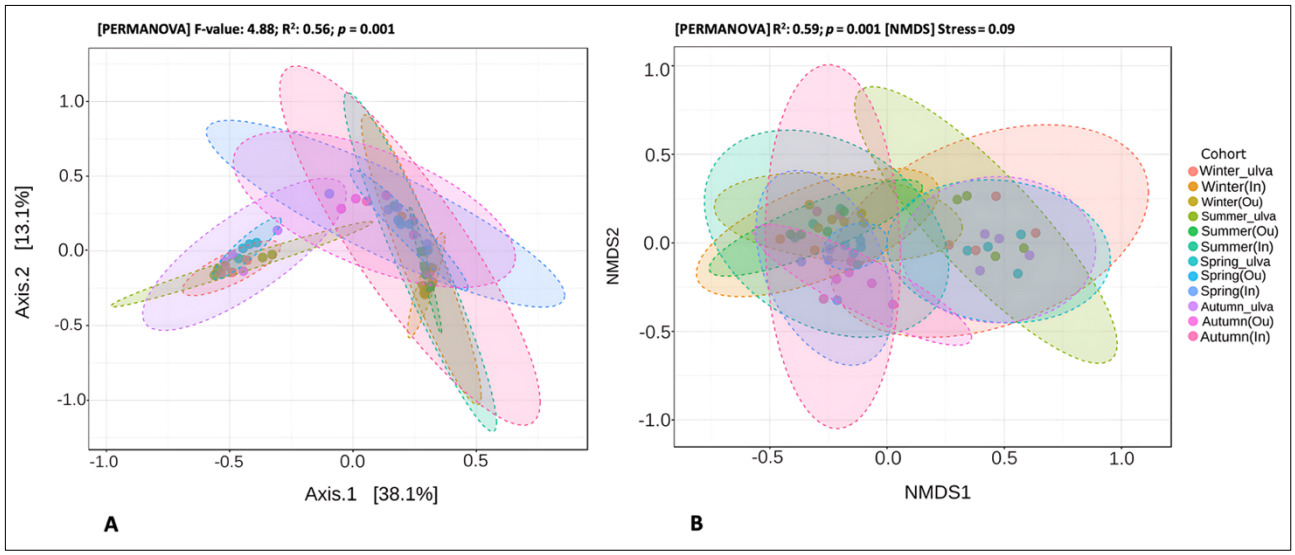

Figure 4.8. Principal co-ordinate analysis (PCoA; A) and Non-metric multidimensional scaling analysis (NMDS; B) at genus level showing the difference in fungal communities of IMTA (AEW) samples based on Bray–Curtis dissimilarity metrics from abalone effluent water (AEW). *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Ou) for respective seasons.

Hierarchical clustering dendrogram analysis at the genus level, based on Bray-Curtis dissimilarity matrices, also demonstrated that fungal communities differed between IMTA (AEW) and non-IMTA (SW) systems, with most of the SW samples (denoted with an H) forming a distinct cluster on the dendrogram (Fig. 4.9), and these samples are more similar to one another. On the other hand, the AEW samples (denoted with a P) had no clear grouping of cohorts. However, these communities also shared similarities in the fungal communities, as the SW and AEW water samples tend to cluster with each other (Fig. 4.9).

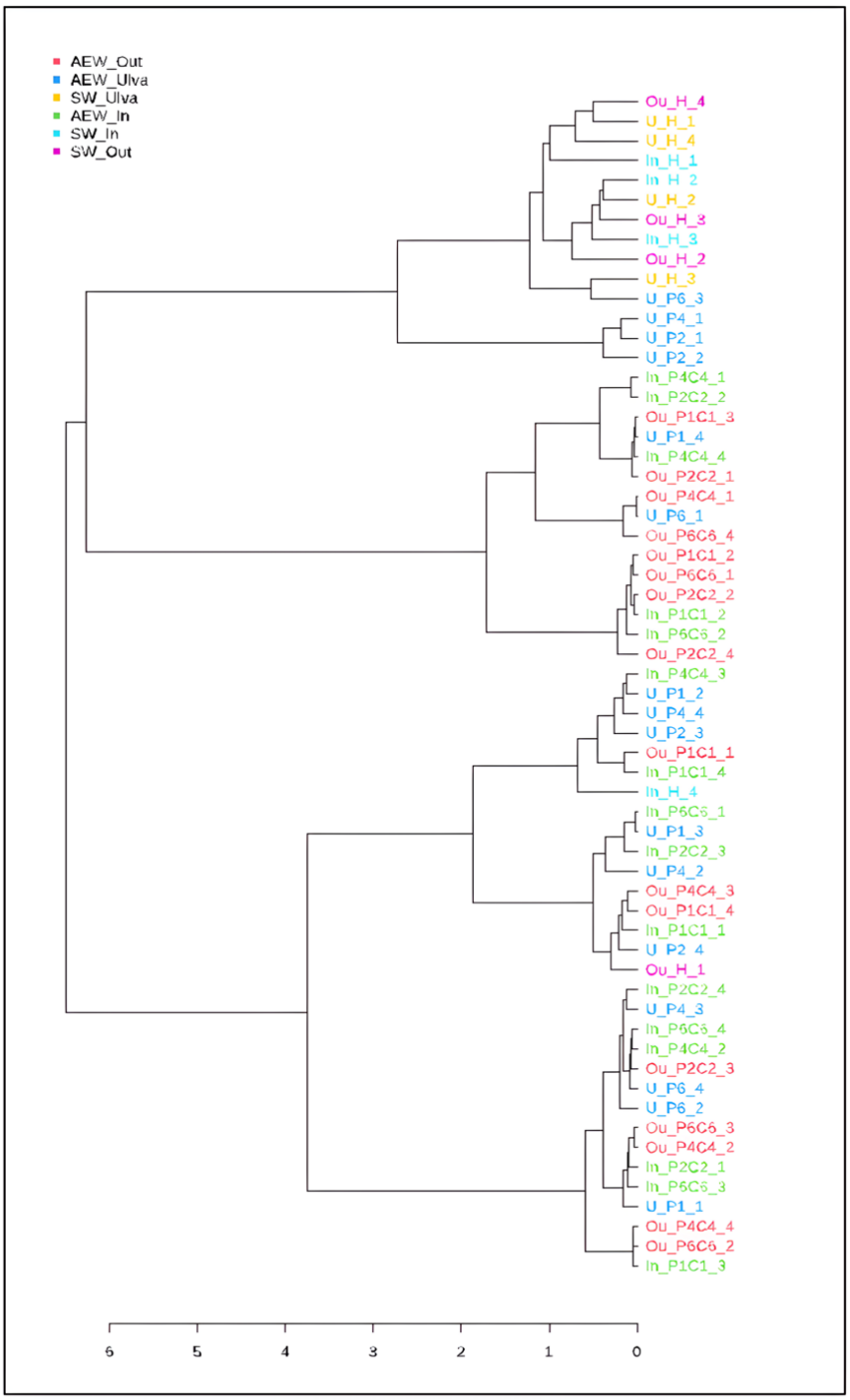

Figure 4.9. Hierarchical clustering dendrogram (Ward algorithm) based on Bray-Curtis dissimilarity matrices at genus level, illustrating the similarity in fungal community composition across cohorts. The organisation of the 60 samples taken from the effluent raceways, seawater tanks and the *Ulva* within each of the systems is depicted. Samples denoted by "H" are from the SW (non-IMTA), while samples denoted by "P" are from the AEW (IMTA) system. *Ulva* samples are denoted by "U", Inlets are denoted by (In), and outlets are denoted by (Ou).

4.3.6 Relative abundance and taxonomic profiling across cohorts

Taxonomic profiling showed the presence of diverse fungal communities across all cohorts, with 54 family-, 63 genus-, and 71 species-level ASVs identified after data filtering (Appendix B; Figures S4.1 – S4.3). The univariate method DESeq2 identified 4 differentially abundant ASVs at the family level, 7 at the genus level and 6 at the species level. Overall, when looking at the relative ASV abundance at the Phylum level within each cohort, some distinct differences between cohorts were observed, particularly amongst the less abundant fungal taxa. In the AEW cohorts, most of the taxa belonged to the phylum Ascomycota (99%), followed by Oomycota (0.28%) and Basidiomycota (0.17%). In the SW cohorts, most of the taxa belonged to Ascomycota (98.95), followed by Oomycota (0.88%) and Basidiomycota (0.12%). Overall, the SW system appeared to have greater fungal relative abundance at the phylum level, compared to the AEW systems. At the class level, the SW cohorts exhibited a higher relative abundance of fungal ASVs compared with the AEW cohorts. The AEW system was dominated by ASVs belonging to the class Pezizomycotina (98%), followed by Saccharomycetes (0.44%) and Dothideomycetes (0.43%). In contrast, the SW system was dominated by Pezizomycotina (43%), followed by Saccharomycetes (18%) and Dothideomycetes (14%). There was also a shift in the relative abundance of fungal taxa as seawater moved from the inlet to the outlet of the *Ulva* paddle raceways in the SW cohorts. The average abundance of Saccharomycetes, Dothideomycetes, Eurotiomycetes, and Sordariomycetes decreased significantly between the inlet and outlet in the latter (SW) system from 25% to 8%, 19% to 12%, 11% to 2.8%, and 12% to 1.4%, respectively. Conversely, no visible shift was observed in the relative abundance of fungal taxa from the inlet to the outlet of AEW (IMTA) cohorts at the class level.

Additional differences were observed in the relative abundance of taxa between the AEW and the SW cohorts at the genus level. The SW cohorts exhibited a higher abundance of fungal genera when compared with the AEW cohorts, particularly for the water cohorts (Fig. 4.10). Additionally, ASV abundance appeared to be lower on the *Ulva* in both systems when compared to the water cohorts in each respective system. The genus *Saccharomyces* was the most abundant fungus associated with *Ulva* in the non-IMTA system (SW_*Ulva*; 56.31%),

whereas its relative abundance was substantially lower in the SW_In (0.8%) and SW_Out (1.07%) of the same system (Fig. 4.10). In line with the higher-order observations, unclassified genera belonging to Pezizomycotina, a subdivision of the Ascomycota, were the second most abundant fungi (28.51%) in the SW_*Ulva* cohort. Conversely, genera belonging to this subdivision were the most abundant fungi in the SW_out (59.33%) and SW_In (28.72%) cohorts from the SW system. Furthermore, the SW_In also had a high abundance of other genera such as *Metschnikowia* (12.78%), *Penicillium* (1.3%), and *Candida* (6.2%), which were in low relative abundance in other SW cohorts (SW_Out and SW_*Ulva*), with the exception of the genus *Metschnikowia* that was absent in the SW_*Ulva* cohort. In the IMTA (AEW) system, all cohorts were dominated by members of the Pezizomycotina genus. Genera belonging to *Saccharomyces* and *Metschnikowia* were also observed in the AEW_In and AEW_Out cohorts, but their relative abundance was substantially lower in comparison to the SW cohorts and completely absent in the AEW_*Ulva* samples. As observed at higher taxonomic levels, there was also a shift in the relative abundance of fungal genera isolated from the Inlet to the outlet of the SW system. Specifically, the relative abundance of *Metschnikowia*, *Candida*, *Aspergillus*, *Alternaria,* and *Aureobasidium* decreased significantly between the inlet and outlet from 13% to 4%, 6% to 0.97%, 5% to 0.75%, 4% to 0.7%, and 4% to 0%, respectively. A similar trend was also observed in the AEW cohorts, although more difficult to see in Figure 4.10, the relative abundance of *Apergillus, Candida, Metschnikowia,* and *Pythium* decreased from the inlet to the outlet of AEW systems from 0.1% to 0.02%, 0.18% to 0.11%, 0.16% to 0.09%, and 0.13% to 0.1%, respectively.

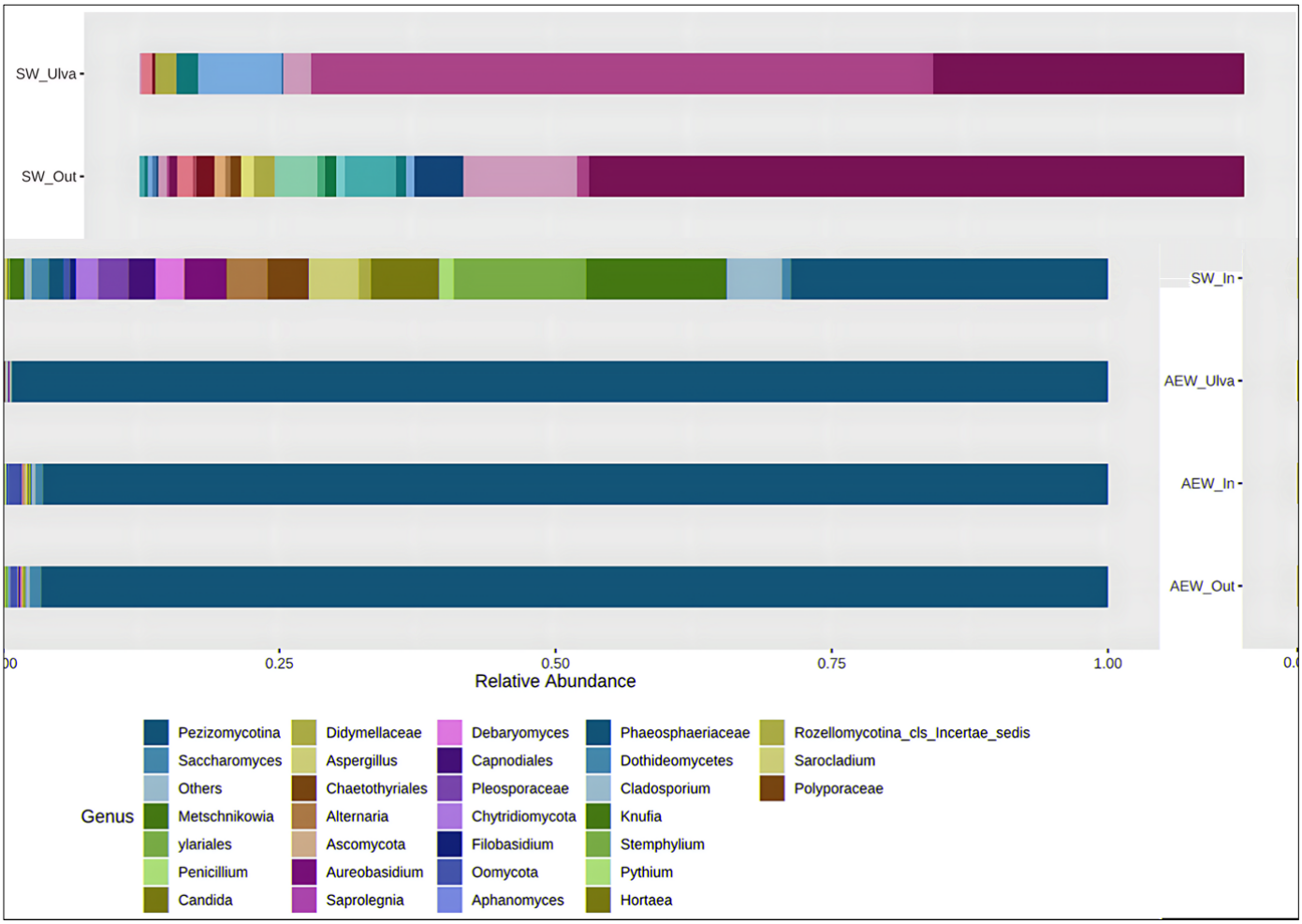

Figure 4.10. Relative abundance (%) of dominant fungal communities at genus level across 60 samples where group ASV abundance is indicated for the 30 most abundant ASVs. Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

A total of 50 ASVs exhibiting a statistically significant disparity in relative abundance were detected between the mycobiomes of the AEW and SW (Fig. 4.11). The fungal communities that differentiated the IMTA systems from non-IMTA system by specific ASVs, were mostly unidentified. Based on the overall fungal community composition (at ASV resolution), it was observed that the SW_*Ulva* cohort had a notably high abundance of ASV2, with the AEW_*Ulva* and SW_Out cohorts following closely behind. The AEW water cohorts, namely AEW_In and AEW_out, had a significantly greater abundance of ASV1 in comparison to the remaining cohorts. Furthermore, ASV4 exhibited a high prevalence in the water cohorts, specifically in the AEW and SW, but was not detected in the *Ulva* cohorts of either system (Fig. 4.11). The

presence of ASV34 was observed at a high frequency within the cohorts from the SW system; however, it was not detected in the cohorts from the AEW system. Similarly, the presence of ASV4 was observed to be abundant in the water cohorts of AEW, whereas it was found to be lacking in the remaining cohorts.

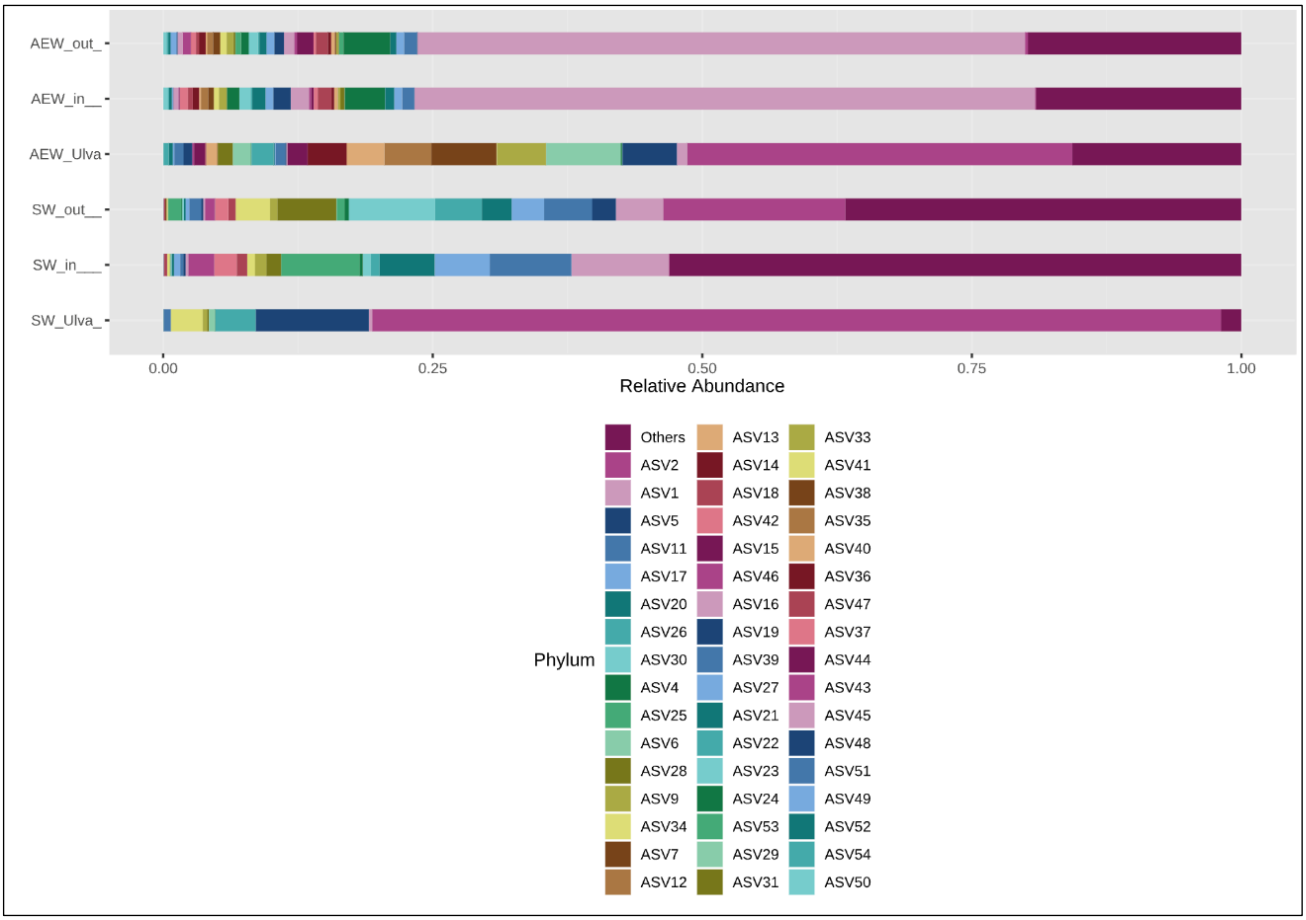

Figure 4.11. Top 50 most abundant ASVs shared between each cohort (AEW_Out, AEW_IN, AEW_*Ulva*, SW_Out, SW, IN and SW_*Ulva*). Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by

The relative abundance and taxonomic profiling of fungi were assessed at the phylum, class, and genus level across seasons. The fungal communities at the phylum level differed in relative abundance in the various cohorts across seasons in the two systems. Fungi belonging to the phylum Ascomycota were the most dominant taxa in all cohorts across seasons. In fact, seawater samples collected from the AEW systems in winter were dominated by Ascomycota

(99%), with the remaining ASVs assigned to the phylum Oomycota (0.65%) and Chytridiomycota (0.05%). Similarly, water samples collected from the SW cohort in summer and the AEW cohort in spring were also dominated by fungi belonging to the phylum Ascomycota but appeared to have a higher relative abundance of Oomycota with 3.86% and 3.97%, respectively, compared to other cohorts. Similarly, water samples obtained from the SW system during autumn were dominated by Ascomycota (97%), with only a few Oomycota (1.5%) and Chytridiomycota (0.72%) ASVs accounted for.

At the class level, the SW system appeared to have a greater relative abundance of taxa compared with the AEW system. The relative ASV abundance in *Ulva* cohorts was lower than in the water cohorts across the different seasons, which is in line with the previous observations. In the SW water cohorts, summer and winter had a relatively higher abundance of taxa compared to summer and winter in the AEW water cohorts. The summer SW cohort was dominated by members of class Pezizomycotina (48%), followed by Dothideomycetes (16%) and Sordariomycetes (13%), while the winter SW cohort was dominated by Pezizomycotina (40%), followed by Dothideomycetes (25%) and Eurotiomycetes (7.9%). On the other hand, the summer AEW cohort was greatly dominated by members of the class Pezizomycotina (98%), followed by Sordariomycetes (1.04%) and Dothideomycetes (0.29%), while the winter AEW cohort followed the same trend with Pezizomycotina (98%) and Sordariomycetes (0.85). Across *Ulva* cohorts, the fungal communities at the class level were dominated by the class Pezizomycotina (> 95%), followed by Saccharomycetes (<1%) and Dothiodeomycets (<1%). Winter, spring, and autumn *Ulva* samples consisted of communities that were attributed to Eurotiomycetes (<1%), while the summer sample consisted of communities attributed to Sordariomycetes (<1%).

The relative abundance of taxa at the genus level was also assessed across seasons. Members of Pezizomycotina appeared to be in high abundance across all seasons in the two systems. (Fig. 4.12). The genus *Metschnikowia* was observed in high abundance in the autumn (SW) cohort (36%), while *Didymellaceae* was observed in high abundance in the winter (SW) cohort (14%). The genera *Saprolegnia*, *Penicillin*, and *Pythium* were also observed, though in low

abundance in the SW cohort collected in summer, spring, and autumn. In order to perform a more comprehensive seasonal analysis, the SW cohort was excluded from the analysis due to a lack of duplicate entries. Consequently, only the AEW samples were evaluated for the purpose of this analysis (Fig. 4.13). The taxonomic composition at genus level exhibited a decrease in relative abundance from the inlet to the outlet within the AEW system across different seasons, with the exception of the winter season. In autumn, *Aspergillus* decreased from 0.73% to 0.05 and *Penicillium* from 0.27% to 0.09%. In spring, *Pythium* decreased from 0.51% to 0.07%. In summer, *Candida* decreased from 0.16% to 0.11% and *Metschnikowia* decreased from 0.21 to 0.08%. In contrast, in winter, genera like *Candida*, *Pythium*, *Saprolegnia*, and *Aphanomyces* increased from the inlet to the outlet from 0.17% to 0.44%, 0.15% to 0.41%, 0.09% to 0.27%, and 0.09% to 0.27%, respectively.

Figure 4.12. Relative abundance (%) of dominant fungal communities at genus level across seasons where group ASV abundance is indicated for the 30 most abundant ASVs. Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

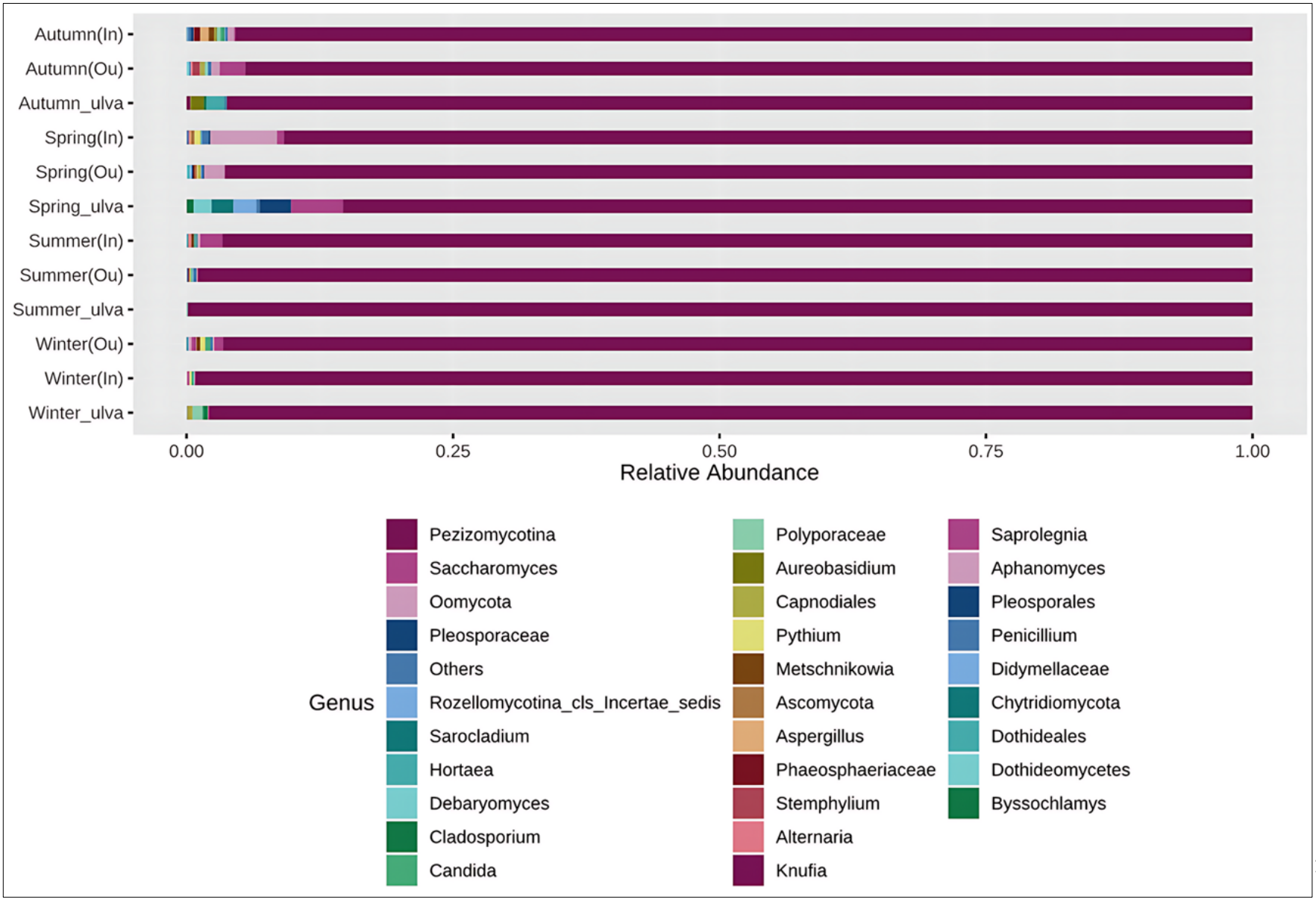

Figure 4.13. Relative ASV abundance (%) from abalone effluent water (AEW) at genus level, across seasons where group ASV abundance is indicated for the 30 most abundant ASVs. Less prominent ASVs were merged and denoted as "Others". Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted with (Out).

In total, six differentially abundant taxa were identified at the genus level when the six cohorts were compared (Fig. 4.14) using the univariate method implemented in DESeq2 (FDR-corrected $p < 0.05$). Members of the subdivision Pezizomycotina were significantly higher in abundance in all the cohorts analysed from the AEW system when compared with the SW system, and within the AEW systems, there was no significant difference in their abundance between the different cohorts (In, Out, and *Ulva*). Conversely, in the SW system, the abundance of Pezizomycotina was lower in the SW_*Ulva* cohort compared with the water cohorts. Similarly, members of the genera *Capnodiales* and *Metschikowia* were high in abundance in the water cohorts collected from both systems, but low in abundance in the *Ulva* cohorts collected from both systems. The SW_*Ulva* cohort generally had a lower differential abundance in most taxa compared to all the other cohorts. Members of the genus *Alternaria* were differentially abundant in the SW cohorts (In and Out), while they were low in abundance in all the other cohorts. Similarly, *Pythium* was differentially abundant in the AEW_In and SW_Out cohorts.

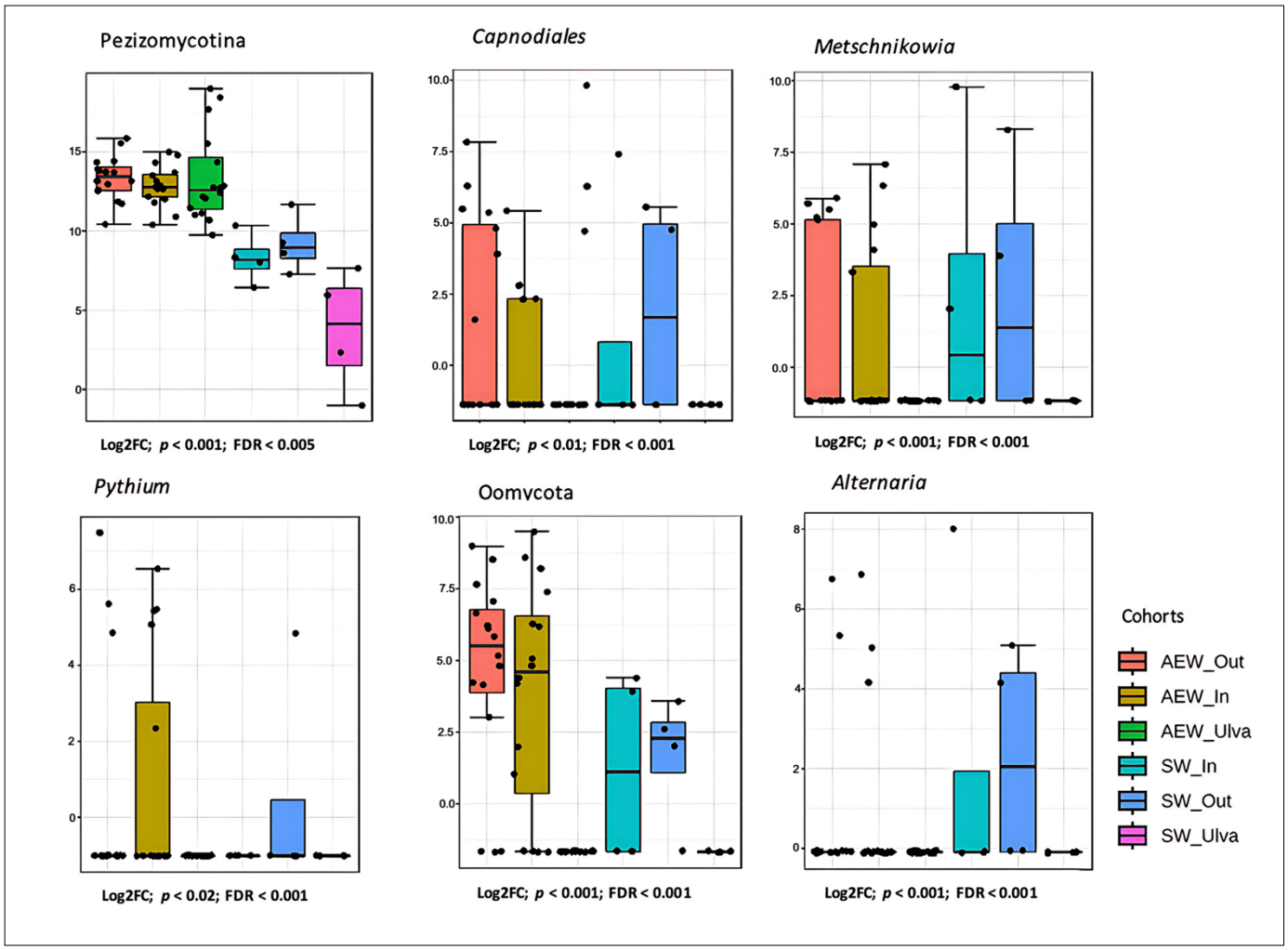

Figure 4.14. Differentially abundant ASVs of AEW, SW and Ulva fungal communities at genus level, where the *p*-value and false discovery rate (FDR) corrected are indicated. Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

At the genus level, a total of 8 differentially abundant taxa were identified across the cohorts from the IMTA and non-IMTA systems across the different sampling periods (seasons) (Fig. 4.15). Members of the genus *Metschnikowia* were differentially abundant in the water cohorts (SW and AEW) during winter, summer, and autumn (SW). Unclassified genera belonging to Oomycota, also known as "water molds" in the kingdom Fungi, were differentially abundant in spring (AEW), while ASVs of the family *Didymellaceae* and subdivision Rozellomycotina were differentially abundant in the winter (SW) cohorts. Moreover, members of the genera *Hortaea* were differentially abundant in summer (AEW and SW), whereas members of *Aureobasidium* were differentially abundant in autumn (SW).

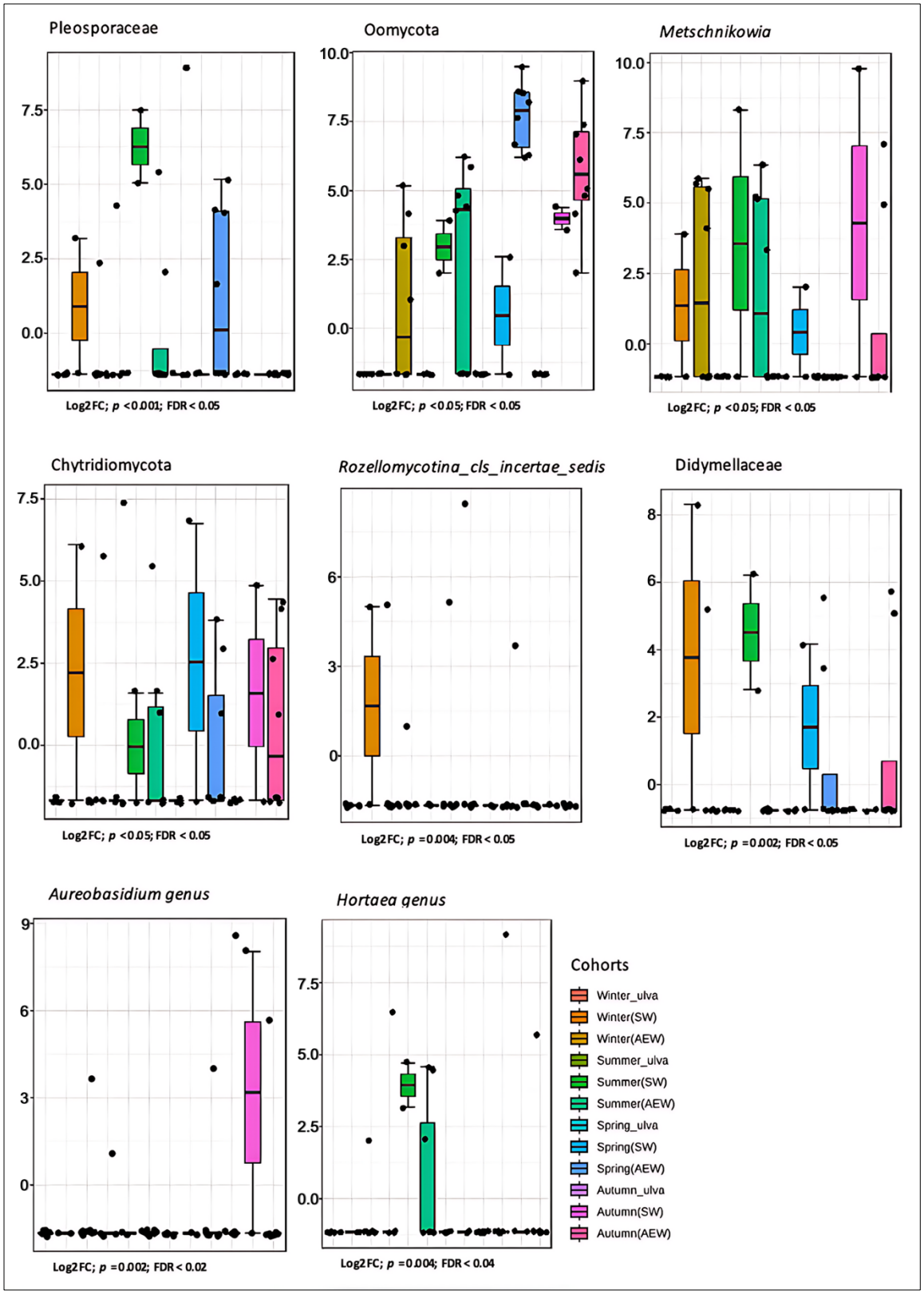

Figure 4.15. Differentially abundant ASVs of samples group by season at genus level, where the *p*-value and false discovery rate (FDR) corrected are indicated. Samples denoted by "SW" are from the seawater (non-IMTA) system, while samples denoted by "AEW" are from the abalone effluent water (IMTA) system. *Ulva* samples are denoted by "*Ulva*", Inlets are denoted by (In), and outlets are denoted by (Out).

4.4 Discussion

To the best of my knowledge, this is the first study to characterise fungal and oomycete communities (mycobiome) associated with an integrated multi-trophic aquaculture (IMTA) system consisting of the green seaweed *Ulva lacinulata* grown in abalone effluent water (AEW) and natural seawater (SW).

4.4.1 Fungal community profiling by ITS2 next-generation sequencing

Data generated from this study, when comparing an IMTA (AEW) to a non-IMTA (SW) system consisting of the same seaweed strain cultivated in seawater (SW), has provided vital information for local abalone producers, such as Buffeljags abalone farm, on biosecurity and mycobiome differences between IMTA and non-IMTA systems. The results showed that the SW and AEW systems at Buffeljags Abalone Farm harboured 169 ASVs that were assigned to identity. The non-IMTA (SW_In and SW_Out) and IMTA (AEW_In and AEW_Out) water cohorts had significantly higher alpha diversity than the *Ulva* (SW and AEW). The fungal phyla in the current study largely belonged to Ascomycota and, to a lesser extent, Oomycota, Chytridiomycota, and Basidiomycota. Most fungal reads were ascribed to unclassified fungi. Currently, there have not been many studies focusing on fungi in seawater, resulting in the majority of sequences that could not be assigned, i.e. unknown or unclassified using NGS. Therefore, it is essential to investigate the diversity and composition of eukaryotic communities in the environment in order to improve and update the available databases. Furthermore, the current study revealed that the nutrient load (refer to Chapter 3; Table S3.3) was correlated with the diversity and abundance of the fungal community. Specifically, a higher nutrient load was observed in the AEW system, which coincided with higher fungal diversity and abundance. This is in line with the findings of Zhang et al. (2021), who demonstrated that nutrients in sediments, particularly the carbon content, play a significant role in influencing the diversity of the fungal community.

The raw sequence reads observed in all cohorts from both systems totalled 19, 919, 573. After the exclusion of unidentified sequences (18, 261, 919), a total of 1, 657, 658 sequence reads were used for subsequent analysis and assigned to 169 ASVs. The rarefaction analysis

indicated sufficient sampling coverage of the samples, with the exception of three samples (U_H_3 and U_H_3), which were excluded from all downstream analyses because of their low count read. The average reads per sample (27, 621) after processing and filtering and the total number of individual ASVs (169 after reads were mapped to custom reference data) obtained in this study are similar to what has been reported for other studies conducted on marine samples. For example, Karla et al. (2022) obtained 193,436 reads and 133 ASVs from marine sediments from the maritime Antarctic. Moreover, Zhang et al. (2021) upon investigating sediment fungal communities in fish, crab, and crayfish ponds using the ITS1 region of the fungal rDNA, obtained 1,283,490 raw sequence reads post-filtering and a total of 848 OTUs. Moreover, a substantial number of sequences read from cohorts in both systems from the present study remained unidentified, totalling 18,261,919. A recent study conducted by Li et al. (2022) assessing the fungal diversity associated with different tissues of green-lipped mussel (*Perna canaliculus*), utilising the ITS1 region, revealed that a significant proportion, specifically over 50%, of the ASVs remained unidentified. Similarly, Zhang et al. (2021) utilised the ITS1 region to explore sediment fungal communities in fish, crab, and crayfish ponds and discovered up to 73.9% of unidentified fungi in pond sediments. Likewise, prior studies have demonstrated that a considerable number of fungal species within terrestrial plant microbiomes remain unidentified (Yao et al., 2019). The large number of unidentified fungi is not surprising given the lack of studies on marine fungi (Amend et al., 2019), the limited number of fungal ITS sequence data available in the currently available datasets and reference databases, and the highly novel phylotypes that are yet to be deciphered in aquaculture ecosystems.

4.4.2 Fungal alpha diversity

When assessing the alpha diversity measures, the fungal community composition was richer in the samples processed from the non-IMTA (SW) system than in the IMTA (AEW) systems (Fig. 4.3). The Chao1 indices, which measure observed richness, found that abalone effluent water (AEW) had a much lower richness than water samples collected from the SW system, indicating that water from the non-IMTA *Ulva* paddle raceway tanks receiving seawater from the adjacent coastline had more diverse ASVs (ANOVA; $p < 0.001$). However, when assessing

ASVs that were not assigned to taxonomy, the majority of which were unidentified taxa, the Chao1 indices had a much higher richness in the IMTA system than in the non-IMTA system (Fig. 4.3), indicating that the AEW systems harboured a higher degree of unique ASVs. This pattern is similar to that observed in chapter 3, pointing to the fact that nutrient-rich water influences fungal growth (Babič et al., 2017). Contrastingly, the fugal communities from the SW system had a higher Shannon and Simpson index, indicating greater species richness and evenness in the water samples collected from the non-IMTA system compared with the IMTA system. This could be because more fungal taxa are introduced in the non-IMTA system given that it doesn't recirculate its water and fresh seawater from the coastline is constantly pumped into the system. Thus, demonstrating a higher fungal diversity than the AEW (IMTA) system. These findings could be attributed to the fact that several procedures and conditions within an IMTA, may affect microbial diversity, since husbandry procedures in IMTA systems and partial recirculation can influence microbial diversity, thereby favouring the proliferation of certain taxa (Attramadal et al., 212). Furthermore, partial recirculation of effluent (50% in the case of this study) can also enhance the accumulation of dissolved and particulate organic matter (DOM and POM, respectively), providing additional nutrients and substrates that may promote the proliferation of certain microorganisms, as documented elsewhere (Bentzon-Tilia et al., 2016).

This can also alter mycobiome composition and lead to reduced diversity and dominance of specific taxa. Interestingly, the number of different fungi present (Chao1 richness estimator) was significantly greater for the water samples collected from the non-IMTA (SW) and the IMTA (AEW) systems when compared to the *Ulva* samples collected from the respective systems (Fig. 4.2 – 4.3), indicating a lower number of distinct ASVs associated with the *Ulva* growing in the two systems. Regular pumping of fresh seawater from the ocean into the *Ulva* paddle raceway system (SW) may explain the increasing presence of unique ASVs in the water columns of this study. Moreover, some algae produce secondary metabolites that are host-specific, seasonally variable, and possess antimicrobial or antifungal properties. Consequently, this may selectively promote growth or alter the microbial structure (Nylund et al., 2010; Nelson et al., 2013). In addition, fungi grow more slowly than bacteria, which may

contribute to the fungal communities being relatively unstable and easily disrupted (Zheng et al., 2020). Therefore, the fungi may have weaker interactions with their algal hosts and tend to move freely in the seawater environment. The results of the present study demonstrate that the *Ulva* cohorts have lower alpha diversity, which indicates that the hosts examined in this study possess sufficient unique characteristics to support different microbial epibiont communities. Macroalgal hosts available for colonisation may also respond to environmental changes, and their overall physiology, especially their chemical signalling behaviour, may exhibit not only temporal but also species-specific fluctuations (Hellio et al., 2004). Thus, spatial variation in fungal assemblages may result from algal behaviour in different environments. For example, in the study of a culturable epibiotic fungi of seaweeds in the Red Sea, Abdel-Gawad et al. (2014) demonstrated that seaweed hosts exert strong selective pressure on the epiphytic fungal population.

Temporal variation in eukaryotic communities in terms of alpha diversity was also detected in the current study, with peaks at specific sampling seasons. When comparing fungal dynamics between the different seasons assessed in this study, consistent patterns begin to emerge. For instance, an increase in Chao1 richness during SW_summer was observed, indicating a high degree of unique ASVs in the SW_summer cohort. Moreover, statistically significantly higher level of phosphate was observed in the summer of SW system (Table 3.2). Fungi, much like other microorganisms, require nitrogen and phosphorus for growth; higher amounts of these nutrients can increase fungal activity and biomass (Carlile et al., 2001). Similarly, higher temperatures during the summer, combined with elevated nutrient levels in the AEW summer cohort, increased fungal diversity. This increase was evidenced by the Shannon diversity index, which accounts for both species' richness and evenness (Fig. 4.4). The availability of light, nutrients, and temperature at different times of the year and in different systems have a direct impact on the development and health of *Ulva* (Lapointe & Tenore, 1981; Sand-Jensen, 1988; Nikolaisen et al., 2011), as well as the composition of the mycobiome. For example, Minich et al. (2018) demonstrated that high temperatures and high CO_2 partial pressure had the greatest effect on the kelp (*M. pyrifera*) mycobiome. During the summer season within the IMTA system, characterised by increased light availability and

slightly elevated water temperatures, the *Ulva* grows well and is healthy. The capacity of other epiphytic fungi and other organisms to grow on seaweed is influenced by healthy *Ulva* and increased biomass.

4.4.3 Fungal beta diversity

Beta analysis of NMDS and PCoA ordination plots, based on the Bray-Curtis distance, revealed distinct differences in the composition of the mycobiome from samples in the different cohorts and systems. PCoA analysis exhibited significant differences in the mycobiome structure of the water cohorts and *Ulva* cohorts in both the IMTA and non-IMTA systems. The mycobiome of seawater (SW) and abalone effluent water (AEW) clustered more closely together but were more different from the fungal communities of the AEW_*Ulva* and SW_*Ulva* cohorts (Fig. 4.6), indicating that similar samples tended to have similar fungal communities ($p < 0.001$). The results of the PCoA were supported by the dendrogram analysis, which also showed that there were distinct differences in the community structure between the AEW cohorts and the SW cohorts as they tended to form different clusters. The NMDS analysis further demonstrated that the *Ulva* cohorts of both systems clustered separately from one another, whereas the water cohorts clustered separately from the *Ulva* and more closely with one another, which is consistent with the PCoA plot. The present study found that there were differences between the fungal communities from the *Ulva* of different systems (AEW_*Ulva* vs. SW_*Ulva*) (Fig. 4.6), suggesting that fungal communities are not easily influenced by phylogeny and the selection of microbial communities may have been influenced by spatial differences. According to Hacquard et al. (2015), the assembly of microbial communities is significantly influenced by environmental or chemical characteristics (e.g., pH or organic carbon availability) rather than phylogeny. Seasonal variations can also influence the composition of epiphytic microorganisms on the surface of macroalgae (Lachnit et al., 2011; El-Said & El-Sikaily, 2013; Korlević et al., 2021). The β-diversity analysis results in this study showed that microbial communities differed in different cohorts (i.e., *Ulva* cohorts and water cohorts). However, there was not a clear distinction among seasons as they all overlapped with one another on the PCoA and NMDS plots. Due to

the large proportion of unassigned ASVs, the impact of season and system type (IMTA vs. non-IMTA) on the composition of fungal communities remains unclear.

4.4.4 Taxonomic classification of fungal communities

Overall, the most abundant groups of fungi associated with the *Ulva* and seawater samples from both the IMTA and non-IMTA systems belonged to the phylum Ascomycota, followed by the Phylum Basidiomycota. Both of these phyla fall within the sub-kingdom Dikarya and are commonly referred to as the 'sac' fungi or ascomycetes and constitute the largest phyla of the Kingdom Fungi. The high abundance of these phyla in the present study is consistent with previous findings, demonstrating that most fungi in marine environments belong to these two phyla (Taylor & Cunliffe, 2016; Wang et al., 2018; Sen et al., 2021). Fungi belonging to these phyla have been found to be overwhelmingly prevalent in several marine environments, including Hawaiian nearshore seawater (Gao et al., 2010), mariculture systems (Guo et al., 2015), nearshore marsh sediments (Mohamed & Martiny, 2011), and mangrove sediments (Arfi et al., 2012). In the present study, the phylum Ascomycota, followed by Basidiomycota and Oomycota, represented the fungal and oomycetes communities' that were most dominant in the seawater (SW) system at Buffeljags abalone, whereas members of the phylum Ascomycota were more prevalent in abalone effluent water (AEW) systems at the farm. Members of the Ascomycota are capable of breaking down organic matter and are symbiotic with Chlorophyta or cyanobacteria (Wu et al., 2022). Its relative abundance in AEW cohorts may be the result of higher concentrations of POM and DOM in the effluent water and the frequency of recirculation. The current study also identified members of the Phylum Chytridiomycota in the SW water cohorts. This phylum comprises several unknown taxa, implying that numerous species of "dark matter fungi" can be found in the ocean.

Many abundant seawater-associated fungal classes found in the current study are common fungi known to grow in association with marine environments. Jin et al. (2014) also demonstrated the presence of Pezizomycotina, Saccharomycetes, and Dothideomycetes in South China sea sponges, suggesting that common properties in seawater may promote the growth of similar organisms (Spatafora et al., 2006; Siriyappagouder et al., 2018). For instance,

Pezizomycotina (dominant in the AEW cohorts), which includes filamentous Ascomycetes, is the largest subphylum of Ascomycota. This subphylum contains approximately 32 000 species (Kirk et al., 2008) that are capable of forming various symbiotic (with mycorrhizae, endophytes and lichens), necrotic (decaying wood and litter), and pathogenic (for animals and plants) ecological relationships (Spatafora et al., 2006). In this study, over 98% of the AEW-derived sequences were identified as Pezizomycotina, while 43% of the sequences obtained from the SW samples mapped to the sub-division Pezizomycotina. Members of the sub-division Pezizomycotina are frequently identified as saprobes, organisms that live off of dead or decaying organic material in a variety of marine environments (Suetrong et al., 2009). A similar dominance of Pezizomycotina was observed in seawater and sea-ice samples from Danborg in Greenland (Hassett et al., 2016). According to Jin et al. (2014), the Pezizomycotina dominated fungal communities in the equatorial Pacific region as a result of selective enrichment in sponge communities (*Theonella swinhoei* and *Xestospongia testudinaria*). In the current study, the dominance of Pezizomycotina in the AEW system (98%) might be attributed to their saprophytic nature, especially because of the elevated levels of POM and DOM in this environment, where they might play a role in the decomposition of organic matter and nutrient recycling. The current study, in additional to previous studies, has demonstrated that water environment factors such as NO_2, NO_3, PO_4, and NH_4 were found to be higher in the aquaculture water effluent than in other locations (De Prisco, 2019; Wu et al., 2022). Additionally, members of the Pezizomycotina were shown to be significantly abundant across all seasons in this study. This is no surprise, as the Pezizomycotina is the largest subphylum of the Ascomycota and contains a large majority of filamentous species, which play important roles in a variety of ecological processes and symbioses (Spatafora et al., 2006).

Members of the Dothideomycetes have frequently been found in various marine environments, including deep sea sediments in India (Singh et al., 2010) Pacific Ocean (Xu et al., 2014e), Arctic fjords (Tao et al., 2015), and the subtropical seas in China (Li et al., 2016), indicating that members of this class are ubiquitous in marine environments. In the current study, Dothideomycetes were identified as one of the dominant fungal classes in the non-IMTA water samples (inlet and outlet). Members of this class are saprophytic and symbiotic,

with some Dothideomycetes previously shown to be plant pathogens and parasites capable of causing disease in a wide range of species, including wheat, barley, seagrass, and marine algae (Suetrong et al., 2009; Ohm et al., 2012). However, their specific role in the physiology and metabolism of marine organisms such as molluscs, and particularly in fish, is poorly understood (Siriyappagouder et al., 2018). They are known to produce a wide range of secondary metabolites and peptides that can contribute to host tissue disruption and fungal colonisation (Stergiopoulos et al., 2013). On the other hand, these ascomycete fungi are also capable of forming endosymbiotic relationships with marine algae, including *Ulva* (Schulz et al., 2008; Zuccaro et al., 2008; Harvey & Goff, 2010; Loque et al., 2010). Macroalgae fungal endosymbionts (FEM) are known to produce molecules with antitumor, antioxidant, anticancer, antimicrobial, antifungal, and cytotoxic properties, as well as other bioactivities (Schulz et al., 2002; Suryanarayanan et al., 2010; Flewelling et al., 2013). FEMs include members of the genus *Aspergillus* that produce antibiotics effective against methicillin-resistant bacteria (Nguyen et al., 2007). In the current study, *Aspergillus* was detected in the non-IMTA seawater cohorts (SW_in and SW_out), specifically in the winter (SW) and autumn (SW) samples. Of the marine-derived endosymbiotic fungi identified to date, the genera *Aspergillus*, *Cladosporium*, and *Penicillium* are common endosymbionts of taxonomically and geographically divergent macroalgae such as *Ulva* (Suraanarayanan et al., 2010; Flewelling et al., 2013). However, the latter endophytes had a low abundance in the current study (5.3% overall) when compared to mycobiome studies conducted on terrestrial plant leaves, such as *Cephalotaxus harringtonia* leaves (Langenfeld et al., 2013). The genera *Aspergillus* and *Penicillium* have been demonstrated to possess extracts that exhibit a relatively broad spectrum of antifouling activity against various biofoulers (Zhang et al., 2018b). In the current study, *Aspergillus* (2.4%) and *Penicillium* (1.4%) strains were detected in low abundance in the SW cohorts, while no traces of these taxa were detected in the AEW. These findings are indicative of the diverse roles of fungal communities in aquatic systems and advocate for an improved understanding of microorganisms in specific environments. Additionally, this study also identified certain genera that are recognised for their ability to promote plant growth. In the non-IMTA and IMTA systems, these genera included *Cladosporium* (Tagawa et al., 2010) and *Pleospora* (Bailey et al., 2002).

The SW and AEW systems also contained potentially pathogenic genera such as *Cryptococcus*, *Pythium*, *Candida*, *Saprolegnia*, and *Aphanomyces* that have previously been shown to be responsible for catastrophic disease outbreaks on aquaculture farms cultivating organisms such as salmon and eel (Hatai & Hoshai, 1994; Scarfe, 2003) and can cause significant economic losses in the natural environment (Auer & Ludwig-Müller, 2015). To better assess potential risks, farms should conduct species-level identification to determine the actual species belonging to each of these genera, especially if farms are considering further diversification and inclusion of other species, such as ocean trout (under investigation by Viking Aquaculture), that may be vulnerable to species within these genera. A species within the genus Aphanomyces, namely *Aphanomyces invadans*, serves as a prime example of a pathogen with a wide range of hosts and the ability to infect susceptible salmonids, including rainbow trout (*Oncorhynchus mykiss*). *A. invadans* is an oomycete pathogen that is responsible for epizootic ulcerative syndrome (EUS) (Baldock et al., 2005), which has affected fish in natural waters and commercial fish farms in Asia, Africa, some regions of Australia, and North America (Sosa et al., 2007; Roberts, 2012). Nevertheless, it is not a disease-causing agent for abalone or *Ulva*. The current study also detected *Cryptococcus* (0.09%), *Pythium* (0.19%), *Candida* (3.38%), *Saprolegnia* (1.3%) and *Aphanomyces* (0.75%) in the SW system and *Cryptococcus* (0%), *Pythium* (0.03%), *Candid*a (0.06%), *Saprolegnia* (0.02%) and *Aphanomyces* (0.01%) in the AEW system. The abundance of these pathogens appears lower in IMTA compared to non-IMTA.

Another good example of a well-known oomycete pathogen is a member of the genus *Saprolegnia*. This genus contains numerous pathogenic species of freshwater and brackish water fish species and their eggs, which account for at least 10% of salmon hatchery and farm mortality rates (Phillips et al., 2008). *Saprolegnia* was once thought to be a secondary pathogen (Diéguez-Uribeondo et al., 2009); however, in recent years, this idea has been unequivocally rejected because it actively suppresses host immunity while an infection is occurring (de Bruijn et al., 2012; Wawra et al., 2012; Belmonte et al., 2014; Minor et al., 2014). More specifically, in Japan, outbreaks of *Saprolegnia parasitica* have resulted in annual losses

of 50% in the production of coho salmon *Oncorhynchus kisutch* and elvers of the eel *Anguilla Anguilla* (Hatai & Hoshai, 1994; Scarfe, 2003).

The current study also detected traces of *Pythium* in seawater (0.9%) and effluent water (0.03%). *Pythium* spp. are widespread and present in agricultural soil and are known to be either plant pathogens or saprotrophs that infect the root systems of a variety of hosts, lowering crop production and quality in affected plants (Schroeder et al., 2013). In aquatic environments, *Pythium* spp. have been identified in moribund and dead fish and crustaceans, but it is still unknown whether these microbes are the principal pathogens of fish or other aquatic organisms (Czeczuga, 2002a; Czeczuga, 2002b; Rahman & Sarowar, 2016). *Pythium* and *Olpidiopsis* pathogens have also been identified in the red algae *Pyropia*. Symptoms of infection begin with the bleaching of the tissue and progress to lesions and holes, eventually leading to the death of the infected host. These infections reduce biomass and product quality, resulting in significant economic losses (Bernard, 2018). The oomycete pathogens *Pythium porphyrae* and *Olpidiopsis porphyrae* are perhaps the most well-studied seaweed diseases due to their detrimental impact on valuable *Pyropia* stocks. Herero et al. (2020) also demonstrated the occurrence and pathogenicity of *Pythium* on *Ulva* species at different salinities. Infections caused by *Pythium* species are likely more common in *Ulva* species that have been subjected to salinity fluctuations, especially at the lower end of the range (Herero et al., 2020). Overall, none of the findings from the current study yielded alarming results. It should be noted that, based on current knowledge, there is no evidence to suggest that the organisms mentioned above pose any significant threat to molluscs or seaweeds. However, the fraction of known and potentially pathogenic fungi should be monitored because they have the potential to cause harmful infections in other marine animals. Moreover, the results from the current study indicate that *Ulva* was capable of modulating many fungal taxa in seawater and effluent water systems, as genera such as *Metschnikowia* and *Aspergillus*, as well as several of the unidentified ASVs, decreased in abundance from the inlet to the outlet of these systems. Thus, the current study accepts the hypothesis that *Ulva* had positive effect on the microbial dynamics by decreasing taxa from the inlet to outlet, positively regulating

the microbial dynamics and reducing potentially harmful fungi and oomycetes and improving the overall microbial health of the system."

Only a few fungal diseases that affect gastropods have been documented in the literature, and in some cases, there is little information available. Grindley et al. (1998) previously described a fungal disease that caused lesions on the inside of the shell of the abalone *Haliotis iris*, *Haliotis australis*, and *Haliotis virginea*. The fungal name has only been tentatively proposed as "Deuteromycotina" (Friedman et al., 1997). Diseases caused by the oomycete *Halioticida noduliformans* are well-documented and have caused mortalities in populations of *Haliotis midae*, *Haliotis rufescens*, and *Haliotis sieboldii* in Japan and South Africa (Murosa et al., 2009; Macey et al., 2011). Additionally, fungi such as *Haliphthoros milfoldensis* (Hatai, 1982), *Halocrusticida awabi* (Kitancharoen, 1994), and *Atkinsiella dubia* have been attributed to *Haliotis sieboldii* (Nakamura & Hatai, 1995), where diseased abalone developed lesions with flat or tubercle-like swelling (Mabuhay-Omar, 2020). In the current study, members of Oomycota were identified from the IMTA (0.28%) and SW (2.3%) systems; however, there is currently no information available regarding the species or potential risks. It is, however, noteworthy to note that their abundance was lower in the SW systems compared with IMTA. The fluctuation in composition for Oomycota members was consistent throughout the seasons, with a relative abundance of less than 0.01. Similar to the composition of Chytridiomycota in the Mariana Trench sediments (Xu et al., 2016), Chytridiomycota in the current study comprised only a small portion of the fungal communities in seawater during winter. A large number of members of Chytridiomycota are still unknown (Chen et al., 2022).

Previously, spatial and seasonal trends in fungal community shifts have been recorded in coastal systems (Mbareche et al., 2017; Tian et al., 2017), whereas a number of studies demonstrate seasonal differences in fungal communities (Duan et al., 2018; Wang et al., 2018). The findings from the current study are in line with these studies, as the total fungal community structure of some taxa differed with season in relation to changing environmental conditions at Buffeljags abalone farm. An important change to note was the autumn increase of *Candida* and *Chaetothyriales* and the winter increase of Chytridiomycota taxa in the SW

system (Fig. 4.12). The current study showed that Buffeljags abalone farm harbours a high level of fungal diversity with an obvious spatial and temporal variation of community composition observed between the AEW (IMTA) and the SW (non-IMTA) systems. Such a great diversity of species, as well as the elucidation of several well-known decomposer representatives, points to the crucial ecological roles played by fungi in aquaculture farms. However, it is important to keep in mind that the metabarcoding-based approach for estimating fungal diversity has several limitations.

4.5 Limitations that currently exists

A few aquatic studies have focused on the links between fungal communities and environmental factors (Guo et al., 2015; Brodie et al., 2018). In their investigation of marine fungal communities in pond farming systems in Yantai, Shandong Province northern China, Guo et al. (2015) found that more than half of the fungal operational taxonomic units (OTUs) were possibly unique. The first-generation sequencing technique (T-RFLP) used in their research led to the observation of 131 OTUs in total. In other environments, higher numbers of fungal OTUs have been identified. For example, 190 OTUs were found in sediments from circular polyethylene tanks, 420 OTUs were found in deep sea sediments, and 1181 OTUs were found in mangrove areas located in Saint Vincent Bay on the west coast of New Caledonia (Luis et al., 2019). Primers, platforms for sequencing, and sequencing processing were all generally different in experimental protocols in previous studies. These could make it difficult to compare the fungi in different aquatic ecosystems, but they are essential to improving our knowledge of the fungi in different environmental samples (Bärlocher & Boddy, 2016). It should be noted that various eukaryotes such as zooplankton, invertebrates, and plant hosts are easily coamplified by amplicon sequencing based on the ITS2 rDNA region used for fungal identification (Scholz et al., 2015). Similarly, in the current study, a high proportion of reads mapped to the brown algae *Microspongium alariae in the* SW (3 717 10) and AEW (98 775) systems. This challenge is exacerbated by the fact that these eukaryotes frequently predominate in metagenomic sequence data from marine environments, which leaves marine fungi with a small representation (Ahmed et al., 2019). The ITS rDNA region primers were developed using sequence alignments from mostly terrestrial samples and are heavily biased

toward terrestrial Dikarya (Basidiomycota and Ascomycota), which results in the low representation of other fungal phyla known to inhabit marine settings (Ahmed et al., 2019). Furthermore, due to the use of culture-dependent methods, ascomycetes and chytridiomycetes account for the vast majority of fungi identified in marine environments, with basidiomycetes accounting for a small proportion (Shearer et al., 2006; Grossart & Rojas-Jimenzes, 2016; Jones et al., 2019). There is a scarcity of molecular studies on fungal communities in aquatic environments, as well as a lack of information about the ecological importance of many of the fungi that NGS may identify or assign to a specific taxon. Pathogenic fungi are studied, as well as fungi associated with specific hosts, for their function and role in the ecosystem, but for many of the environmental community members (i.e., water mycobiome), very little information is available regarding what they do, how they regulate their metabolism, and what benefit/problem they may cause to animals or plants.

4.6 Conclusions and recommendations

As expected, effluents from recirculated aquaculture systems are an extraordinary habitat, harbouring higher fungal diversity than seawater. It is plausible to assume that fungi, as heterotrophic microbes, use nutrient peaks in IMTA systems. Therefore, the current study provides a glimpse into the aquatic fungi present in the effluents and *Ulva* seedling ponds of an abalone aquaculture system. Metabarcoding techniques provide a roadmap for a deeper understanding of the distribution patterns, diversity, and abundance of fungi. In addition to the use of metabarcoding analyses, one of the primary technical drawbacks of this study was that the majority of fungi were either unidentified or mapped to other eukaryotic organisms. In order to improve the taxonomic assignment of fungal variants in the future, it is evident, based on the results of this study's large number of unidentified fungi, that accurate and comprehensive reference databases are essential. Furthermore, additional assessments of the ITS2 barcoding gene are required, and other barcoding genes may need to be considered. In conclusion, it has been demonstrated that these systems host various fungal communities such as *Fusarium*, *Aphanomyces*, *Pythium,* and several other species that can cause disease in plants and animals. This suggests that more research is needed to understand the potential risks associated with these fungi in integrated systems.

Chapter 5: Conclusions and future perspectives

5.1. Overview of study findings

Abalone (*Haliotis* spp.), a marine mollusc (marine snail), is a valuable export product, especially in East Asian countries like Japan and China. There are five *Haliotis* species found throughout South Africa's coastline (Muller, 1986); however, only one species, *H. midae,* is harvested or cultivated for economic purposes. Due to the decline in wild fisheries, South Africa began large-scale cultivation of *H. midae,* and today it is one of the world's top producers of cultivated abalone outside of Asia and Chile (FAO, 2014, 2017; DAFF, 2016). The successful cultivation of abalone is heavily reliant on the accessibility and utilisation of algae for several reasons, including the settlement of larvae, a source of feed for post-settled animals, juveniles and adult stages, and the bioremediation of farm effluent. The challenges associated with near- and off-shore aquaculture, such as the high energy of the coastline and the limited availability of sheltered bays, have been significant drivers for the development of integrated abalone – *Ulva* cultivation systems in South Africa. In certain geographical areas (e.g., Eastern Cape Province), the scarcity of wild seaweed (s), mainly *Ecklonia maxima*, as a natural feed for cultured abalone further highlights the need for alternative farming methods. Additionally, the adoption of these integrated systems offers advantages such as recirculating water and reduced pumping costs (Bolton et al., 2016). Integrated multi-trophic aquaculture (IMTA) is a sustainable aquaculture method that incorporates the co-cultivation of more than one species, such as fish, shellfish, and seaweeds, in a single system. These production systems aim to mimic natural ecosystems by developing a symbiotic interaction between species, often at different trophic levels, in which the waste products of one species serve as nutrients for another. However, feed supply, effluent water treatment and management, and biosecurity concerns have hindered the adoption of this sustainable approach in some countries, including South Africa, where only a limited number of producers cultivate abalone in integrated systems with *Ulva* spp. to improve the economic and environmental sustainability of their farming operations (Robertson-Andersson, 2003; Primavera, 2006). Bacterial and fungal communities likely play a significant role in IMTA systems, as they are responsible for organic matter decomposition and nutrient cycling. However, little is known about the

composition and function of the microbial communities in abalone – *Ulva* IMTA systems and the potential biosecurity risks or threats associated with the practice of seawater recirculation and the use of effluent-grown seaweed(s) as a supplementary feed. There are a number of ways to monitor microbiomes in complex environments, and in recent years, DNA metabarcoding has emerged as a highly effective method to characterise microbial communities in these environments, including IMTA systems. Therefore, the current study characterised the bacterial and fungal communities in abalone-*Ulva* IMTA systems and compared this to a non-IMTA system on the same farm using a metagenomics approach.

The objective of **Chapter 2** was to optimise the preparation of samples (filtration and DNA isolation methods) for next-generation sequencing and bioinformatic pipelines for 16S rDNA and ITS2 gene analysis of bacteria and fungi, respectively. The study demonstrated how extraction technique affect the yield and quality of total genomic DNA and, consequently, may also affect the estimated richness of ASVs detected in downstream bioinformatics analysis. The Qiagen QIAamp Micro and Qiagen Plant Mini kits were found to generate a lower yield of DNA compared to the Heat lysis method of Greeff et al. (2012). However, both kits exhibited DNA purity that was superior to the Heat lysis method and met the necessary standards for NGS analysis. Three bioinformatics pipelines were compared (QIIME2, mothur, and PIPITS), which are the three commonly used bioinformatics pipelines for processing 16S rDNA and ITS2 gene amplicon sequencing data, to determine whether and how each pipeline affects the number of reads assigned to taxonomy and the computational time to process the data. For the 16S rDNA gene data, it was demonstrated that QIIME2 and mothur were similar in terms of the number of reads, the number of unclassified reads at the genus level, and the number and type of phyla and genera identified. However, QIIME2 was superior to mothur based on the computational time, the number of reads assigned to taxonomy, and the number of unclassified reads. For the ITS2 gene analysis, QIIME2 (forward vs. merged reads) and PIPITS/QIIME2 (merged reads) were compared. The alpha diversity measure Chao1 showed no significant difference between the mycobiome detected with QIIME2 and PIPITS ($p > 0.05$), and the beta-diversity results showed no significant difference ($p > 0.05$) for the merged reads (QIIME2 and PIPITS), QIIME2 (forward reads) analysis demonstrated that samples clustered

according to sample type (p = 0.05), whereby *Ulva* and water samples clustered separately from one another. Moreover, fewer ASVs were identified in total for QIIME2 using merged reads, and QIIME2 retained more reads when using forward only as opposed to the merged reads (Table 2.5). The higher read counts detected by QIIME2 when using forward reads only were preferred, since less information was lost and raw read counts were more accurate. Consequently, the subsequent analysis of the 16S analysis in **Chapter 3** and the ITS2 analysis in **Chapter 4** were carried out using the QIIME2 software for analysis.

Bacterial communities in IMTA systems are diverse and complex, and their composition can be influenced by numerous biotic (biological) and abiotic (physical) factors and stressors such as water quality, temperature, nutrient availability, and the presence of other organisms (Duarte et al., 2019). Certain bacteria can protect fish and shellfish against disease (Huang et al., 2018), whereas the presence of some microorganisms has been shown to cause diseases in numerous aquaculture settings (Wang et al., 2007; Ma et al., 2008). Therefore, the sustainability of aquaculture systems requires a comprehensive understanding of the bacterial community to address potential biosecurity concerns and improve system management. **Chapter 3** characterised the microbiome of effluent water from an integrated abalone-*Ulva* aquaculture system where 50% of the water is recirculated (IMTA) between the abalone and *Ulva* raceway systems and compared this data to an seawater from *Ulva* seedling system that does not receive abalone effluent and water is not recirculated (non-IMTA). Using both culture-based (traditional plate count method) and non-culture-based (next-generation sequencing of the 16S rDNA, V4 hypervariable region) approaches, the current study assessed the biosecurity implications of recirculating water and using effluent-grown *Ulva* as a supplementary feed for the abalone on the farm.

The culture-based technique found notable differences in bacterial abundance between the non-IMTA and IMTA systems. Specifically, the abalone effluent water exhibited significantly higher levels of culturable bacteria in its inlets and outlets, as well as on the *Ulva*, compared to the samples taken from the non-IMTA system. Additionally, it was observed that *Ulva* has the potential to substantially reduce the bacterial load in abalone effluent, as evidenced by a

significant decrease in the number of culturable bacteria per mL of seawater from the inlet to the outlet of the *Ulva* paddle raceway (observed on both TSA and TCBS agar plates) in both the IMTA and non-IMTA systems. The latter findings were further supported by the non-culture-based approach. Alpha (within sample) diversity analyses, based on Chao1, Shannon, and Simpson indices, showed that the SW (non-IMTA) and AEW (IMTA) water cohorts displayed a higher degree of bacterial diversity (p = 0.01) than the *Ulva* cohorts, indicating a higher degree of unique ASVs in the water columns (AEW and SW) (Fig. 3.7). The significantly higher bacterial diversity in the water columns may be attributed to the constant pumping of seawater from the ocean to the abalone system, as it has been previously demonstrated by Brailo et al., (2019) that environmental seawater can be a natural source of microbial communities in aquaculture systems. This practice could be responsible for the increasing diversity of unique ASVs in the water column. Moreover, it is expected that the seawater would have a higher bacterial abundance and diversity than the *Ulva* due to *Ulva*'s ability to modulate microbial populations, as demonstrated previously by Kong et al. (2023). The provision of formulated and fresh feeds to abalone within the IMTA systems also has the potential to elevate nutrient loads, which can promote the growth of microbes and the expansion of microbial activity. Seasonal variation in the bacterial microbiome was also detected in the systems investigated in this study, where there was an increase in the number of unique ASVs and alpha diversity during the summer and spring months in the AEW system, corresponding with an increase in temperature over these periods.

The non-metric multidimensional scaling (NMDS) analysis revealed a high degree of overlapping ASVs across the six cohorts. The partial overlap between some of the water cohorts (AEW and SW) in the NMDS analysis at genus and family level implies that the communities of most of the sampling points shared similar taxa. Nevertheless, the clear distinction at genus level observed between the water and *Ulva* cohorts shows that notable differences exist between the bacteria present on the *Ulva* and in seawater. Another important observation to note was the distinct separation observed between samples obtained from the *Ulva* collected in the two systems (AEW *Ulva* and SW *Ulva*), revealing the presence of a distinct bacterial microbiome associated with *Ulva* cultivated in the IMTA and

non-IMTA systems (Fig. 3.9). Species previously identified as opportunistic pathogens of fish and abalone were identified in both system types (Nicolas et al., 2002; Cheng et al., 2004; Piñeiro-Vidal et al., 2008; Travers et al., 2008; Olsen et al., 2017), but had a low prevalence across all cohorts and were differentially abundant in the AEW cohort, but none of the abundant species were known pathogens. While opportunistic bacteria, including *Vibrio*, have been detected in the systems, it is important to note that they are naturally occurring in marine environments and are typically part of the microbiota associated with healthy ecosystems, thus posing a minimal biosecurity threat (Kennedy et al., 2006; Castex et al., 2014; Gao et al., 2019). Additionally, culture-based and non-culture-based methods used in this study demonstrated positive changes in the microbiome from the inlets to the outlets of both the non-IMTA and IMTA *Ulva* raceways systems. Most notably, the total number of *Vibrio* species declined between the inlets and outlets of both system types, suggesting that the macroalga *Ulva* has a strong modulatory effect on *Vibrio* species in the seawater (Fig. 3.2). It is worth noting that the inhibitory effect seems to be influenced by the presence of nutrients in the system, as a more pronounced reduction in *Vibrio* species was detected in the effluent water system. The results presented here are in line with the findings of Lu et al., (2008), where a reduction in the abundance of *V. anguillarum* following exposure to *U. clathrata* was enhanced by the addition of nitrogen and phosphorus to the seawater. The high levels of organic matter, primarily from fish bioproducts and uneaten feed, in combination with temperature and salinity in integrated aquaculture systems, such as IMTA, can play a significant role in the rate of bacterial proliferation (Asplund et al., 2011; Resendeet al., 2015; Lin et al., 2016). However, *Ulva* and its associated microorganisms, act as natural disinfectants and antifouling agents, where they effectively lower the risk of disease by inhibiting the proliferation of opportunistic bacteria within biofilms on biofilters. The full extent of *Ulva*'s disinfection capacity warrants further investigation and to enhance the system's health, it is advisable for farmers to practice good husbandry, including periodic tank drainage and cleaning between *Ulva* harvests, which can effectively prevent the excessive formation of biofilms. The composition of bacterial communities in IMTA systems can be influenced by the types of organisms cultivated in the IMTA. Inclusion of seaweeds, for example, has been shown in this study (Fig. S3.1), and elsewhere (Wang et al., 2016), to facilitate the proliferation

of specific bacterial taxa that are commonly found on the seaweed, such as Rhodobacteraceae and Flavobacteriaceae. Similarly, previous studies have demonstrated that the inclusion of abalone in an IMTA can facilitate the proliferation of specific bacterial taxa that are associated with the abalone gut microbiota, including *Vibrio* and *Pseudoalteromonas* (Liu et al., 2018). Furthermore, putative metabolic functions assigned to the bacterial communities demonstrated an upregulated function of metabolism in all cohorts. The findings of this study highlight the significance of continuous surveillance of microbial communities across several farm locations, particularly during periods of increased microbial activity, in order to support the sustainability of Integrated Multi-Trophic Aquaculture (IMTA).

Fungal communities in IMTA systems are less well-studied than bacterial communities, regardless of their importance in the cycling of nutrients and the decomposition of organic matter. Fungi are known for their ability to degrade complex organic compounds, such as lignin and cellulose, and are therefore important in the breakdown of plant and animal waste products (Pointing et al., 2001; Deshmukh et al., 2016). **Chapter 4** assessed the spatial and temporal variation of the fungal and oomycete composition of the effluent water in an integrated abalone-*Ulva* system, where 50% of water is recirculated (IMTA), and compared it to the seawater from *Ulva* raceway system that does not receive abalone effluent and water is not recirculated (non-IMTA). The assessment of the fungal communities associated with the IMTA and non-IMTA systems using next-generation sequencing of the ITS2 region revealed the presence of 169 individual ASVs, excluding unassigned reads. For the analysis of diversity that was assigned to taxonomy, the alpha (within sample) diversity analyses, based on Chao1, Shannon, and Simpson indices, displayed a higher degree of fungal diversity ($p < 0.001$) in the non-IMTA cohort than in other cohorts, which may have been explained by the regular pumping of fresh seawater from the ocean over the abalone and into the effluent raceways and the increasing presence of unique ASVs in the water column. However, a truer representation of alpha diversity, which included diversity that was not assigned to taxonomy, indicated that the richness estimator Chao1 was significantly greater for the IMTA system when compared to the non-IMTA system, indicating unique ASVs in the nutrient-rich IMTA system. Similarly, the richness estimator Chao1 was significantly greater in the water samples

of both system types (SW and AEW), when compared with the *Ulva* samples in the two systems. This finding is consistent with the trend observed in Chapter 3, wherein the level of diversity in the IMTA system was notably higher compared to the non-IMTA system. The observed difference in fungal diversity between the IMTA system and the non-IMTA system can be attributed to the elevated nutrient levels present in the effluent system (Robertson-Andersson, 2003; Probyn, et al., 2016). This finding coincides with previous findings that have demonstrated that fungi tend to proliferate in environments characterised by high nutrient availability (Sen et al., 2022). The temporal variation in eukaryotic communities in terms of alpha diversity was also detected in our study, where higher temperatures during summer increased fungal diversity in the SW_summer cohort. Beta (between sample) diversity analyses, including principal coordinate analysis (PCoA), showed a separation between water columns (AEW and SW) and a separate cluster of (*Ulva*_AEW and *Ulva*_SW), indicating that similar samples tended to have significantly different fungal communities (R^2 = 0.44, p < 0.001). The non-metric multidimensional scaling (NMDS) was consistent with PCoA, whereby water cohorts still clustered separately with one another, demonstrating that mycobiome differed in different cohorts.

The most abundant groups of fungi associated with *Ulva* and seawater samples in this study belonged to the Ascomycota, followed by Basidiomycota. This is consistent with previous findings that showed most fungi in marine environments belong to these two fungal phyla (Taylor & Cunliffe, 2016; Wang et al., 2018; Sen et al., 2021). Differential abundance analysis (DESeq2) showed six differentially abundant pathogenic taxa, though in low prevalence, that included *Cryptococcus* (0.09%), *Candida* (3.38%), *Saprolegnia* (1.3%) and *Aphanomyces* (0.75%) in the non-IMTA system and *Cryptococcus* (0%), *Pythium* (0.03%), *Candid*a (0.06%), *Saprolegnia* (0.02%) and *Aphanomyces* (0.01%) in the IMTA systems. In spite of this, when animals are exposed to stressful conditions, such as the stresses associated with aquaculture practices, fungal species that ordinarily do not act as disease-causing agents may become pathogenic. The composition of fungal communities in IMTA systems can also be influenced by the presence of other organisms. For example, the presence of seaweeds in IMTA systems can promote the growth of certain fungal taxa, such as *Aspergillus* and *Penicillium,* which are

known to be associated with seaweed surfaces and have been demonstrated to possess extracts that exhibit a relatively broad spectrum of antifouling activity against various biofoulers (Zhang et al., 2009; Zhou et al., 2014). Overall, the result from this chapter suggests that the presence of certain taxa is more likely to be caused by farm activities and environmental parameters such as temperature. Additional characterisation of environmental parameters (which were briefly analysed in the study) are needed to give comprehensive overview of the interactions emerging from an eventual network between microorganisms and their hosts. Understanding when and how two microorganisms are co-occurrent or co-abundant and what environmental parameters are at the time, can provide insight into the metabolic relationship between the two. As a result, to explore the co-occurrence patterns of fungi and oomycetes, a more detailed analysis of the environmental parameters would be a prerequisite to establishing evidence of the co-occurring networks between fungi. Currently, there is limited knowledge about the bacterial and fungal characterisation of abalone – *Ulva* IMTA systems, and the current study represents a critical contribution to the current body of knowledge in aquaculture and environmental sciences. Several South African commercial aquaculture farms already practice integrated aquaculture and grow *Ulva* in abalone effluent and feed this back to abalone, thus recycling nutrients sourced from feed and excreted by the animals (Bolton et al., 2009). Identification of pathogens can be done using targeted methods (culture-based or molecular). Molecular methods can inform whether the microbiome(s) configuration/structure might promote the pathogens outbreak and can be used to modulate the water and *Ulva* microbiome, for example through inoculation of probiotics or prebiotics. Probiotics, including *Vibrio midae* and *Debaryomyces hansenii*, have been studied for their potential to enhance abalone growth and health (Venter et al., 2018). According to Van Wyk (2004), probiotics are a viable treatment option that can effectively inhibit the proliferation of pathogenic bacteria, reducing the likelihood of disease outbreaks while improving the overall health of abalone. As a result, microorganisms, especially specific algae species and probiotics, are critical components of IMTA systems for abalone farmers, providing benefits such as larval settlement cues, food sources, and better growth and health. This study contributes to the knowledge of the microorganisms in the IMTA systems and can be a step

forward the introduction of molecular characterisation techniques in IMTA systems as an aid in decision making about biosecurity measures.

5.2 Future perspectives

Understanding microbiomes in IMTA systems and distinguishing between health-promoting microbial communities and an unbalanced microbiome structure that can facilitate the proliferation of pathogens is crucial for the long-term operation of aquaculture systems that farmers should implement. To accomplish this objective, it is important to build long-term monitoring programmes that are designed to follow and analyse the dynamics of microbial communities in relation to disease outbreaks, environmental changes or variations in farm productivity. The utilisation of metagenomic sequencing techniques could allow for the examination of microbial composition and functionality, leading to valuable insights. For example, researchers can infer microbial metabolic pathways involved in nutrient cycling, degradation of pollutants, or synthesis of bioactive compounds. Using shotgun sequencing and metatranscriptomics in Integrated Multi-Trophic Aquaculture (IMTA) systems can improve our understanding of microbial communities, especially fungi and bacteria. Shotgun sequencing enables comprehensive taxonomic profiling by detecting a diverse range of microorganisms, including rare species, and provides insights into their functional potential based on genetic content. This information is critical for understanding ecosystem processes and their potential uses in biotechnology and environmental remediation. Moreover, for future studies, it is important to emphasise the importance of studying the abalone. This is a valuable aspect that requires further exploration. Establishing a comprehensive understanding of the microorganisms associated with the abalone microbiome is essential, as it will improve our understanding of the interaction between microorganisms in the IMTA and the abalone microbiome. By making this connection, we can enhance our knowledge of the interactions and impacts within these complex aquatic farming systems, with potential implications for productivity.

Metabarcoding methods such as ITS and 18S are opening the way to a more in-depth understanding of fungal distribution patterns, diversity, and abundance. However, one of the

major technical constraints in the current study was that the majority of sequences were unclassified. Moreover, there is a need for an adaptation of a new design considering the fact that amplicon sequencing based on ITS2 readily coamplifies other eukaryotes or invertebrates. The findings of this study highlight the necessity for more precise and comprehensive reference databases to enhance the taxonomic assignment of fungal ASVs in subsequent studies. The utilisation of advanced bioinformatics techniques facilitates the process of pattern identification, while joint research efforts contribute to the development of multidisciplinary knowledge. Comparative studies between healthy and unhealthy systems can be used to discover essential microbial indicators, whereas multi-omics techniques can be used to provide entire microbial functions. Moreover, experiments involving controlled modifications of the microbiome can be employed to evaluate the effects on the overall health of a system. The implementation of education and standardised protocols plays a crucial role in maintaining data consistency within the context of aquaculture. Additionally, regulatory engagement serves as a means to ensure that the promotion of microbiome health is effectively integrated into the established standards of the aquaculture industry. These strategies, especially when used collectively, enable the aquaculture industry to make well-informed decisions, enhance the overall health of the system, and assure long-term sustainability.

5.3 Study limitations

Several limitations should be considered while analysing the water microbiome of integrated multi-trophic aquaculture (IMTA) systems. To begin with, this study focused solely on the water columns and the *Ulva* due to *Ulva*'s biofiltering capacity and concerns about potential biosecurity issues associated with feeding abalone the *Ulva* grown in effluent water. Direct sampling of abalone tissue, which is a crucial component of the IMTA system, was not included. This omission limits our understanding of microbial interactions related to abalone health and performance. However, this study will inform which microorganisms are present in their culture facilities and how they are transferred across different system components. The current study identified a microbial community that included the genera *Psychrilyobacte*r, *Vibrio*, and to a lesser extent, *Mycoplasma*. These results are in line with

previous studies by Danckert et al. (2020) and Gobet et al. (2018), who demonstrated that the core microbiome of *Haliotis laevigata*, *Haliotis rubra* and *Haliotis tuberculata* were dominated by genera *Psychrilyobacter*, *Vibrio*, and *Mycoplasma*. The absence of mortality of abalone observed in the previous studies suggest that these bacteria are vital to maintaining a functional digestive tract of abalone. To advance our understanding and practices in abalone aquaculture, future studies should include long-term monitoring of microbiome changes in the aquaculture system and the abalone, functional analyses of key bacterial roles to support abalone health. In addition to the current limitation, the sampling frequency was low and the sample size for the SW system was limited, which may have limited capturing temporal fluctuations and nutrient levels, and may have impacted microbial community dynamics, clouding our understanding of baseline microbial profiles. These limitations highlight the necessity for future studies to use more thorough sampling methodologies that include all important components of IMTA systems and consider seasonal differences. Another key limitation of the study was the availability and lack of available fungal databases, as many fungal species have not been identified or described, resulting in gaps in the current databases. Collaboration between researchers and database curators, and further exploration and characterisation of marine fungi is necessary and critical for expanding our understanding of fungal diversity and its impact on microbiome dynamics.

5.4 Conclusion

In conclusion, the use of DNA metabarcoding presents a robust approach towards the characterisation of bacterial and fungal communities within IMTA systems, as well as a thorough examination of their functional potential. The research presented in this thesis has highlighted the significance of microorganisms such as bacteria and fungi in IMTA systems and detected microorganisms such as *Vibrio, Pythium,* and *Aphanomyces* in the water systems that have the potential to be pathogenic. Moreover, despite concerns expressed by certain commercial abalone farmers regarding the potential risks associated with recirculation within aquaculture feed systems, there is currently no scientific literature reporting any instances of disease outbreaks attributed to the utilisation of effluent-grown *Ulva* as feed for abalone. Future studies should continue to employ this technique or make

use of new advanced techniques in order to acquire a more comprehensive understanding of the functions of microorganisms in IMTA systems and their potential to be enhanced to achieve optimal productivity and sustainability and to correlate microbiome composition with the health status of the animals and productivity of the farm.

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Appendix A: Supplementary Figures for Chapter 3

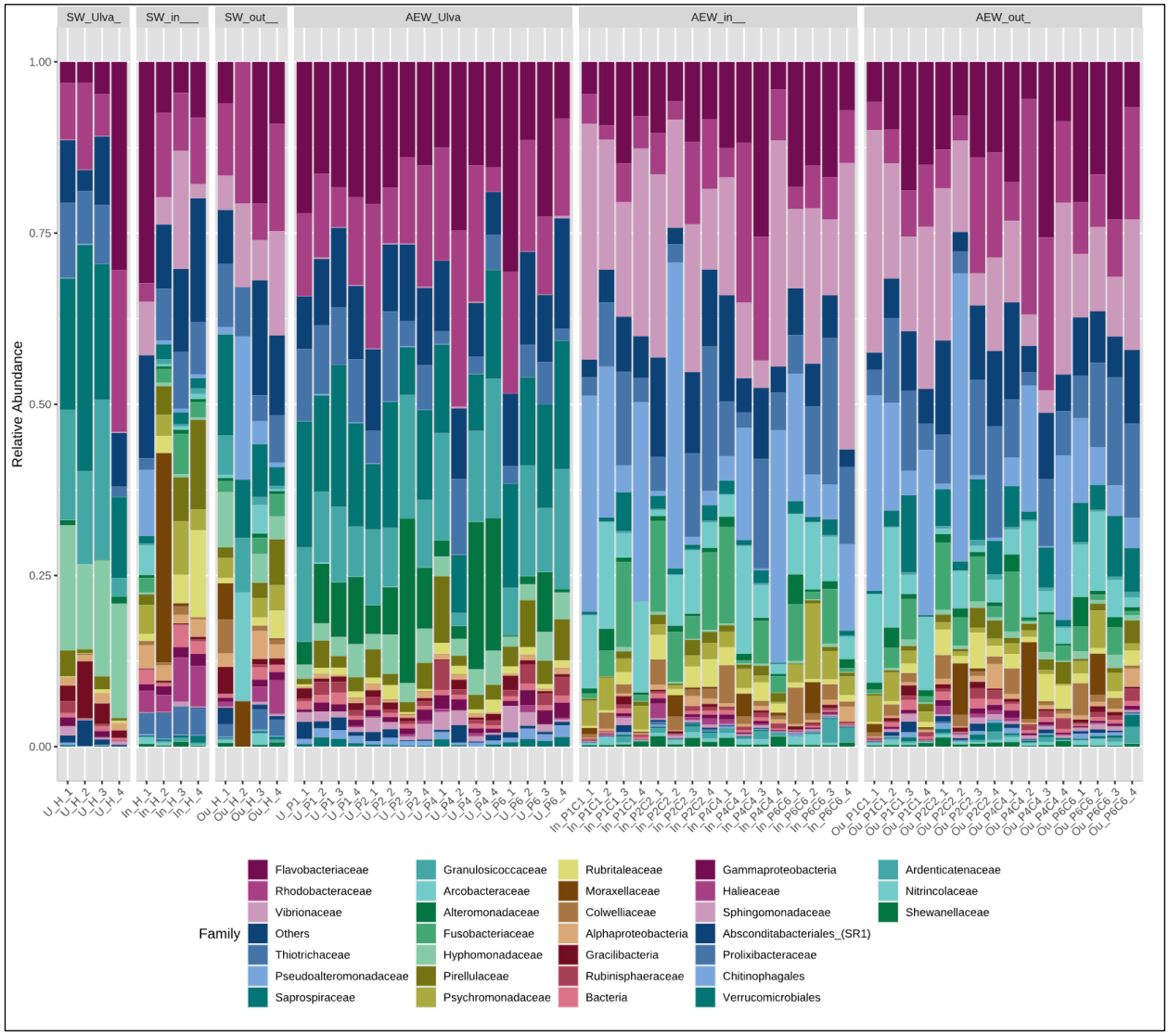

Figure S3.1 Sample-wise relative ASV abundance (%) at family level, across 60 samples. Less prominent ASVs were merged and denoted as "Others" Sample codes need to be described. Samples denoted with "H" are from the SW (non_IMTA), while samples denoted "P" are from the AEW (IMTA) system. *Ulva* samples are denoted with" U", Inlets are denoted with (In), and outlets are denoted with (Ou).

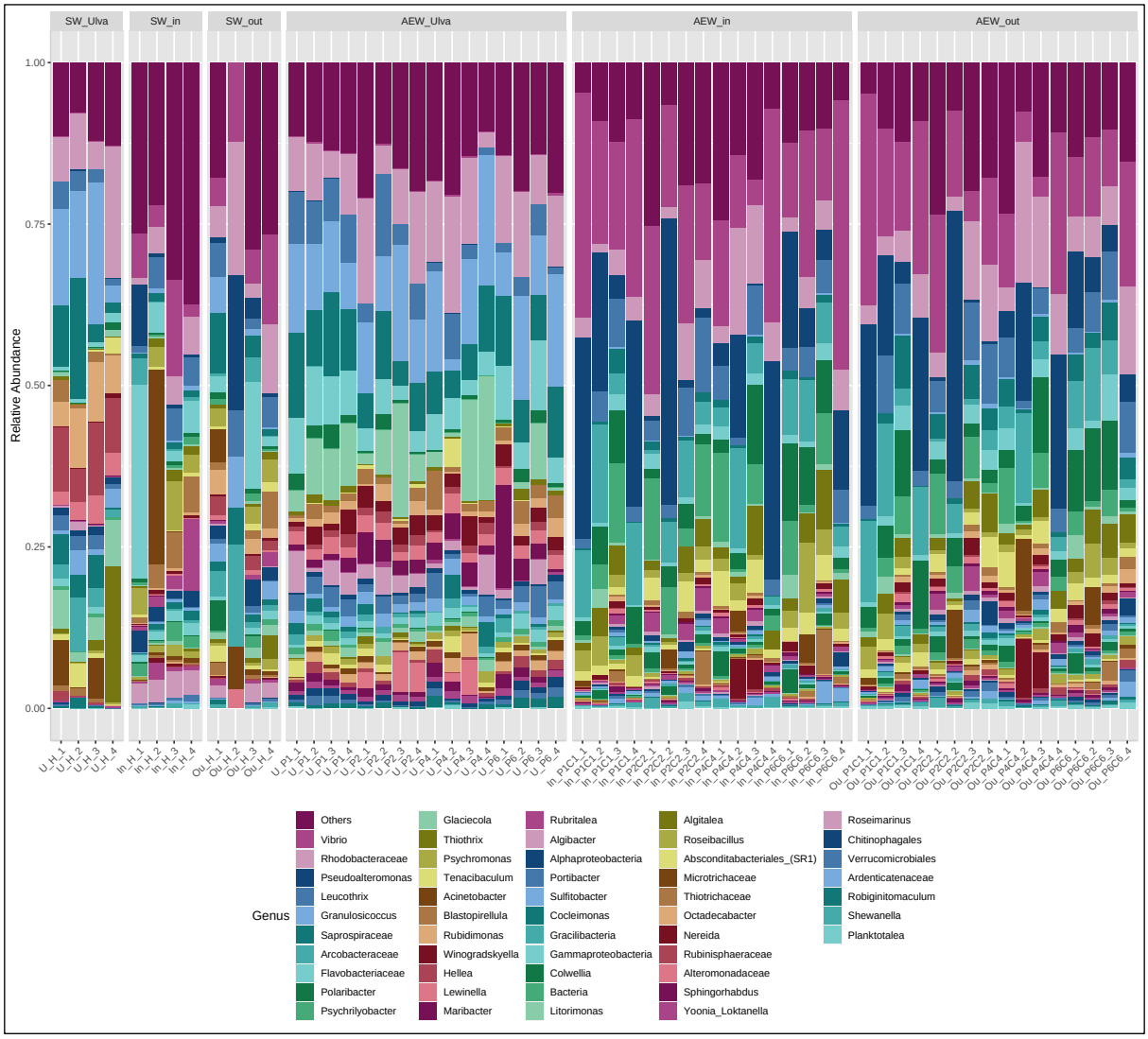

Figure S3.2. Sample-wise relative ASV abundance (%) at genus level, across 60 samples. Less prominent ASVs were merged and denoted as "Others" Sample codes need to be described. Samples denoted with "H" are from the SW (non_IMTA), while samples denoted "P" are from the AEW (IMTA) system. *Ulva* samples are denoted with" U", Inlets are denoted with (In), and outlets are denoted with (Ou).

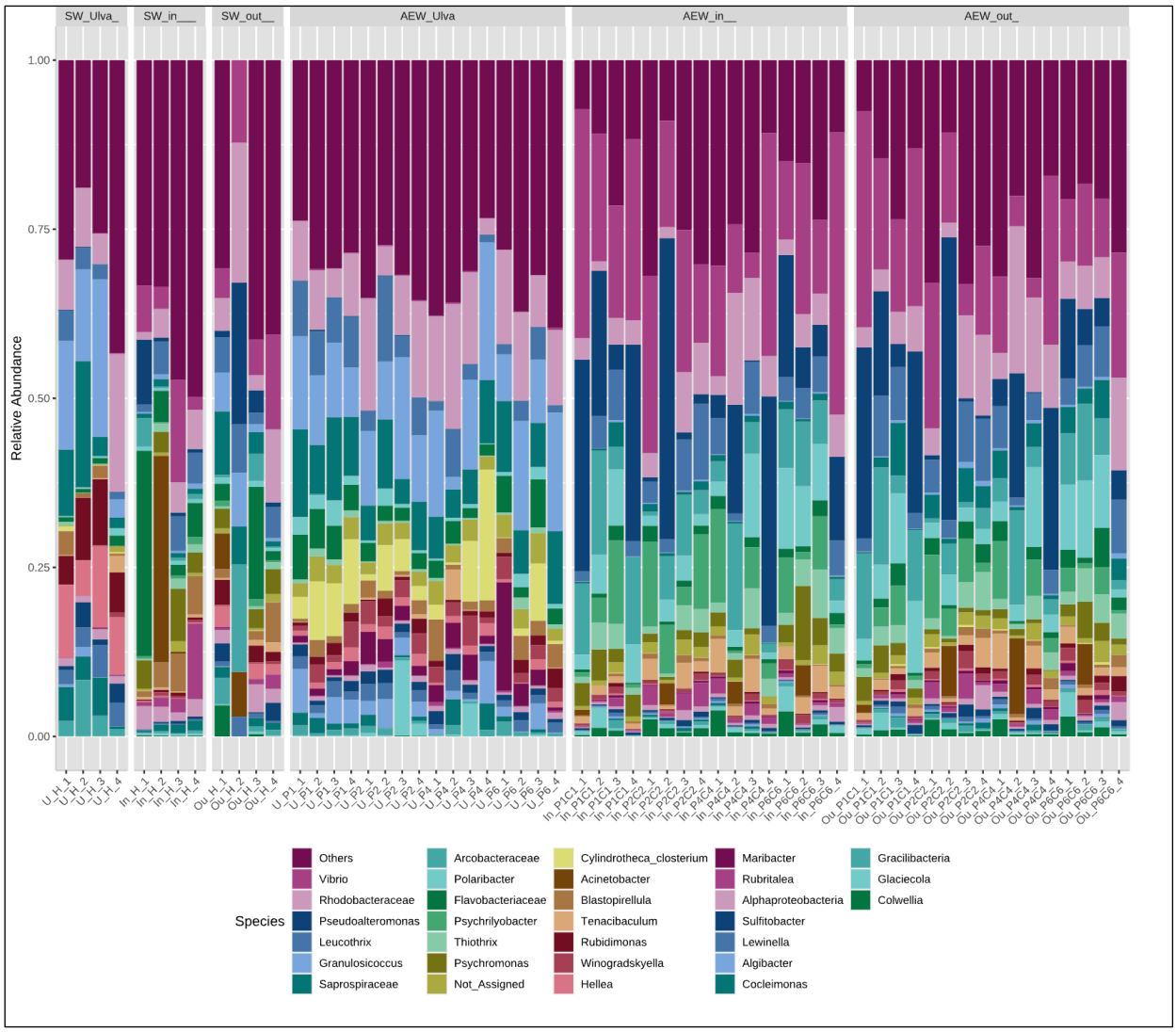

Figure S3.3. Sample-wise relative ASV abundance (%) at species level, across 60 samples. Less prominent ASVs were merged and denoted as "Others" Sample codes need to be described. Samples denoted with "H" are from the SW (non_IMTA), while samples denoted "P" are from the AEW (IMTA) system. *Ulva* samples are denoted with" U", Inlets are denoted with (In), and outlets are denoted with (Ou).

Table S3.1. A comprehensive description of samples

Sample_id	SampleType	Description
Ou_P6C6_1	AEW_Out	Abalone Effluent outgoing water
U_P1_1	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P1_2	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P1_3	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P1_4	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P2_1	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P2_2	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P2_3	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P2_4	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P4_1	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P4_2	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P4_3	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P4_4	SW_*Ulva*	Seawater *Ulva*
U_P4_4	SW_*Ulva*	Seawater *Ulva*
U_P6_2	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P6_3	AEW_*Ulva*	Abalone Effluent *Ulva*
U_P6_3	AEW_*Ulva*	Abalone Effluent *Ulva*
U_H_1	SW_*Ulva*	Seawater *Ulva*
U_H_2	SW_*Ulva*	Seawater *Ulva*
U_H_3	SW_*Ulva*	Seawater *Ulva*
U_H_4	SW_*Ulva*	Seawater *Ulva*
In_P1C1_1	AEW_In	Abalone Effluent incoming water
In_P1C1_2	AEW_In	Abalone Effluent incoming water
In_P1C1_3	AEW_In	Abalone Effluent incoming water
In_P1C1_4	AEW_In	Abalone Effluent incoming water
In_P1C1_4	AEW_In	Abalone Effluent incoming water
In_P2C2_2	AEW_In	Abalone Effluent incoming water
In_P2C2_3	AEW_In	Abalone Effluent incoming water
In_P2C2_4	AEW_In	Abalone Effluent incoming water
In_P4C4_1	AEW_In	Abalone Effluent incoming water
In_P4C4_2	AEW_In	Abalone Effluent incoming water
In_P4C4_3	AEW_In	Abalone Effluent incoming water
In_P4C4_4	AEW_In	Abalone Effluent incoming water
In_P6C6_1	AEW_In	Abalone Effluent incoming water
In_P6C6_2	AEW_In	Abalone Effluent incoming water
In_P6C6_3	AEW_In	Abalone Effluent incoming water
In_P6C6_4	AEW_In	Abalone Effluent incoming water
In_H_1	SW_In	Seawater Incoming water
In_H_2	SW_In	Seawater Incoming water

In_H_3	SW_In	Seawater Incoming water
In_H_4	SW_In	Seawater Incoming water
Ou_P1C1_1	AEW_Out	Abalone Effluent outgoing water
Ou_P1C1_2	AEW_Out	Abalone Effluent outgoing water
Ou_P2C2_1	AEW_Out	Abalone Effluent outgoing water
Ou_P2C2_2	AEW_Out	Abalone Effluent outgoing water
Ou_P4C4_1	AEW_Out	Abalone Effluent outgoing water
Ou_P4C4_2	AEW_Out	Abalone Effluent outgoing water
Ou_P6C6_2	AEW_Out	Abalone Effluent outgoing water
Ou_H_1	SW_Out	Seawater outgoing water
Ou_H_2	SW_Out	Abalone Effluent outgoing water
Ou_P1C1_3	AEW_Out	Abalone Effluent outgoing water
Ou_P1C1_4	AEW_Out	Abalone Effluent outgoing water
Ou_P2C2_3	AEW_Out	Abalone Effluent outgoing water
Ou_P2C2_4	AEW_Out	Abalone Effluent outgoing water
Ou_P4C4_3	AEW_Out	Abalone Effluent outgoing water
Ou_P4C4_4	AEW_Out	Abalone Effluent outgoing water
Ou_P6C6_3	SW_Out	Seawater outgoing water
Ou_P6C6_4	AEW_Out	Abalone Effluent outgoing water
Ou_H_3	SW_Out	Seawater outgoing water
Ou_H_4	SW_Out	Seawater outgoing water

Table S3.2. An overview of filtering and quality trimming of individual samples performed on DADA2

Sample-id	Input	Filtered	Denoised	Merged	Non-chimeric
Ou_P6C6_1	455741	375712	369737	344427	326168
U_H_1	341601	296544	294212	281823	279542
U_H_2	387929	351543	349932	338337	336783
In_P1C1_1	366517	319136	316727	302125	264496
In_P1C1_2	447228	390771	387067	366815	339874
In_P2C2_1	383650	315782	309758	290986	287645
In_P2C2_2	405437	338602	334955	320915	303342
In_P4C4_1	489627	404140	397772	375239	365117
In_P4C4_2	417932	364803	360906	342805	327772
U_P1_1	462525	407551	404469	386175	380787
In_P6C6_1	943814	799040	791327	743832	686037
In_P6C6_2	384152	327420	323716	306082	284957
In_H_1	385561	344072	338232	320121	316106
In_H_2	380492	326656	322357	306182	302030
Ou_P1C1_1	414926	360331	357587	338219	297913
Ou_P1C1_2	397331	345732	341584	325782	316562
Ou_P2C2_1	396678	328122	321482	301220	296214
Ou_P2C2_2	443922	373520	369899	355645	337314
Ou_P4C4_1	432045	363782	357236	336497	328332
Ou_P4C4_2	383065	332401	329334	319033	309717
U_P1_2	434302	390622	387709	376371	373083
Ou_P6C6_2	395204	346511	343209	327541	313306
Ou_H_1	345764	308017	303316	291768	287480
Ou_H_2	1750	951	697	437	437
U_P1_3	397221	333651	330144	319187	315051
U_P1_4	432107	378865	375731	362306	357897
U_P2_3	373136	318116	315937	306703	305072
U_P2_4	301347	250001	247358	237565	236027
U_P4_3	405966	338902	336886	327074	325000
U_P2_1	396538	348319	345185	333479	331271
U_P4_4	406691	339634	337389	324016	317973
U_P6_3	337346	256399	253528	240779	236582
U_P6_4	367210	293416	290505	280094	277860
U_H_3	416756	378299	376080	367158	363240
U_H_4	229883	170929	169264	162800	160373
In_P1C1_3	425979	363405	357562	333763	323315
In_P1C1_4	369999	304437	301212	282192	249998

In_P2C2_3	339971	298246	292934	276257	267570
U_P2_2	437398	392609	390036	374488	366134
In_P2C2_4	253280	202272	197600	182375	179637
In_P4C4_3	367667	304369	299048	279911	273001
In_P4C4_4	432684	351023	347138	330108	307835
In_P6C6_3	343524	284110	279731	260818	249083
In_P6C6_4	410995	316069	312500	291932	265915
In_H_3	323848	220701	215643	191733	184654
In_H_4	372324	306834	300632	273298	270141
Ou_H_3	427377	328685	321864	295413	292232
Ou_P1C1_3	410350	358288	351899	328382	316814
Ou_P1C1_4	428209	365919	362140	339278	296894
U_P4_1	401773	351783	349491	339759	338046
Ou_P2C2_3	340340	277532	271540	253029	250283
Ou_P2C2_4	275979	225067	219563	205969	200888
Ou_P4C4_3	383069	291704	286505	265815	261435
Ou_P4C4_4	427199	348141	343460	325673	309931
Ou_P6C6_3	341349	291209	286263	264530	253935
Ou_P6C6_4	667997	543353	535477	505573	480141
Ou_H_4	376201	328071	321742	297040	293680
U_P4_2	426627	368071	364122	349132	344331
U_P6_1	406545	355906	352635	338658	334602
U_P6_2	394880	349482	346698	336668	333984

Table S3.3. Seasonal average variations in physicochemical parameters (temperature and pH), nitrogen compounds (nitrate—NO3, nitrite—(nitrate—NO^-_3, nitrite—NO^-_2, ammonium—NH_4^+, and phosphate $PO4^{3-}$) in the abalone effluent water (AEW) and seawater (SW) aquaculture farming systems across autumn (a), winter (b), summer (c) and spring (d)

(A) AUTUMN

	H1			**C1**			**C2**			**C3**			**C4**		
Parameters	**Inlet**	**Outlet**	**Centre**	**Inlet**	**Outlet**	**Centre**	**inlet**	**Outlet**	**Centre**	**Inlet**	**Outlet**	**Centre**	**Inlet**	**Outlet**	**Centre**
Temp (°C)	12,8			16,1	15,9		16,1	16		19,3	19		17	17,2	
pH	7,93			8,08	8,01		7,58	7,64		8,24	7,99		8,02	7,71	
DO (mg/L)	8,35			9,8	8,8		9,4	8,8		7,2	7		7,4	6,7	
NO_2^- (mg/L)	0,30	0,25	0,27	0,41	0,30	0,72	0,49	0,68	2,04	2,8	1,40	2,58	1,32	0,75	1,31
NO_3^- (mg/L)	13,96	18,33	18,04	6,17	11,97	3,81	15,27	24,85	8,15	24,04	19,39	32,89	33,15	22,0	26,26
NH_4 (mg/L)	1,18	0,94	0,73	0,13	1,83	0,38	6,86	2,45	2,64	22,95	4,92	9,95	12,37	5,57	4,72
PO_4 (mg/L)	1,16	0,56	0,45	0,81	0,85	2,11	1,57	0,74	1,86	5,80	2,02	6,94	1,86	1,39	1,46

(B) WINTER

	H1		**C1**		**C2**		**C3**		**C4**	
Parameters	**Inlet**	**Outlet**	**Inlet**	**Outlet**	**Inlet**	**Outlet**	**Outlet**	**Inlet**	**Inlet**	**Outlet**
Temp (°C)	15,6		14,3	14,4	15,7	15,9	14,2	14,3	14,3	14,4
pH	8,12		7,84	7,68	8	7,79	7,64	7,7	7,72	7,62
DO (mg/L)	7,79		8,2	7,7	8	7,8	8,1	8,3	9	8,7
NO_2^- (mg/L)	0,291	0,178	0.95	0,62	0,63	0,98	1,33	1,26	1,956	2,41
NO_3^- (mg/L)	5,39	4,09	5,69	12,55	12.22	6	7,79	16.76	27.37	19,79
NH_4 (mg/L)	-2,62	2,11	11,41	1,63	8,35	3,27	10,77	23,44	17,07	8,11
PO_4 (mg/L)	0,51	0,27	1.29	0,85	1.18	8,74	1,68	1.43	3,83	3,56

Appendix A

(C) SUMMER

	H1			C1			C2			C3			C4		
	Inlet	**Outlet**	**Centre**	**Inlet**	**Outlet**	**Centre**	**inlet**	**Outlet**	**Centre**	**Inlet**	**Outlet**	**Centre**	**Inlet**	**Outlet**	**Centre**
Parameters															
Temp (°C)	16,3	16,4	16,4	17,7	17,6	17,6	19.5	19,1	18,6	16,1	16,2	16,2	18,5	17,1	15,6
pH															
DO (mg/L)															
NO_2^- (mg/L)	0,029	0,100	0,12	0,12	0.22		0,16	0,11	0,19	0,53	0,97	0,67	0,30	0,09	0,10
NO_3^- (mg/L)	-1,67	-3,24	-0,68	-1.15	-3.36		0,93	2,69	-3,99	-3,42	-2,12	-3,05	0,09	-3,09	-3,38
NH_4 (mg/L)	0,19	-0,09	2,25	16,02	5,33		11,68	6,42	6.77	25,97	16,82	10,02	3,15	1,16	0,29
PO_4 (mg/L)	2,41	1,44	1,35	3.68	3.87		2,62	2,44	2,38	7,87	4,31	5,64	5,91	5,28	6,21

(D) SPRING

	H1			C1			C2			C3			C4		
	Inlet	**Outlet**	**Centre**	**Inlet**	**Outlet**	**Centre**	**inlet**	**Outlet**	**Centre**	**Inlet**	**Outlet**	**Centre**	**Inlet**	**Outlet**	**Centre**
Parameters															
Temp (°C)	14,5			14,6	14,7		16,1	16,2		17,1	17,3		16,3	16,6	
pH	7,9			7,53	8,04		8,09	7,69		8,18	8,02		7,9	7,71	
DO (mg/L)	8,76			7,8	7,8		9.6	8,5		7,8	7,8		7,5	7	
NO_2^- (mg/L)	0,19	0,34	0,14	0,76	0,27	0,31	0,31	0,72	1,27	0,66	0,55	0,54	1,10	1,19	0,41
NO_3^- (mg/L)	4,52	4,45	5,33	1,21	-0,98	0,31	19,29	11,59	12,31	27,51	24,53	28,29	22,61	18,77	7,58
NH_4 (mg/L)	0,35	0,13	0,11	3,66	0,49	0,07	6,67	4,81	3,14	10,21	3,31	2,03	2,79	0,63	2,49
PO_4 (mg/L)	1,06	0,79	0,88	0,92	2,52	3,09	2,03	2,19	2,24	4,53	3,81	3,94	4,93	5,33	4,27

Appendix B: Supplementary Figures for Chapter 4

Table S4.2. An overview of filtering and quality trimming of individual samples performed on QIIME2

Sample_id	Input	Filtered	Percentage of input passed filter	Denoised	Non-chimeric	Percentage of input non-chimeric
Ou_P6C6_1	504294	413505	82	409971	407195	80,75
U_P1_1	479303	383587	80,03	381689	379736	79,23
U_P1_2	275875	225202	81,63	223730	223593	81,05
U_P1_3	336262	248063	73,77	246398	244391	72,68
U_P1_4	1601741	1235079	77,11	1232326	1230985	76,85
U_P2_1	775088	665259	85,83	664540	659848	85,13
U_P2_2	739336	611994	82,78	610840	600342	81,2
U_P2_3	68578	43682	63,7	42851	42190	61,52
U_P2_4	182345	130892	71,78	129803	129366	70,95
U_P4_1	666966	541681	81,22	540646	537093	80,53
U_P4_2	382133	314598	82,33	312976	311503	81,52
U_P4_3	98538	66743	67,73	65757	65669	66,64
U_P4_4	95828	65417	68,27	64477	64090	66,88
U_P4_4	347283	288993	83,22	285695	285029	82,07
U_P6_2	312808	258329	82,58	256960	256829	82,1
U_P6_3	168481	111952	66,45	110227	108880	64,62
U_P6_3	199041	138497	69,58	137110	136424	68,54
U_H_1	291200	245060	84,16	244554	244384	83,92
U_H_2	223217	187017	83,78	186628	186549	83,57
U_H_3	343696	229974	66,91	229164	228911	66,6
U_H_4	529392	412362	77,89	411990	411360	77,7
In_P1C1_1	470246	389615	82,85	388650	386061	82,1
In_P1C1_2	502820	419211	83,37	417246	414198	82,38
In_P1C1_3	303578	258479	85,14	254555	251483	82,84
In_P1C1_4	237087	197910	83,48	195196	193733	81,71
In_P1C1_4	201325	168081	83,49	163378	161748	80,34
In_P2C2_2	523782	428154	81,74	425405	423484	80,85
In_P2C2_3	278758	239807	86,03	236983	235458	84,47
In_P2C2_4	436908	345521	79,08	343620	341205	78,1
In_P4C4_1	565660	472463	83,52	470027	466815	82,53
In_P4C4_2	512896	427682	83,39	425465	423824	82,63
In_P4C4_3	298120	258739	86,79	256159	254036	85,21

In_P4C4_4	711625	618191	86,87	610421	601624	84,54
In_P6C6_1	563461	479329	85,07	476867	473716	84,07
In_P6C6_2	522219	428422	82,04	426233	423470	81,09
In_P6C6_3	304837	259217	85,03	256437	254737	83,56
In_P6C6_4	283634	245498	86,55	242265	231051	81,46
In_H_1	440557	365823	83,04	361788	348655	79,14
In_H_2	263643	210187	79,72	208391	199683	75,74
In_H_3	425159	311617	73,29	308758	276231	64,97
In_H_4	445566	326365	73,25	324660	310247	69,63
Ou_P1C1_1	428997	359819	83,87	357908	356016	82,99
Ou_P1C1_2	462366	376324	81,39	374332	372476	80,56
Ou_P2C2_1	522661	425043	81,32	422604	419623	80,29
Ou_P2C2_2	581428	478321	82,27	476218	470430	80,91
Ou_P4C4_1	562858	453626	80,59	451072	446665	79,36
Ou_P4C4_2	535254	449794	84,03	448128	445709	83,27
Ou_P6C6_2	478955	398860	83,28	397308	395201	82,51
Ou_H_1	471033	359471	76,32	356876	351221	74,56
Ou_H_2	538869	433485	80,44	431086	399360	74,11
Ou_P1C1_3	333933	281565	84,32	273773	270144	80,9
Ou_P1C1_4	294005	250266	85,12	247666	244200	83,06
Ou_P2C2_3	258144	223135	86,44	219287	217928	84,42
Ou_P2C2_4	352314	301973	85,71	296112	292656	83,07
Ou_P4C4_3	297137	259089	87,2	256321	252369	84,93
Ou_P4C4_4	282167	246891	87,5	241465	234647	83,16
Ou_P6C6_3	196142	167977	85,64	164762	164057	83,64
Ou_P6C6_4	747185	625965	83,78	614917	594007	79,5
Ou_H_3	210595	170832	81,12	168154	157725	74,89
Ou_H_4	307469	248843	80,93	246472	242704	78,94

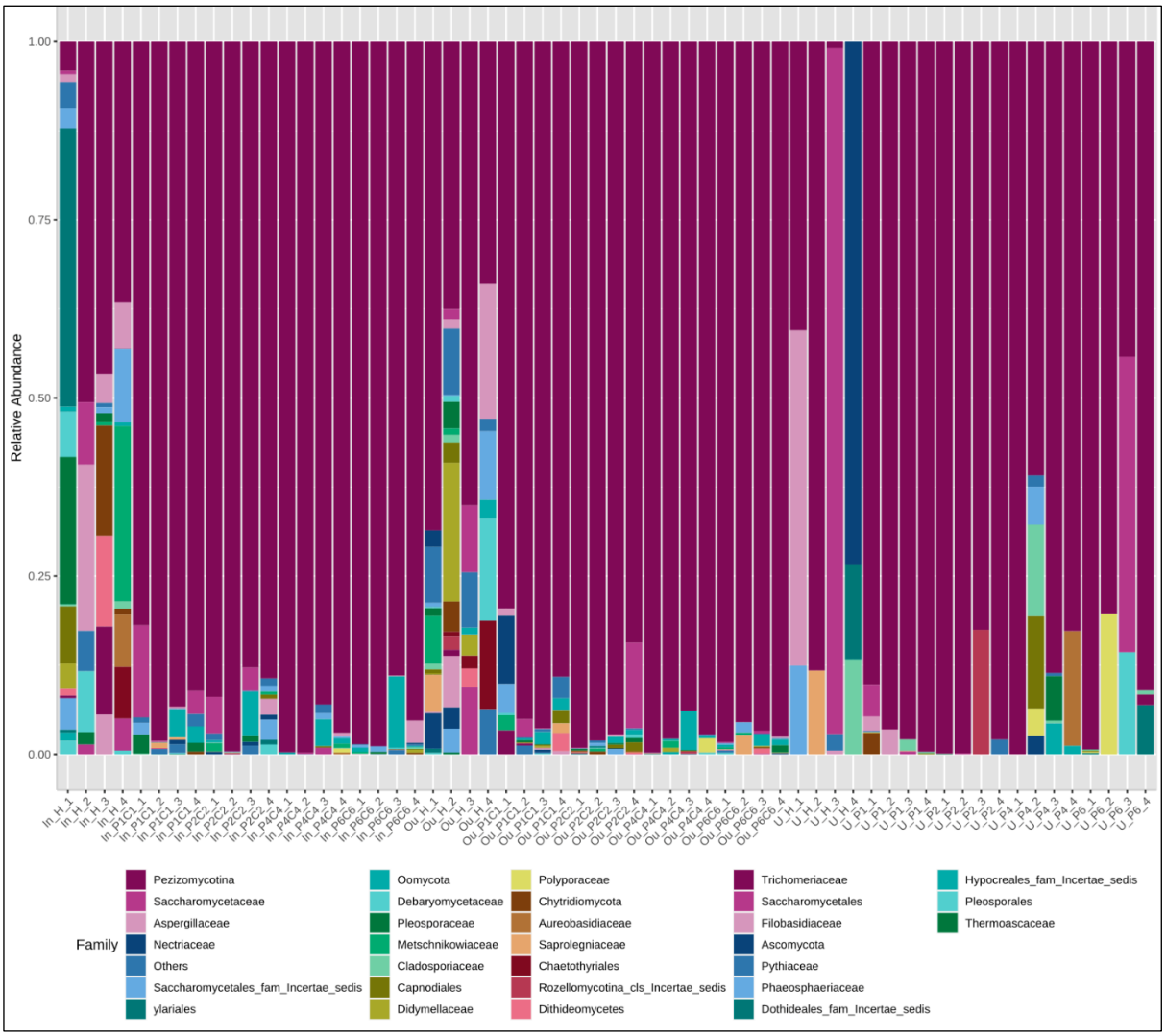

Figure S4.1 Samples-wise relative (%) ASV abundance of fungal communities at family level for the 30 most abundant ASVs. Samples denoted with "SW "are from the seawater (non_IMTA) system, while samples denoted "AEW" are from the abalone effluent water (IMTA) system *Ulva* samples are denoted with "*Ulva*", Inlets are denoted with (In), and outlets are denoted with (Out).

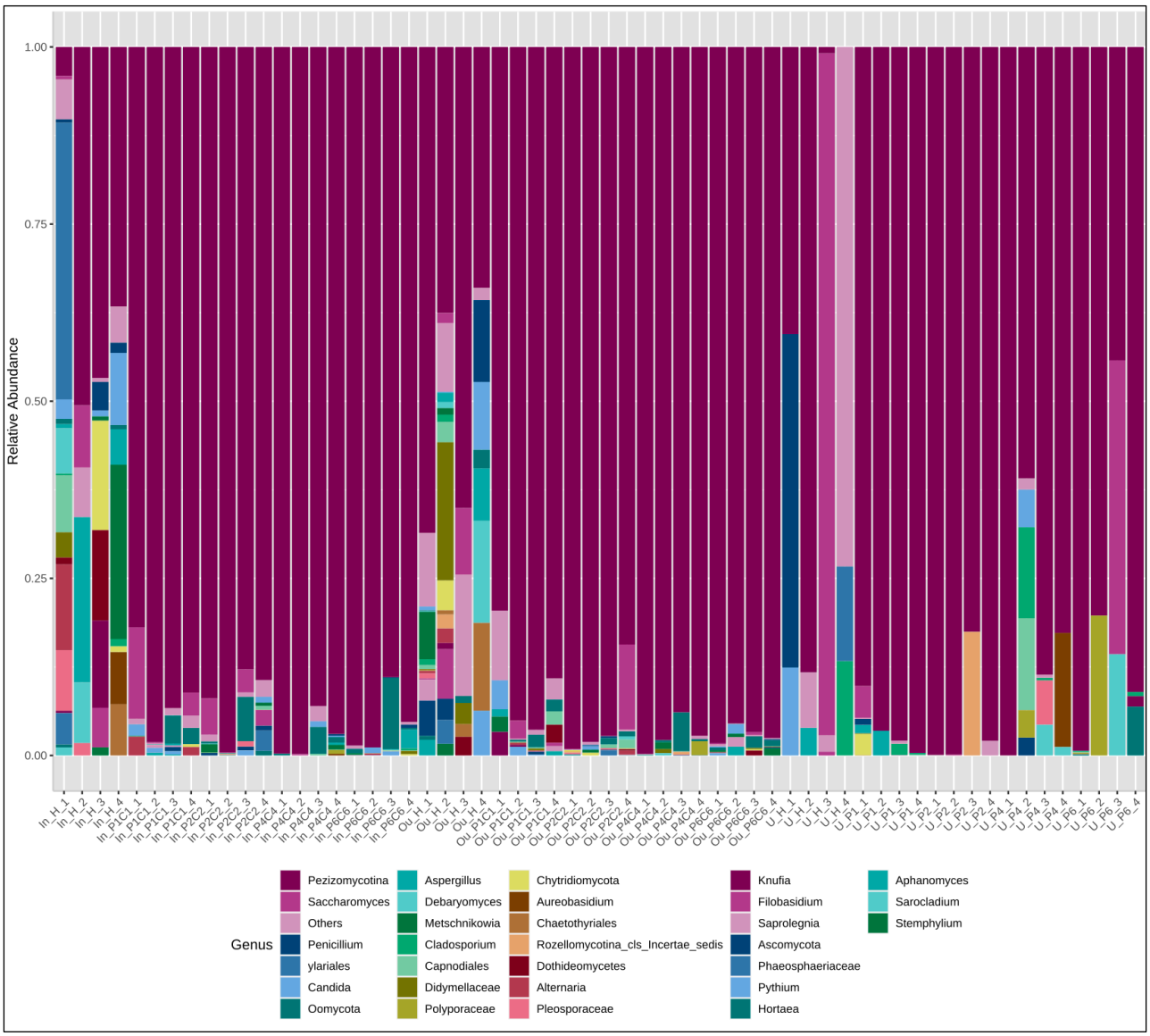

Figure S4.2 Samples-wise relative (%) ASV abundance of fungal communities at genus level for the 30 most abundant ASVs. Samples denoted with "SW "are from the seawater (non_IMTA) system, while samples denoted "AEW" are from the abalone effluent water (IMTA) system *Ulva* samples are denoted with "*Ulva*", Inlets are denoted with (In), and outlets are denoted with (Out).

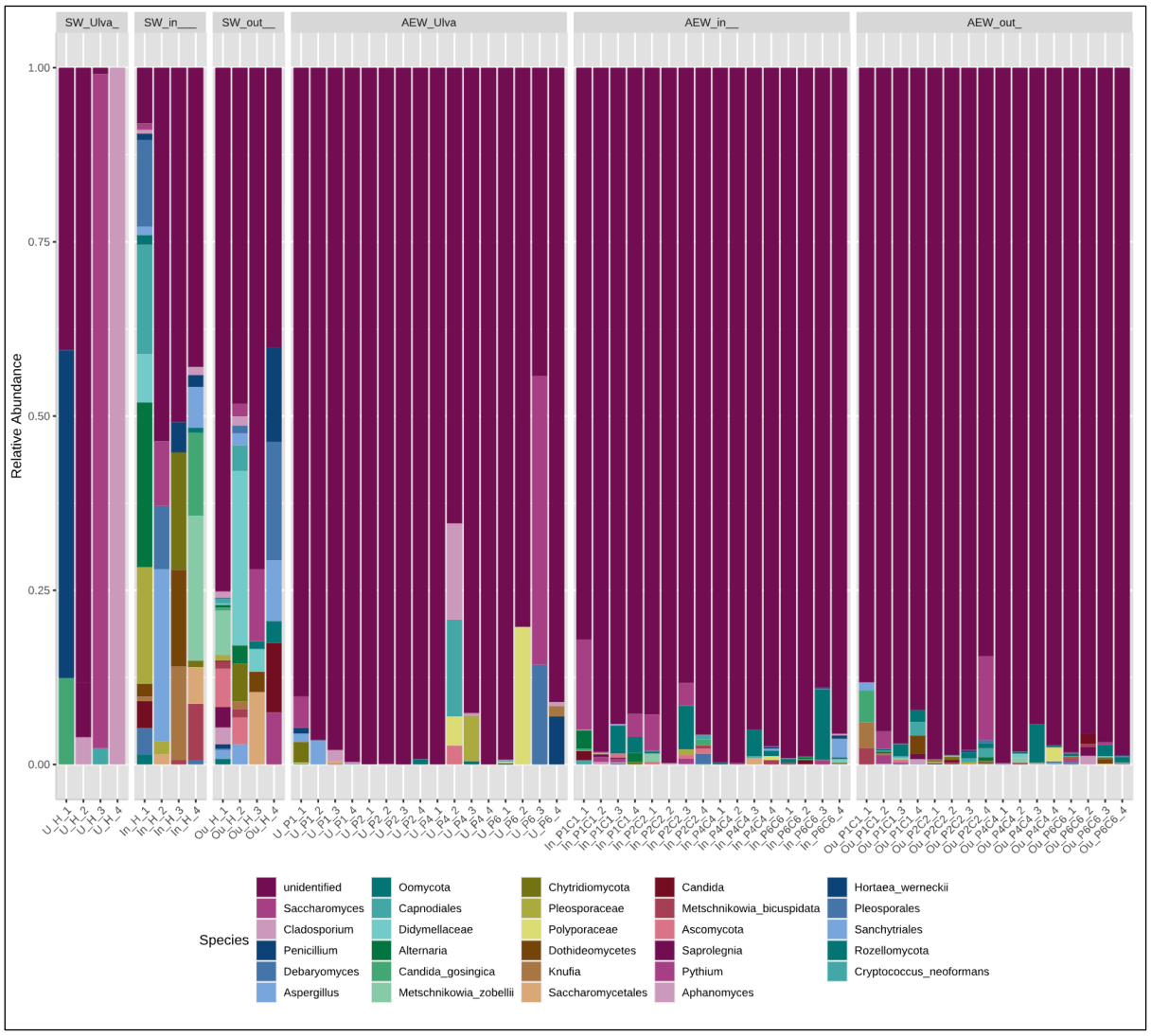

Figure S4.3 Samples-wise relative (%) ASV abundance of fungal communities at species level for the 30 most abundant ASVs. Samples denoted with "SW "are from the seawater (non_IMTA) system, while samples denoted "AEW" are from the abalone effluent water (IMTA) system *Ulva* samples are denoted with "*Ulva*", Inlets are denoted with (In), and outlets are denoted with (Out).