MICROORGANISMS ASSOCIATED WITH ULVA GROWN IN ABALONE EFFLUENT WATER: IMPLICATIONS FOR BIOSECURITY

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Dissertation presented in the partial fulfilment of the requirements for the degree of
Master of Science (BIO 5006W Applied Marine Science)
Department of Biological Science
University of Cape Town
February 2019

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DECLARATION

I know the meaning of plagiarism and declare that all of the work in the dissertation (or thesis), other than that which was carried out by the University of the Western Cape Next Generation Sequencing Facility and that which is properly acknowledged, is my own work as assisted by my supervisors. This thesis has not been submitted in this or any form to another university. Experimental work discussed in this thesis was carried out under the supervision of Prof. J. J. Bolton of the Department of Botany, University of Cape Town and Dr. B.M. Macey of the Department of Agriculture, Forestry and Fisheries.

Signed by candidate

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Mr Kristin de Jager

Date:

DEDICATIONS

I dedicate this thesis to all things green, as without words they strive to answer questions we have never asked.

ABBREVIATIONS

ANOVA	Analysis of variance
ASW	Autoclaved seawater
AEW	Abalone effluent wastewater
CFU	Colony forming units
DAFF	Department of Agriculture, Forestry and Fisheries
dH_2O	Distilled water (Millipore)™
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene-diamenetetra-acetic acid
ES	Effluent system
FS	Fertilised system
FSW	Fertilised seawater
FTS	Flow through system
IMTA	Integrated multitrophic aquaculture
LEfSe	Linear discriminate analysis effect size
ML	Maximum likelihood
MRA	Marine Research Aquarium
NGS	Next generation sequencing
NMDS	Non-metric multidimensional scaling analysis
OUT	Operational Taxonomic Unit
PCoA	Principal co-ordinate analysis
rbcL	Ribulose-1,5-bisphosphate carboxylase
RAS	Recirculating aquaculture system
RNA	Ribonucleic acid
TCBS	Thiosulfate Citrate Bile Sucrose Agar
TSA	Tryptone Soy Agar

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PREFACE

The following aspects of this thesis have been presented:

CONFERENCE PRESENTATIONS:

Kristin N. De Jager, Brett M. Macey, John J. Bolton. Micro-organisms associated with *Ulva* cultivated in an integrated aquaculture system with abalone, and implications for biosecurity. Presented at World Aquaculture Society meeting in Southern Africa, Cape Town, Western Cape, South Africa, 2017.

John J. Bolton, Brett M. Macey, Mark D. Cyrus, Kristin N. de Jager, Joseph. De Prisco, Teejaswani. Bachoo. Growing *Ulva* (Chlorophyta) In Commercial Integrated Systems With Abalone In South Africa For Feed And Bioremediation: An Update. Presented at 23rd International Seaweed Symposium, Jeju Island, Korea. May 2019.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr Brett Macey and Professor John Bolton for their continued assistance and encouragement throughout the project. I would also like to thank Vuyokazi Kutu and Mark Cyrus for their help and support in and out of the lab. A special thanks goes to I&J abalone farm in Danger Point for allowing me to take *Ulva* and water samples utilised in the project. Finally, I would like to thank Dr Bronwyn Kirby and her fellow associates at UWC for assisting with the molecular sequencing approach as well as Stellenbosch University for performing bacteria and *Ulva* sequencing.

ABSTRACT

Macroalgae such as *Ulva* are living hosts that are known to perform essential roles in marine ecosystems, and are extensively utilised for several aquaculture operations, including in the integrated production of high value goods such as abalone. Intensive aquaculture operations along the coastline release effluent water into the surrounding coastal waters, which has adverse impact on the environment. As a means to bioremediate abalone effluent, several commercial abalone farms in South Africa use *Ulva* as a biofilter, after which the *Ulva* may serve as a feed source for the abalone. Advancements in gene sequencing technology has enabled the assessment of large scale 16S rRNA gene libraries of near full-length sequences. However, studies concerning the epiphytic bacterial communities present on macroalgae grown in effluent systems are scarce, and as a result several commercial farmers have become sceptical about utilising effluent grown *Ulva* as feed. This study addresses the biosecurity implications associated with the use of *Ulva* as a biofilter and feed within an integrated multitrophic aquaculture (IMTA) system along with the abalone *Haliotis midae* by assessing the bacterial communities associated with *Ulva* and its environment. Water and *Ulva* samples were collected from an integrated abalone farm along the Western Cape of South Africa and assessed via a culture and a non-culture-based approach. Samples were collected from both fertilised seawater tanks and abalone effluent wastewater raceways. The water samples were collected at the inlets and outlets of each tank/raceway and the *Ulva* was collected from within each system. The culture-based approach utilised three selective media for the isolation and quantification of culturable bacteria, namely Tryptic Soy Agar (TSA, a general growth media), thiosulfate-citrate-bile-sucrose (TCBS) agar (vibrio selective growth media), and *Ulva*n agar plates, where the primary carbohydrate of *Ulva* was utilized as the main carbohydrate source. Post isolation, selected bacteria underwent 16S rDNA gene analysis for identification. The non-culture-based approach utilised the next generation Illumina 16S Metagenomic Sequencing platform (MiSeq). Moreover, the *Ulva* was sequenced using the *rbcL* gene to identify the species grown in the aquaculture system. Phylogenetic analysis of *Ulva* suggests that it falls within the *U. rigida* clade.

The sequenced *Ulva* cultivated at I&J abalone farm shared close similarity with *Ulva rigida* (KP233772) and *Ulva scandinavica* (EU484416) on the GenBank database, and hence was referred to with the name of its corresponding molecular synonym, i.e. *U. rigida.* The culture-based results indicate that bacterial numbers were significantly higher in the raceways receiving abalone effluent water when compared with the *Ulva* tanks receiving seawater that were fertilized. Bacterial abundance on all three selective media types was higher on the *Ulva* cultured in the abalone effluent raceways than on the *Ulva* cultured in the fertilized seawater tanks. Moreover, it was observed that the *Ulva* has the potential to significantly reduce the bacterial load of abalone effluent water raceways. Rarefaction results from the non-culture-based approach indicate that the *Ulva* in both the fertilised seawater and abalone effluent raceways had significantly lower bacterial alpha diversity than the water columns themselves. Principal co-ordinate analysis (PCoA) at phylum level showed that bacterial communities on the *Ulva* and in the water,

columns shared similar phyla diversity. Alternatively, PCoA at genus level demonstrated that microbial communities residing on the *Ulva* (both effluent and fertilised seawater grown *Ulva*) had significant differences compared with the water samples obtained from both the inlets and outlets of the effluent and fertilised seawater systems. When assessing the differential abundant bacteria on the *Ulva,* general marine bacteria appear in high abundance and potentially pathogenic bacteria such as *Vibrio* appear in low abundance. Moreover, the presence of the *Ulva* within the wastewater seemed to decrease the bacterial abundance of *Vibrio* within the fertilised seawater tanks as well as the abalone effluent raceways. Despite the presence of potentially pathogenic bacteria within the abalone effluent raceways, the *Ulva* does not seem to act as a sink for potentially pathogenic bacteria indicating that feeding effluent grown *Ulva* to abalone is not of significant biosecurity concern. Even though several commercial abalone farmers consider recirculation within aquaculture feed systems high-risk technology, no papers have reported disease outbreaks due to the use of effluent grown *Ulva* as abalone feed.

These results provide a general basis for the dynamic changes in the bacterial community profiles in a commercial abalone farm associated with utilising effluent grown *Ulva* as a feed additive for abalone. This effort to profile the bacteria associated with *Ulva* and its environment under fertilised and effluent conditions provides deeper insight on understanding the biosecurity implications of incorporating effluent grown *Ulva* into abalone feed.

SECTION 1. GENERAL INTRODUCTION

1.1 Brief history of the cultivation of abalone in South Africa

Abalone (*Haliotis* spp.) are highly sought-after species of marine mollusc (marine snails) that provides a high value export product; particularly to countries in East Asia, such as Hong Kong and China. Six species of *Haliotis* have been identified along the coastline of South Africa (Muller, 1986), of which the largest, *H. midae,* is the only species that is commercially exploited (Fig. 1). In South Africa, large-scale cultivation of *H. midae* began in the mid-1990s, primarily due to a decline in wild fishery harvests (Hecht & Britz, 1990), but also as a result of the development of a market for small "cocktail" sized abalone (approximately 50 mm shell length) for sale in the East Asia (Macey & Coyne 2005). Commercial abalone farms in South Africa are land-based and are concentrated in two main areas along the coastline of the Western Cape Province (Troell, et al., 2006), in close proximity to the sea, where they have access to large quantities of fresh sea-water.

Since the mid-1990s, the industry has expanded to over 17 commercial farms, stretching from Port Nolloth on the Atlantic coast of South Africa to Haga Haga, north of the city East London, on the Indian Ocean coast (Fig. 2; DAFF, 2016). In 2016, South Africa had a reported production of approximately 1,480 t (DAFF, 2016) of abalone, making S. Africa one of the largest producers of cultured abalone outside of Asia and Chile (FAO, 2014; FAO, 2017). Increasing demand for cultured abalone and the subsequent increase in production by local producers has however been faced with several obstacles, including feed supply, effluent water treatment/ management, and disease, all of which require innovative approaches to ensure that further expansion of the industry is conducted in an economically and environmentally sustainable manner (Robertson-Andersson, 2003; Primavera, 2006).

Figure 1. (A) Abalone, *Haliotis midae*, cultured in land-based aquaculture systems in South Africa. (B) Abalone and *Ulva* sp., one of the main seaweeds fed to commercially grown abalone in South Africa.

In most countries, including South Africa, cultivation of abalone relies heavily on the availability and use of algae, for settlement of larvae, as feeds for post-settled animals, and as feed for the juvenile and adult stages. Abalone are herbivores by nature, and along the Western and South-Western coasts of South Africa the brown seaweed, *Ecklonia maxima,* is one of the primary feeds of both wild and farmed abalone. Intensive harvesting of *E. maxima*, coupled with diminishing access to wild socks of this seaweed in some concession areas, and the limited availability of other suitable seaweeds as a natural feed for cultured abalone is one of the primary obstacles faced by the local abalone sector and has been a key driver for the development of land-based seaweed cultivation and development of formulated feeds. In 2007, over 6,000 t of *E. maxima* was harvested as feed for the expanding abalone industry (Andersson, et al., 2007). Demand for kelp resources have increased substantially over the years and certain concession areas cannot sustain the growing industry, as indicated by the harvesting of these kelp concession areas at 60-99% of their mean sustainable yield (Troell, et al., 2006). More recently, Rothman et al (2020) reviewed the trends in seaweed resource use in South Africa and Namibia, stating that kelp harvests for abalone feed reached a plateau of between 4000 and 5000 t fresh per annum. Increased demand for kelp concession areas, concerns of overharvesting and a lack of sufficient quantities of wild seaweed in the vicinity of certain abalone farms has partly attributed to the formulation of alternative compound feeds, such as the locally produced formulated feed Abfeed® (Britz, et al., 1994; Andersson, et al., 2007), and the initiation of land-based seaweed cultivation.

The development of formulated feeds and land-based seaweed cultivation has stimulated development of the abalone farming sector and enabled farming along the southeast coast of the country, such as Wild Coast Abalone near East London, where the farms do not have access to sufficient quantities of natural seaweed, such as the kelp (Troell et al., 2006). High temperatures along the southeast coast, during certain months of summer have also been associated with health problems in abalone, sometimes resulting in a condition called “bloat” (Macey 2005), that is exacerbated when using protein rich formulated feeds during this period, further stressing the requirement for the use of cultivated seaweeds as feeds. The demand for macroalgae and their products cannot be supplied entirely by natural populations (Gutierrez, et al., 2006; Buschmann, et al., 2008), hence great effort has gone into research focusing on farming macroalgae, as feed for abalone and for bioremediation of farm effluent water.

Figure 2. Map highlighting the South African abalone facilities, indicated in red, as well as the abalone facilities growing Ulva, indicated in green.

1.2. The global seaweed industry: species, production and markets

Macroalgae play a key role in global aquaculture development, and are currently dominating the mariculture sub-sector, with production comparable to that of aquacultured marine molluscs, crustaceans and fish combined (FAO, 2014; Olsen, 2015). Global macroalgae production in 2005 was valued at more than 3.8 billion US dollars, with an estimated production of 13.5 million tonnes (FAO, 2014). The international macroalgal industry has grown considerably since 2005 and doubled to over 31 million tonnes by 2016, of which 96.5% by volume was cultivated (FAO, 2018). Asides from the current production within established regions, it is predicted that as technology and strains improve, new countries without an algal culturing background will begin to produce and supply macroalgae, and further expand commercial markets (Tseng & Borowitzka, 2003).

A large majority of global seaweed production consists of the Rhodophyceae (red algae) and Phaeophyceae (brown algae). Zemke-White & Ohno (1999) noted that at least 32 chlorophytes, 125 rhodophytes and 64 phaeophytes showed commercial value. These numbers are however believed to be largely underestimated as numerous species are collectively listed from a given genus, without listing the individual species (e.g. *Ulva* spp.), and there is also a lack of reporting for medicinal species, especially for those used in traditional medicines. Of the 221 reported species, four genera, *Laminaria* (682,581 t dry wt), *Porphyra* (130,614 t dry wt), *Undaria* (101,708 t dry wt.) and *Gracilaria* (50,165 t dry wt.), made up 93% of globally cultured macroalgae (Zemke-White & Ohno, 1999).

Seaweed harvesting in South Africa started during the Second World War, with the collection of red seaweeds (*Gracilaria* and *Gelidium*), and later with the collection and harvesting of brown kelps (*Ecklonia maxima* and *Laminaria pallida*) (Robertson-Anderson, et al., 2006). Green macroalgae have since become increasingly important from an economic perspective, and have been identified as a source of sustainable biomass for both feed for animals (including humans) and biotechnology, including bioremediation, integrated multitrophic aquaculture and other biorefinery processes (Neori, et al., 2003; Bolton, et al., 2009; Bikker, et al., 2016; Bolton, et al., 2016). The macroalgae industry in South Africa, with an estimated value of at least R35 million (DAFF, 2014), is still small relative to the global algal industry. In 2014, South Africa produced approximately 2000 t of seaweed, accounting for approximately 0.007% of global seaweed production (FAO, 2014), and this is mainly attributed to the production of *Ulva* spp.

Macroalgae of the genus *Ulva* have received interest within the global aquaculture sector since the early 1900s (Neori, et al., 1991; Lee, 1999; Neori, et al., 2003). *Ulva* species are primarily grown as a source of feed and biomass due to their high vitamin, trace metal and dietary fibre content (Lahaye & Jegou, 1993; Robertson-Andersson, 2003; Bolton, et al., 2009). In addition, *Ulva* have shown commercial usage in several other industries, including use as a functional feed for humans and animals, in integrated mariculture with marine invertebrates, for effluent bioremediation and for a variety of other medicinal applications including disease resistance (Abbott, 1978; Koh-ichi, et al., 1987; Robertson-Andersson, 2003; Cyrus, et al., 2014; Bolton, et al., 2016). In South Africa, *Ulva* is primarily grown as feed for abalone as well as for its bioremediation potential of farm effluent water (Bolton, et al., 2016; Neveux, et al., 2018).

1.3 General overview of Ulva cultivated in South Africa

1.3.1 Taxonomy

The genus *Ulva* was first described in 1753 by Linnaeus in his Species Plantarum, after which it has undergone several reviews (Round, 1984; Bold & Wynne, 1985; Van den Hoek, et al., 1988). As presently circumscribed, the genus includes 127 known species, of which the global diversity and species-level delimitation is uncertain (Guiry & Guiry, 2017). *Ulva* has since remained in the class Ulvophyceae along with other green algae including Acetabularia, Codium, Caulerpa, and Cladophora (Guiry & Guiry, 2017). Historic taxonomical studies on the speciation and phylogenetic relationships within the family *Ulvaceae* were largely dependent on anatomical, morphological and cytological data to characterise species (Bold & Wynne, 1985; Joska, 1992; Stegenga, et al., 1997; Woolcott, 1999). More recently, phylogenetic studies on *Ulva* have incorporated the use of the plastid (rbcL) and nuclear small subunit rDNA (18S) genes, as well as the nuclear internal transcribed spacer [ITS] regions, mitochondrial sequences and complete genome sequencing (Lewis & McCourt, 2004; Mahendran & Saravanan, 2017). The rbcL and 18S genes have both been widely used as established DNA barcodes for several plant groups, including seaweeds, and have formed the basis of resolving numerous phylogenetic and taxonomic matters (Hayden & Waaland, 2002; Hayden, et al., 2003; Lewis & McCourt, 2004). ITS regions have also been extensively used for plant molecular phylogenetic studies, with Kandjengo (2002) being the first to carry out a molecular study on South African species of *Ulva* based on the ITS4 and ITS5 gene sequences. Despite extensive use of the ITS gene region, the ITS gene can produce false phylogenetic inferences (Buckler, et al., 1997), and genes that have a faster rate of change, such as the 5.8S or rbcL gene, may prove more useful for deeper sequencing (Judd, et al., 1999). Despite this, uncertainty also remains surrounding the use of the rbcL gene due to the presence and variability of introns which complicate the ability to amplify

and sequence reads due to the large fragment sizes processed within a single bidirectional read (Hanyuda, et al., 2000). Moreover, green algae are prone to acquiring intron sequences, of which their abundance within the rbcL gene is poorly understood and requires further evaluation (Hanyuda, et al., 2000). The above factors negatively affect the universality of rbcL barcode markers for green marine macroalgae classification (Hayden & Waaland, 2004). Nonetheless, phylogenetic studies utilising rbcL and ITS genes have been and continue to be utilised within the *Ulvales* (Leskinen & Pamilo, 1997; Blomster, et al., 1998; Woolcott, 1999; Kandjengo, 2002; Guidone, et al., 2013; Kazi, et al., 2016). Joska et al*.,* (1992) revised the *Ulva* along the South Western Cape using morphological characters and identified the following species; *U. capensis* Areschoug, *U. fasciata, U. lactuca, U. rhacodes* and *U. uncialis,* the latter of which was removed as Stegenga et al. (1997) considered it was a dwarf form of *U. rigida*.

1.3.2 Morphology and reproduction

In their mature form, *Ulva* are relatively simple in appearance, yet exhibit a wide range of morphological, anatomical and cytological characteristics (Cocquyt, et al., 2010; Wichard, et al., 2015), consisting of either a distromic foliose or a monostromic (formerly regarded as *Enteromorpha*) tubular thallus (Hayden, et al., 2003). *Ulva* are characteristically epilithic, but some may be epiphytic. *Ulva rhacodes* and *U. anandii,* for example, can attach to substrates such as rocks and kelps via a discoid holdfast (Amjad & Shameel, 1993; Stegenga, et al., 1997)*,* whereas others, such as *U. armoricana, U. rigida* and *U. lactuca* survive detached in free floating communities (Dion, et al., 1998). The morphological characters of *Ulva* are known to display considerable variation in response to environmental perturbations (Titlyanov, et al., 1975; Steffensen, 1976; Tanner, 1986; Malta, et al., 1999). Variation regarding the size and thickness of the thalli of both *U. fenestrata* and *U. californica,* have been attributed to factors such as seasonality, salinity, wave exposure, temperature and even latitude (Titlyanov, et al., 1975; Tanner, 1986; Choi, 2010). Tanner (1979) additionally noted that the temperature-related morphological discrepancies observed for both species may explain why distinctly different morphotypes exist in different seasons. Morphological characters may additionally vary with stressors such as grazing, age, its attachment status, as well as the reproductive state of the *Ulva* (Malta, et al., 1999), thus making it very difficult to accurately identify different *Ulva* species based on morphological characters alone.

In the generative form, *Ulva* display a multitude of reproductive modes, and typically alternate between isomorphic, unisexual haploid gametophytes and diploid sporophytes that are both multicellular and vegetative (Hoek, et al., 1995). The mechanics of algal sporulation are not yet fully understood, however, nutrient availability, temperature, lunar cycle, and certain species of bacteria are believed to be factors impacting propagule release (Mohsen, et al., 1974; DeBusk, et al., 1981; Niesenbaum, 1988; Weinberger, et al., 2007). Not all species of *Ulva* reproduce sexually, and sterile strains that only display vegetative growth phases have been

identified. A sterile mutant of *U. pertusa* as well as another uncharacterised sterile *Ulva* sp. have been documented and used in mariculture (Migita, 1985; Hirata, et al., 1993; Sato, et al., 2006). Fragmentation holes have been known to form during sporulative propagule release (Gao, et al., 2010), which may result in a considerable loss of biomass, of up to 60 % total mass each month, depending on seasonality (Niesenbaum, 1988), which has even been known to change the water colour (Gao, et al., 2010). Alternatively, during vegetative reproduction, cells may slough off and form new algae, with the majority of cells possessing the capability of becoming reproductive (Bonneau, 1978).

1.3.3 *Ulva* cultivars maintained on South African Abalone farms

Ulva grown on abalone farms in South Africa survive detached without a holdfast and display vegetative reproduction, enabling relatively simple culture conditions (Robertson-Anderson., 2003). Sterile strains that propagate through vegetative reproduction are of great cultivar importance as they have the potential to replace sporulative cultivars, which may lose biomass (Robertson-Andersson, 2003). To successfully cultivate *Ulva* on an industrial scale, Marine Growers in Port Elizabeth and I&J in the Western Cape were among the first commercial abalone farms to establish parental cultivars (Steyn, 2000; Robertson-Andersson, 2007). Currently, there are at least 5 abalone farms with parental cultivars that are cultivating *Ulva* at a commercial scale. Parental cultivars consist of a small portion of seaweed material that may be cultivated in isolated tanks, which receive clean seawater. These tanks may be used to seed the primary systems in cases of contamination, or if the harvest was insufficient to restock the primary cultivation system. Parental cultivars additionally aid in selecting for larger thalli which are preferred for cultivation (Robertson-Andersson, 2003). Both parental cultivars stocked at I&J and Marine Growers were sourced locally from either Simons Town (Robertson-Andersson, 2003) or Saldanha Bay (Steyn, 2000). Robertson-Anderson., (2003) identified 5 species from the original stocking material utilised at I&J based on morphological and anatomical characteristics: namely *U. lactuca, U. capensis, U. fasciata, U. rhacodes* and *U. rigida*; which coincided with the *Ulva* sp. reported to reside along the Western Cape at that time (Joska, 1992; Kandjengo, 2002). Robertson-Anderson., (2003) went on to note that *U. lactuca* and *U. capensis* were dominant and *U. rigida* was reported to occur in low densities, whereas *U. fasciata* and *U. rhacodes* were epiphytic and attached to *Gracilaria* that had blown over from other tanks. Species dominance within the cultivars is however not fixed and switches in dominance have been found to occur. Low light environments, for instance, induced by shading with 80% shade cloth, has been shown to alter species dominance from *U. lactuca* to *U. capensis*; however when the shade cloth was removed a year later, the species dominance gradually returned to *U. lactuca* (Robertson-Andersson, 2003).

Recently, Bachoo (2018) reviewed and characterized the *Ulva* species cultured on 5 local abalone farms, including I&J abalone farm, and deduced that the *Ulva* on all five farms belonged to the same species (*U. rigida*) when using the *rbcL* and ITS genes. Bachoo (2018) went on to note, that using morpho-anatomical features alone are not suitable for species level identification of *Ulva* as there was a large difference in the *Ulva's* morphology that could be environmentally induced. Understanding morphology and speciation is essential for value chain enhancement as specific morphotypes may be more favourable for certain applications, such as weaning, settlement and growth. I&J, for example, has in the past preferentially selected for *U. lactuca* as it is optimal for high light environments and grows unattached (Robertson-Andersson, 2003), whereas Marine Growers Ltd. previously chose *U. rigida* over *U. fasciata* due to its higher SGR, nutrient value, and resistance to fragmentation (Steyn, 2000).

1.4 Uses and benefits of Ulva grown in integrated aquaculture systems

1.4.1 Ulva as a feed additive

Research into the use of commercially cultured macroalgae as a feed for abalone began in the late 1990's when *Gracilaria* was used as a feed for abalone along the southeast coast (which has no kelp forests) (Fourie, 1994; Smit, 1997), and later when *Ulva* (Robertson-Andersson, 2003) grown in abalone effluent water was used as a supplementary feed. At present, several farms in South Africa are successfully cultivating *Ulva* for use as a supplementary feed for cultured abalone: including Wild Coast Abalone, Buffeljags, Abagold, Diamond Coast and I&J West Coast Abalone (Bolton, et al., 2009). The incorporation of small amounts of dried *Ulva* (15-20% w/w) into formulated feed that is then fed to abalone and urchins and has since been shown to significantly improve the chemosensory properties/ palatability of artificial diets, enhance feed consumption, improve growth rates, increase digestible protein intake, and intensify pigmentation of specific organs, such as the gonads of the sea urchin *Tripneustes gratilla* (Daume, et al., 2003; Daume, 2006; Strain, et al., 2006; Smit, et al., 2010; Mos, et al., 2011; Mulvaney, et al., 2013; Cyrus, et al., 2014). *Ulva* grown in abalone effluent water has been shown to increase the growth rates of abalone compared with abalone fed wild seaweeds (Mulvaney, et al., 2013) and this effect has been attributed to the higher protein content (roughly 20%-30% crude protein) of farmed seaweeds relative to their wild counterparts (roughly 10%-15%) (Daume, et al., 2003; Bolton, et al., 2009; Shuuluka, et al., 2013). Nonetheless, studies assessing the impact of effluent grown *Ulva's* associated microbiome on abalone growth are scarce and require further assessment as microbiomes are known to impact feed intake and metabolism (Macey & Coyne, 2006).

The incorporation of *Ulva* into feed has been shown to have probiotic effects, enhancing health and disease resistance of shrimp and fish fed diets supplemented with seaweed, while simultaneously upregulating innate immune defence factors, such as the agglutination index, phagocytic rate and bacterial clearance activity (Koh-ichi, et al., 1987; Selvin, et al., 2004; Daume, 2006; Cruz-Suarez, et al., 2009; Selvin, et al., 2011). These probiotic effects have been partially attributed to the release of bioactive marine secondary metabolites (MSMs) from the seaweeds, such as acrylic acid (Burkholder & Sharma, 1969), which stimulate the release of non-specific immune defence factors in response to invading bacterial pathogens. However, the exact modes of action of the MSMs are still unknown (Selvin, et al., 2004). Several authors have also reported antibacterial activity from a number of *Ulva* sp., including *U. lactuca* and *U. reticulata* (Egan, et al., 2000; Vallinayagam, et al., 2009; Ravikumar, et al., 2016). Antibacterial activity testing has demonstrated that *Ulva* can inhibit the growth of both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Vibrio anguillarum, Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae*) bacteria, and results have shown that *Ulva* has higher antibacterial activity compared to other green macroalgae species, such as *Chaetomorpha aerea* (Lu, et al., 2008; Seenivasan, et al., 2010).

1.4.2 Ulva and Ulva extracts for improving larval settlement

In abalone mariculture, abalone larvae are spawned from either wild-collected broodstock or farmed broodstock that have been conditioned in rearing systems. After spawning there is a short larval phase, after which the juveniles actively seek a suitable settlement substrate. On several farms in South Africa, juvenile abalone are settled onto vertical PVC plates that have been seeded naturally with microorganisms present in the incoming seawater and plates are subsequently colonised by a consortium of different species of bacteria, algae and diatoms. The success of this process is however limited, as the diatom and bacterial biofilm colonizing the settlement plates is unpredictable and different algae/bacteria may induce varying settlement rates, resulting in inconsistent and poor (1% - 10% of larvae) settlement success (Daume, et al., 2003). Larval settlement and post-settlement survival of larvae is regarded as a limiting factor in the production of most cultured species, including abalone and sea urchins (Hannon, et al., 2013; Cyrus, et al., 2015). Both abalone and sea urchins, as well as other invertebrates, are known to settle on specific substrates in response to specific chemical cues, such as those produced by red coralline algae (Morse & Morse, 1984; Daume, et al., 1999), and often settle on the species on which they feed. Red corallines may be optimal for settlement of abalone; however, their use is not amenable for commercial scale production of abalone. Highly inductive chemical settlement ques, such as histamine, have been identified and are known to induce settlement in abalone, however are not necessarily optimal for post-settlement survival (Mos, et al., 2011). Consequently, a lot of research has been dedicated towards the testing of alternative substrates, including a diverse array of algal, bacterial and diatom species as

well as synthetic compounds/ biofilms, for improved larval settlement (Slattery, 1992; Daume, et al., 1999; Gapasin & Polohan, 2004; Daume, 2006).

The presence of macroalgae in seawater has been shown to significantly improve settlement success and metamorphosis of several marine invertebrate larvae (Rodriguez, et al., 1993), including abalone (Morse & Morse, 1984; Daume, et al., 2000; Huggett, et al., 2005), sea urchins (Pearce & Scheibling, 1991; Dworjanyn & Pirozzi, 2008) and chitons (Barnes & Gonor, 1973). Abalone hatcheries in both Japan and Australia have successfully utilised germlings of the crustose green alga *Ulvella lens* to settle larvae of the abalone *Haliotis discus hannai* and *H. laevigata*, respectfully (Takahashi & Koganezawa, 1988; Daume, et al., 2007). Several chemical inducers have been extracted from *U. lens* (including glycoglycerolipids), which can promote larval settlement and metamorphosis of the sea urchin *S. intermedius* (Takahashi, et al., 2002). More recently, in South Africa, *Ulva* and *Ulva* extracts have been investigated for improving larval settlement of the urchin *Tripneustes gratilla* and the abalone *Haliotis midae* (pers. comm.). The *Ulva* and some of the extracts utilized in the latter studies have been shown to contain glycolipids similar to those found in *U. lens* (Etwarysing, et al., 2018).

In addition to macroalgae and their extracts, thin films dominated by a single species of bacteria or benthic diatoms have been shown to enhance settlement. Daume et al. (1999) noted that bacteria and diatoms can influence larval settlement but are not the key drivers and suggested that algae are the primary force for settlement induction. Nonetheless, it has been shown that bacteria alone are capable of inducing both settlement and metamorphosis of numerous marine invertebrates, including abalone, barnacles, oysters, polychaetes, starfish and sea urchins (Fitt, et al., 1990; Johnson & Sutton, 1994; Johnson, et al., 1997; Unabia & Hadfield, 1999; Huggett, et al., 2006). Cyanobacteria, such as *Synechococcus* spp., are capable of inducing metamorphosis of the abalone *Haliotis rufescens* (Morse, et al., 1984). Similarly, Hugget et al. (2006) investigated the effects of biofilms on settlement of the sea urchin *Heliocidaris erythrogramma* and exposed larvae to 250 different strains of bacteria, of which many, incl. *Pseudoalteromonas, Vibrio, Shewanella, Photobacterium* and *Pseudomonas*, were capable of inducing settlement equivalent to coralline algae. Research into biotics may offer safe alternatives to enhance settlement, metamorphosis and larval rearing as the amenability of bacteria to large-scale cultivation make them useful candidates to produce such inducers. Isolating specific bacteria and diatoms, and thereafter creating monocultures thereof, could potentially be used to inoculate settlement tanks allowing for greater control. This has not been looked at in detail by industry and further investigation is required to test the efficacy in larger scale systems. Many of the biofilms used for settlement on South African abalone farms, for instance, are maintained through passive seeding from seawater, of which their activity is known to increases with age (Roberts, 2001; Roberts, et al., 2007). Hence, understanding the bacterial compositions within the water column is essential for disentangling and beginning to manage settlement and health on a biotic level.

1.4.3 Use of Ulva in IMTA systems and for bioremediation of farm effluent water

The high stocking densities associated with most commercial aquaculture farms, including abalone farms, produces effluent water that often has high concentrations of ammonia and other dissolved and solid waste products. These waste products have been shown to impair growth and promote stress/ disease, and in extreme cases even cause mortalities (Primavera, 2006). Integrated aquaculture systems offer an environmentally friendly means to bio-remediate nutrient-rich effluent water generated from monoculture facilities (Chopin, et al., 2001). In doing so, integrated aquaculture, using seaweeds, addresses some of the negative environmental impacts associated with high density monoculture, by creating an ecosystem-based management approach to aquaculture (Soto, et al., 2008). *U. ohnoi,* for example, has been noted as an ideal species to target bioremediation of land-based aquaculture activities since the 1900s in Australia (Cotton, 1910), whereas in South Africa *Ulva* spp. have been grown successfully in integrated systems since 2002; where *Ulva* is grown in abalone effluent and fed back to the abalone as a supplementary feed, thus recycling nutrients sourced from feed and excreted by the animals (Bolton, et al., 2009; Bolton, et al., 2016).

A primary advantage of integrated multi-trophic aquaculture is that it involves the biofiltration of effluent waste water from aquaculture systems (such as abalone, prawns and fish) using extractive organisms from lower trophic levels (such as algae and bivalves), that are capable of utilising dissolved particulates and converting the inorganic nutrients into useful biomass that can then be used as feeds or secondary crops (Robertson-Andersson, 2003; Bolton, et al., 2009), medicines (Mao, et al., 2006; Hong, et al., 2007), cosmetics (Farasat, et al., 2014), fertilizers (Selvam & Sivakumar, 2013) and other biorefined goods (Buchholz, et al., 2012). Extractive aquaculture practices using macroalgae, in conjunction with Integrated Sequential Biorefinery (ISBR) practices, will become increasingly important as feed resources become scarce. The use of macroalgae as biofilters shows great promise as it reduces the impact of nutrient sequestration into the environment (Vandermeulen & Gordin, 1990; Chung, et al., 2002; Neori, et al., 2004; Nielsen, et al., 2012; Lawton, et al., 2013; Aníbal, et al., 2014), resulting in improved ecosystem health, social acceptability and economic stability (Soto, et al., 2008). Furthermore, the use of *Ulva* as a biofilter has the advantage in that it allows for partial wastewater recirculation within recirculating aquaculture systems (RAS), substantially reducing pumping and energy costs on the farm. Robertson (2007) also reported that 25% water recirculation, using *Ulva* for bioremediation, had no significant effect on abalone health. Partial recirculation systems also provide secondary protection against harmful algal and dinoflagellate blooms (Robertson-Andersson, et al., 2008), which are increasing in prevalence along the coast of South Africa and in 2017 caused one of the largest mass mortalities on three South African abalone farms (Pitcher, et al., 2019).

Several published studies suggest there is large potential for land-based integrated seaweed cultivation in South Africa (Fourie, 1994; Morgan, 2000; Robertson-Andersson, 2003; Njobeni, 2006), and practices for culturing *Ulva* have been well defined (Robertson-Andersson, 2003; Lüning & Pang, 2003). In South Africa, *Ulva* is primarily farmed in specially designed land-based raceways that receive effluent wastewater from abalone (*H. midae*) tanks, enabling high *Ulva* production rates throughout the year at a relatively low cost. These systems do have energy inputs, such as electrical costs for pumps and paddlewheels, but these costs are often justified by the production of a supplementary feed and the bio-remediatory benefits (Nobre, et al., 2010). Similarly, cultivation of *Palmaria mollis* (Pacific dulse) has been integrated with *H. rufescens* (Red abalone) and is reported to be a self-sustaining system (Evans & Langdon, 2000). Larger three stage systems have also been created that incorporate fish (sea bream), seaweed (*Ulva lactuca*) and abalone (*Haliotis discus hannai*), whereby fish waste water is used as a nutrient source for the algal culture and the resultant algae is then used as feed for abalone (Neori, et al., 2003). Currently 5 farms grow *Ulva* for feed in SA, but only two farms (i.e. Buffeljags and Diamond Coast abalone farm; pers. comm. Nick Loubser, Viking Aquaculture) use the *Ulva* for bioremediation purposes that enable partial water recirculation.

Numerous facets of land-based, abalone aquaculture systems integrated with seaweeds have been assessed in South Africa, including nutrient recycling within the system, changes in water chemistry, the growth of farmed animals (abalone and urchins) in the integrated systems and/or fed effluent grown macroalgae, and socio-economic evaluations (Troell, et al., 2006). Nonetheless, very little attention has been devoted to assessing potential biosecurity risks associated with the use of effluent grown *Ulva* as a supplementary feed or feed additive. There has also been limited research on the potential health benefits of growing animals in integrated systems and the effects of integration on the microbiome. Nonetheless, managing aquatic environments is complex due to the intimate relationship that exists between microorganisms and their hosts, especially in open flow-through aquaculture systems (Cripps & Bergheim, 2000; Olafsen, 2001), as water quality and disease control are directly impacted by the bacterial communities entering and residing within the system and these complex interactions are also affected by changes in biotic and abiotic factors (Moriarty, 1997; Thompson, et al., 2002). Due to the closely-knit association between bacteria and their microenvironment, it is vital to understand how communities assemble and function on the host(s) as well as within the integrated system.

1.5 Role of bacteria in integrated abalone seaweed systems and the beneficial associations between bacteria, Ulva and abalone

1.5.1 Host-bacterial interactions

Marine macroalgal surfaces provide a suitable habitat for a diverse and complex consortium of microbial communities, including bacteria, diatoms, fungi and countless other organisms (Bolinches, 1988; Snoeijs, 1994; Bengtsson, et al., 2010; Godinho, et al., 2013). Bacteria are generally believed to be primary colonisers of new surfaces (Bryers & Characklis, 1982) and are highly capable of influencing subsequent colonisation by other micro- and macroscopic organisms (Crisp & Ryland, 1960; Henschel & Cook, 1990; Garrett, et al., 2008). The consistent colonization of healthy algae and abalone by bacteria suggests that a positive relationship exists between these hosts and their associated epiphytic bacteria. This is not surprising, given that macroalgae and abalone have naturally developed in a world of microbes, with numerous complex interactions having evolved between these hosts and their associated holobiont. Several macroalgal-bacterial interactions have been documented, ranging from beneficial and pathogenic associations (Armstrong, et al., 2001; Dale & Moran, 2006; Goecke, et al., 2010) to communal relationships that entail bacteria residing within the host without influencing host functionality (Macpherson & Harris, 2004). Two contrasting theories exist on how microbial communities assemble and establish themselves on the host; the hologenome theory and the competitive lottery model. The hologenome theory proposes that a stable core microbial community exists and acts as a selective force for evolution (Zilber-Rosenberg & Rosenberg, 2008); whereas the competitive lottery model proposes that the community forms randomly as a result of its surrounding environment, whereby it is more likely for individuals to co-exist in a highly variable environment (Chesson & Warner, 1981).

The hologenome theory considers the holobiont, which was initially proposed for corals (Rohwer, et al., 2002), and since then has been suggested for algae (Barott, et al., 2011). The holobiont may be defined as an association of the host (coral or alga) and its symbionts (bacteria, fungi, viruses, protozoa and archaea), whereby they function as an ecological unit (Rohwer, et al., 2002; Knowlton & Rohwer, 2003). The hologenome theory is based on the following four generalisations; “(1) All animals and plants establish symbiotic relationships with microorganisms. (2) Symbiotic microorganisms are transmitted between generations. (3) The association between host and symbionts affects the fitness of the holobiont within its environment. (4) Variation in the hologenome can be brought about by changes in either the host or the microbiota genomes; under environmental stress, the symbiotic microbial community can change rapidly” (Zilber-Rosenberg & Rosenberg, 2008). Lachnit (2009) further suggested that phylogeny matters more than habitat for epibacterial communities on

macroalgae, which correlates with the stable core bacterial communities across space and time proposed by Tujula et al. (2010).

Not all authors agree with the hologenome theory proposed for algal holobionts and lottery competitive systems have been proposed. The lottery competitive system was first theorised by Sale (1978) for coral reef fish and assumes that space is a limiting resource, whereby unoccupied space is colonised by the nearest recruit, and that once colonized there is strong priority for the colonizer (Sale, 1977; Sale, 1978). Burke et al. (2011) provided an in-depth analysis of the bacterial community of *U. australis,* whereby they constructed 16S rRNA gene libraries from *U. australis* (six samples and 5293 sequences) and from seawater (10 samples and 10884 sequences) at different time points and locations within the Sydney coastal area and compared the bacterial communities in order to establish the extent of specificity in community composition between the two habitats (*Ulva* vs. surrounding seawater). Burke et al. (2011) noted that the bacterial libraries were highly variable and that consistent bacterial sub-populations were not detected, suggesting that competition may explain algal surface colonisation patterns. Burke et al. (2011b) also noted that the bacterial community assembly was based on functional genes rather than taxonomic identity and concluded that epibacterial assemblages on *U. australis* are determined by a “lottery” model as opposed to symbiotic interactions (Burke, et al., 2011a; 2011b). The lottery model may stand for most associated bacteria (Ghaderiardakani, et al., 2017), nonetheless several specific bacteria have been consistently isolated from *Ulva* that are essential for settlement, growth and morphogenesis (Spoerner, et al., 2012), suggesting that a core holobiont does exist (Hollants, et al., 2013). A study by Paver et al. (2013) indicated that the algal holobiont can impact bacterial community succession within the water systems, demonstrating the importance of understanding the holobiont in aquatic systems.

Both abiotic factors (e.g. temperature, light, salinity, aeration, pH, nutrient availability, water movement, stocking density and seasonality) and biotic factors (e.g. microhabitat production, polymers and nutrient release, attachment mechanisms, surface competition, defences and secondary metabolite chemistry) are known to impact bacterial interactions and composition in the water column as well as on the host (Taylor, et al., 1997; Wieczorek & Todd, 1998; Goulter, et al., 2009). Bacterial diversity on seaweeds is also known to change with the developmental age of the seaweed and vary with the location on the seaweed. Bengtsson et al. (2012) demonstrated that bacterial diversity, both richness and evenness, increases with the age of the kelp *Laminaria hyperborea,* and is consistent with the primary succession of its bacterial community.

Bacteria are known to perform a range of metabolic functions that influence the composition of the holobiont, including the cycling of nitrogen, carbon, lipids, fatty acids, sulfur and other heavy metals (Lesser, et al., 2004; Wegley, et al., 2007; Raina, et al., 2009; Krohn-Molt, et al., 2013). The holobiont may deter potentially pathogenic bacteria via the production of antibiotics and other inhibitory compounds (Lemos, et al., 1985; Ritchie, 2006; Nissimov, et al., 2009). The wellbeing of the holobiont is associated with the configuration of its biotic elements, whereby disruptions such as nitrogen (Siboni, et al., 2012) and temperature fluctuations (Case, et al., 2011) often lead to proliferation of pathogenic organisms associated with disease (Thurber, et al., 2008; Bourne, et al., 2008). Interestingly, Zilber-Rosenberg & Rozenburg (2008) suggested that the genetic wealth of microbial diversity may play an important role in adaptive processes and evolution as the symbiont can aid the host during periods of rapid environmental change.

1.5.2 Role of bacteria in morphogenesis and reproduction of Ulva

Ulva grown axenically and in combination with specific bacterial strains has confirmed the dependence of this alga on specific bacteria to adopt a normal morphology; where the absence of associated bacteria may render it as an undifferentiated callus (Provasoli & Pintner, 1980; Nakanishi, et al., 1996; Marshall, et al., 2006; Singh, et al., 2011; Spoerner, et al., 2012; Wichard, et al., 2015). Nakanishi et al. (1996) identified several bacterial genera that are involved in inducing normal morphogenesis in *U. pertusa* including; *Cytophaga, Flavobacterium, Escherichia, Pseudomonas, Halomonas and Vibrios*. The density of bacteria on the algal surface has also been shown to elicit changes in either morphology and/ or growth (Marshall, et al., 2006). Marshall et al. (2006) demonstrated that out of twenty isolated strains of periphytic bacteria, thirteen returned *U. linza* to its normal foliose morphology, five significantly increased growth (by up to 243%), and one increased growth but did not influence morphology. Spoener et al. (2012) suggested that single isolates were not capable of inducing normal morphology, and that synergistic effects of bacteria were essential for *U. mutabilis and U. linza* thalli development (Vesty, et al., 2015). The rhizoid cells of *U. mutabilis* display chemotaxis toward *Roseobacter* sp., which appear to co-operate with *Cytophaga,* whereby the *Roseobacter* induce cell division, similar to cytokinins. Conversely, *Cytophaga* stimulate rhizoid and basal stem cells, and are more comparable to auxins (Spoerner, et al., 2012). Interestingly, *Roseobacter* may be replaced with α-proteobacteria, including *Sulfitobacter* sp. and γ-proteobacteria such as *Halomonas* sp., however *Cytophaga* and *Flavobacterium* spp. appear obligatory in controlling morphogenesis (Nakanishi, et al., 1996; Matsuo, et al., 2003; Matsuo, et al., 2005), suggesting that specific genes may determine the associations between *Ulva* and their associated bacteria (Spoerner, et al., 2012).

Bacteria have even been shown to induce algal morphogenesis when separated by a semi-permeable membrane, suggesting that diffusible compounds are released (Spoerner, et al., 2012). Matsuo et al. (2005) was the first to document “thallisin”, a morphogenetic compound isolated from bacteria of the *Cytophaga*-Flavobacterium-Bacteroidetes group, which is believed to be indispensable for development of a normal foliose morphology in macroalgae such as *Gayralia oxyspermum* (Gao, et al., 2006).

Moreover, algal-associated bacteria have been found to positively impact the metabolic processes of alga and diatoms by providing cofactors essential for vitamin synthesis and growth (Haines & Guillard, 1974; Rébeillé, et al., 2007; Xie, et al., 2013). Oligotrophic and halophilic bacteria such as *Halomonas* sp. for instance have been found to enhance growth of the green microalga *Dunaliella bardawil*, by increasing iron availability via iron solubilisation (Keshtacher-Liebson, et al., 1995). In addition, Croft et al (2005) surveyed 326 algal species, of which 171 species required exogenously produced cobalamin (vitamin B_{12}) for growth and went on to propose that bacteria were the source of cobalamin production (Croft, et al., 2005).

1.5.3 Impacts of bacteria on algal sporulation and settlement

Primary colonisers, such bacteria, have been shown to both positively and negatively influence the release, settlement and permanent attachment of algal spores (Fletcher & Callow, 1992; James, et al., 1996; Patel, et al., 2003; Ista, et al., 2004). Patel et al. (2003) screened ninety-nine strains of marine bacteria isolated from natural biofilms on rocks and the surface of *Ulva* (as *Enteromorpha*) for spore settlement, and identified a total of 35 unique strains belonging to three groups; γ-*Proteobacteria* (including *Pseudoalteromonas* (14 strains), *Vibrio* (5 strains), *Shewanella* (5 strains), *Halomonas* (three strains) and *Pseudomonas* (One strain)), *Cytophaga-Flavobacteria-Bacteriod* (CFB) group (6 strains), and α- *Proteobacteria* (1 strain). The latter study demonstrated that spore settlement was strain- but not taxon- specific and varied with the age of the biofilm. Most strains belonging to the genus *Vibrio* and *Shewanella* induced algal spore settlement, whereas species of *Pseudoalteromonas* had a broader effect, including spore lysis, paralysis and settlement inhibition (Patel, et al., 2003). In a more recent study, Joint et al. (2007) suggested that cross-kingdom cross-talk between algae and bacteria can influence algal settlement, growth and morphology as bacterial cells in biofilms are capable of utilising bacterial “quorum sensing” to aid in attachment site selection (Joint, et al., 2002). Joint et al. (2007) for instance demonstrated that *Ulva* (as *Enteromorpha*) zoospore settlement is strongly enhanced by the presence of *Vibrio anguillarum,* which can express quorum sensing signal molecules such as N-acyl homoserine lactones (AHL’s), whereby mutants lacking the AHL gene were not capable of enhancing settlement. Tait et al. (2005) further demonstrated that *Ulva* zoospores settled directly on bacterial microcolonies, which are sites of concentrated AHL production (Tait, et al., 2005).

1.5.4 The role of algal associated bacteria on abalone larval and adult digestive processes, growth and health

In the aquatic environment, there is a direct and constant interaction between the host animal and the immediate environment, and it is suggested that the types of microorganisms colonizing the digestive tract of the host are obtained directly from feed(s) and from the surrounding water (Macey, 2005). Bacteria residing on ingested macroalgae and within the digestive tract of abalone have been shown to significantly improve growth rates, gastrointestinal health, carbohydrate assimilation and disease resistance of farmed abalone (Erasmus, et al., 1997; Macey & Coyne, 2005; Daume, 2006; Macey & Coyne, 2006). Newly settled abalone are often incapable of digesting diatoms and extracellular materials and bacteria present within biofilms are suggested to be a primary source of nutrition for these juveniles (Daume, 2006). This notion is supported by findings from Garland et al. (1985), who demonstrated that post larval *H. rubra* consume bacteria present on coralline red algae. The latter authors suggested the bacteria are either utilized directly as a food source or contribute indirectly towards metabolic processes. This is especially important for juvenile animals with an undeveloped digestive system, whereby the polysaccharolytic enzymes secreted by gut bacteria improve the hosts digestive efficiency and development of the gut/host. Post-larvae of *H. discus hannai* for instance have been noted to only start producing endogenous polysaccharolytic enzymes on day 17 (Takami, 1998). Vitalis et al. (1988) noted the importance of assessing the enzymatic activity of resident bacteria in hosts that feed on nutrient poor diets, such as kelp, as bacteria are known to improve host digestive efficiency by providing enzymes required for digestion.

Abalone are known to possess a wide variety of endogenous digestive enzymes that are capable of breaking down the complex polysaccharides present in their natural seaweed diets, such as α-amylase, cellulose, agarose, laminarinase and alginate lyases (Knauer, et al., 1996; Erasmus, et al., 1997; Suzuki, et al., 2003; Macey & Coyne, 2005; Garcia-Esquivel & Felbeck, 2006; Nikapitiya, et al., 2009). In 1997, Erasmus et al. investigated the role of bacteria in the digestion of seaweeds by gnotobiotic (bacteria-free) abalone (*H. midae)* and demonstrated that gut bacteria make a significant contribution towards the pool of digestive enzymes in this animal, facilitating digestion of complex polysaccharides and assimilation of nutrients. The latter authors identified several abalone gut bacteria with high extracellular polysaccharolytic activity, contributing towards the digestion of alginate, laminarin and agarose, amongst others. Sawabe et al. (2003) isolated and identified *Vibrio halioticoli,* present in the gut of *H. diversicolor aquatilis* and several other abalone species, including *H. midae,* that plays a role in the conversion of alginate to acetic acid (Sawabe, et al., 2003). Moreover, several studies have indicated that gut associated flora and digestive enzymes change with alterations in diet, environment and the developmental stage of the abalone (Harris, et al., 1986; Knauer, et al., 1996; Erasmus, et al., 1997; Tanaka, et al., 2003; Zhao, et al., 2012). Improved knowledge

of the microbiome associated with abalone and *Ulva* grown in integrated recirculating aquaculture systems may aid in the selection of probiotic bacteria that could further enhance digestibility and overall health of the cultured abalone and possibly the integrated systems.

Algae are known to possess several mechanisms to prevent fouling epibionts, including sloughing of cells, mucus production and the production of secondary metabolites (Keats, et al., 1997; Steinberg, et al., 1997; Dworjanyn, et al., 1999; Ibtissam, et al., 2009). Extracts obtained from *U. lactuca* have been shown to have antibacterial activity against *Staphylococcus aureus, Bacillus subtilis* and methicillin-resistant *Staphylococcus aureus* (Kim, et al., 2007; Tan, et al., 2012). The biologically active steroid, 3-O-β-D glucopyranosyl-stigmasta-5,25-dien, which possesses both anti-inflammatory and anti-microbial activity, has been isolated from this seaweed (Awad, 2000). Both *U. lactuca* and *U. reticulata* have also shown to exert antifungal activity against pathogenic fungi, including *Aspergillus niger, Saccharomyces cervisiae* and *Mucor indicus* (Aruna, et al., 2010).

Apart from *Ulva's* inherent biocidal activity, it has been suggested that epiphytic bacteria living on the surface of the algae provide additional protection against secondary colonisation and aid in combatting epibiosis (Boyd, et al., 1999; Egan, et al., 2001; Rao, et al., 2007). *Ulva reticulata* has been shown to possess bioactive compounds that inhibit settlement of the polychete *Hydroides elegans,* which may be dependent on not only the macroalgae itself but also on its associated epibiota (Dobretsov & Qian, 2002). *U. australis* is also dominated by a diverse bacterial community (Longford, et al., 2007; Burke, et al., 2011), which is suggested to inhibit the settlement of fouling organisms (Egan, et al., 2000). Both *Pseudoalteromonas tunicata* and *Phaeobacter* sp. strain 2.10, commonly associated with *U. australis,* produce several extracellular compounds that inhibit the attachment of fouling organisms (Rao, et al., 2007). Interestingly Rao et al. (2007) noted that despite the low abundance of *P. tunicata* and *Phaeobacter* sp. strain 2.10 on marine eukaryotic hosts ($<1 \times 10^3$ cells cm^{-2}; Skovhus 2004), their numbers are sufficient to inhibit fouling by other marine bacteria, fungi, invertebrate larvae and algal spores. Natural antifouling compounds and settlement inhibitors derived from algae and bacteria could serve as a viable alternative to organotin compounds (e.g. tributyltin (TBT) and triphenyltin) which were banned in 2008 by an international treaty on the application of antifouling (AF) coatings to ships (Antizar-Ladislao, 2008). The antifouling properties of bacteria associated with seaweeds could also have a positive impact on organism cultured in integrated RAS and improve the general productivity and health of the system as a whole. The use of probiotics and other feed additive, such as seaweeds, for improved nutrition and disease management is becoming increasingly important as aquaculture moves towards becoming more environmentally sustainable. This preventative approach, as opposed to treatment and the indiscriminate use of broad-spectrum antimicrobials, traditionally used by farmers, is of paramount importance for preventing further spread of antimicrobial resistance and degradation

of the environment (Alderman & Hastings, 1998; Gram, et al., 2001; Cabello, 2006). *Ulva fasciata* for instance has shown promise in aiding in the management of shrimp bacterial diseases (Selvin, et al., 2004; Selvin, et al., 2011) and *U. clathrata* is known to have an inhibitory effect on the bacteria *V. anguillarum* 65 (Lu & Liu, 2008). Itami et al. (1998) demonstrated that bacteria can enhance disease resistance in the shrimp *Penaeus japonicus* and bacteria isolated from the digestive tract of *H. midae* and incorporated in feeds as probiotics have been shown to improve growth and health of cultured abalone (Macey & Coyne, 2005; (Ten Doeschate & Coyne, 2008).

1.6 Diseases in aquaculture and the biosecurity concerns associated with the use of effluent grown Ulva as a feed or supplementary feed

Diseases are considered to be one of the major barriers preventing the successful development, continuation and trade of aquacultured organisms globally (Verschuere et al., 2000; Macey, 2005). Due to the intensive nature of most aquaculture operations, such as high stocking densities in association with grading, cleaning and feeding, etc., the health of the cultured organisms is often compromised and the spread of infectious disease agents within the system is often exacerbated. Infectious diseases caused by bacteria, viruses and eukaryotic pathogens (fungal like organisms and parasites) have been shown to significantly disrupt the aquaculture sector and international trade (Stentiford et al., 2007). Of the infectious disease outbreaks reported to date, bacterial diseases are the most prevalent (Dhar et al., 2014), particularly when taking into consideration production diseases that are commonly associated with the rearing of fish and shellfish.

Disease may be defined as “a continuing disturbance” to the organisms “normal structure and function” such that it is “altered in growth rate, appearance, or economic importance” (Andrews, 1976). Both infectious and non-infectious diseases have been identified from cultured marine organisms, whereby the former involves transmissible infectious agents (such as other algae, epiphytes, bacteria, fungi, mycoplasma and viruses) (Craigie & Correa, 1996; Egan, et al., 2014), and the latter is induced by environmental perturbations (such as temperature extremes, light intensity, water quality, salinity and pollution) (Dring, 2005). Moreover, both obligate and opportunistic bacterial pathogens have been identified, whereby obligate pathogens require a host to complete replication and can only live for a limited duration outside the host, while opportunistic pathogens may cause disease when the host is under stress. Stress factors associated with grading trauma, anaesthetics, and gradual salinity alterations in recirculation systems, etc. can exacerbate disease (Handlinger, et al., 2002; Handlinger, et al., 2005). Global climate change has also raised concerns regarding the emergence and proliferation of some, but not all, pathogenic marine organisms (Karvonen, et al., 2010). The virulence of some pathogenic bacteria such as *Vibrio*

cholerae, Ichthyopthirius multifiliis, and *Flavobacterium columnare* (Long, et al., 2005), are known to increase with increasing ocean surface temperatures, whereas other bacteria (*Ichthyobodo necator*) have the opposite reaction, or no reaction at all (*Chilodonella* spp.) (Karvonen, et al., 2010).

Until recently, the primary infectious agent of cultured abalone in South Africa was the sabellid worm *Terebrasabella heterouncinata* (Simon, et al., 2004; Ruck & Cook, 1998), however an oomycete (*Halioticida noduliformans;* Macey et al., 2011), protozoa (*Perkinsus;* Lester & Davis., 1981) and several opportunistic bacteria (Bower, et al., 1994) have also been identified. Microbial diseases of abalone have been identified in multiple culture facilities worldwide, including South Africa; California, USA; Mexico; Australia; New Zealand; Canada and France (Bower, 2010). In 2006, an oomycete pathogen, *H. noduliformans*, caused significant mortality in both adult (up to 90% mortality) and juvenile (~30% mortality) abalone on South African abalone farms. This fungus-like organism causes a disease known as abalone tubercle mycosis (Macey, et al., 2011). Numerous bacteria, including several *Vibrio* spp. (including *V. carachariae, V. harveyi, V. splendidis, V. flavialis II* and *V. parahaemolyticus*), *Flavobacteria, Clostridium* (*C. lituseberense*) and *Klebsiella* (*K. oxytoca*) are also known to induce disease in abalone (Paillard, et al., 2004;Cai & Wang, 2006; Cai, et al., 2008). Many of these bacteria are regarded as opportunistic pathogens, whereas certain species of *Vibrio*, such as *V. harveyi*, are regarded as significant pathogens of both marine invertebrates and vertebrates (Austin & Zhang, 2006). Systemic infections induced by bacterial exotoxins often result in tissue necrosis and death. In Japan, disease outbreaks in *H. diversicolor supertexta* induced by *V. carachariae* resulted in the formation of white spots consisting of necrotic muscle fibres on the abalone foot which resulted in high levels of mortality (Nishimori, et al., 1998). Similarly, in Australia, disease outbreaks among cultured *H. rubra, H. laevigata* and their hybrids were linked to two *Vibrio* species (*V. harveyi* and *V. splendidis* I) and a *Flavobacterium*-like bacterium (Handlinger, et al., 2005). Nonetheless, *Vibrio* spp. are not of major concern for abalone larval culture as the larval period is short and stringent sanitisation is enough to avoid difficulties. Furthermore, abalone cultured under high density conditions are known to display lesions and erosions on the epithelial layer of their foot and epipodium which are often infected by various bacteria (Diggles & Oliver, 2005). Moreover, clear associations between maturation, spawning processes and immune status of *H. tuberculata* and susceptibility to *V. harveyi* infection have been made, whereby higher mortalities were reported in mature individuals at temperatures above 19ºC (Travers, et al., 2008). Asides from disease, microbiota in aquaculture systems can produce off-flavour-causing compounds in aquacultured species (Burr, et al., 2012). Anderson, et al. (2006) noted the South African abalone industrys concerns regarding a bad taste and smell in some abalone that were fed *Ulva. lactuca,* which he attributed to Dimethylsulfoniopropionate (DMSP) accumulation in the abalone. Moreover, it was hypothesized that the DMSP could be converted to DMS in the muscle of the abalone during canning. Microbial activity within the system may

mediate the conversion of DMSP to DMS via microbial DMSP lyase (Ronald & Thomson, 1964), suggesting that bacteria may promote bad tastes in downstream processes such as canning.

Diseases have also been documented in several algae, including *Ulva*, *Porphyra, Gracilaria* and *Laminaria* (Meili, 1991; Weinberger, et al., 1994; Del Campo, et al., 1998; Ding & Ma, 2005). *Gracilaria gracilis* previously harvested from the west coast of South Africa (Saldanha Bay) often suffered from complete or partial die-offs that were attributed to bacterial infection that was suspected to be caused by *Pseudoalteromonas* sp. (Schroeder et al., 2003). A disease known as "ice-ice" has been identified in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Largo, et al., 1995). "Ice-ice" disease is generally caused by "unfavourable factors" such as environmental perturbations (e.g. temperature), which enable normal (resident) bacteria to become pathogenic . For *Eucheuma,* two bacterial pathogens, belonging to the *Vibrio-Aeromonas* complex and the *Cytophaga-Flavobacterium* complex have been identified which are capable of inducing ice-ice disease if the alga is subjected to stress (Largo, et al., 1995b). Bacteria that display motile behaviour, such as *Vibrio*, are known to be efficient macroalgal surface invaders, and are believed to penetrate the inner region of seaweeds through hydrolytic enzyme action (Araki, et al., 1999; Largo, et al., 1995; Michel, et al., 2006). Several alginate, laminarin as well as other polysaccharide-degrading enzymes have since been isolated from *Vibrio* species (Badur, 2015), and other marine bacteria (Michel & Czjzek, 2013), that play a role in their pathogenicity against seaweeds. These studies suggest that bacteria may also pose a risk to the commercial cultivation of macroalgae, as high stocking densities and unfavourable conditions may exacerbate impacts associated with diseases caused by opportunistic bacteria.

As previously highlighted, there are several commercial abalone farms in South Africa that are practicing integrated aquaculture, where *Ulva* is grown in abalone effluent water or fertilized seawater and subsequently fed back to abalone as a supplementary feed. Even though this practice has occurred successfully in South Africa for several years, limited information exists on the microbial communities associated with *Ulva* grown in these systems and more particularly the potential role of effluent-grown *Ulva* as a vector of pathogenic microorganisms for cultured abalone. As a consequence of the perceived biosecurity risk associated with feeding effluent grown seaweed back to animals on a farm, a few local abalone farms are no longer partially recirculating their seawater and instead are cultivating *Ulva* in separate paddle-raceways systems receiving fertilized seawater. Conversely, other farms continue to grow *Ulva* in abalone effluent water and feed this back to abalone; a practice that has occurred for over 15 years without any disease consequences (Bolton, et al., 2009). Due to the enormous benefits of growing *Ulva* in integrated systems, there is presently a growing interest in generating more knowledge on the diversity and relative abundance of microorganisms associated with *Ulva* and abalone grown in integrated systems; with or without abalone effluent water input.

1.7 Methods for the detection, quantification and assessment of microbial community structure in complex ecosystems

There are a number of direct and indirect methods that can and have been used for the detection and quantification of microorganisms in complex ecosystems (Macey and Coyne, 2006; Greeff et al., 2012). Direct methods such as culture-based techniques that assess the number of colony forming units of an organism(s) per unit area, volume or mass are still the most widely used, but these methods often underestimate the number of microorganisms in the environment due to difficulties in selection of suitable culture media and conditions (Macey and Coyne, 2006; Greeff et al., 2012). Some microorganisms may also be strongly associated with biofilms, requiring active removal. Regrettably, culture-based studies do not provide explicit proof of an association between the host and the pathogen (Largo, 2002), but do nonetheless provide useful information on changes in total number of bacteria, or specific groups of bacteria (e.g. *Vibrio* spp.) under different environmental conditions. In correlation with the 'great plate count anomaly' (Staley & Konopka, 1985), it is assumed that the enumeration of culturable bacteria, on average, yields less than 1 to 0.1% of the total bacterial count. Nonetheless, culturable bacteria are useful for obtaining a broader insight on the abundance of certain pathogenic/ probiotic indicator bacteria that may be present within the system. Potentially harmful or beneficial conditions induced by bacteria in aquatic habitats may be detected by assessing the presence and abundance of certain culturable groups of known indicator bacteria (Muroga, 2001; Paillard, et al., 2004; Osuolale & Okoh, 2015).

More recently, indirect molecular based techniques have revolutionized the detection, enumeration and characterization of microorganisms in complex ecosystems. Within the past decade, there has been a boom in the field of microbial ecology, which has been brought about by advancements in DNA sequencing technology and molecular marker development, enabling comprehensive documentation of bacterial communities in complex ecosystems, including the marine environment and aquaculture systems (Giovannoni & Stingl, 2005; Xu, 2006). Indirect methods are advantageous in that they do not depend on culturability or viability of the target organisms, are generally more sensitive and specific and can often generate data in a short period of time (Greeff et al., 2012). Nonetheless, selection of a suitable molecular marker, sampling method and appropriate DNA extraction protocol is crucial to the success of these methods. Other drawbacks of molecular based techniques are that they are often costly, required specialized equipment as well as expertise to perform the techniques and analyse the data.

Bacteria colonising macroalgae and abalone have been successfully characterised using 16S rDNA gene sequencing approaches (Burke, et al., 2009; Lachnit, et al., 2009). Sanger sequencing has been used to study bacterial communities and was first developed by Frederick Sanger et al. (1977). Sanger sequencing is particularly useful

for smaller-scale projects and for producing long contiguous DNA sequence reads (>500 nucleotides). Bacterial metagenomic studies commonly target the 16S ribosomal DNA (16S rDNA) gene for classification of strains down to genus and species level. The 16S rDNA gene region is approximately 1,500 base pairs (bp) in length and contains nine variable regions (V1 – V9), interspersed between conserved regions. In 2011, Burke et al. used the 16S rRNA gene to identify 5293 bacterial strains from six *Ulva australis* samples, providing insight on the diversity and phylogenetic profile of bacterial communities inhabiting green *Ulvacean* algae.

More recently, high volume “Next-Generation” sequencing (NGS) technology has been used to study microbial communities. Multiple NGS platforms exist, which use different sequencing technologies to sequence thousands to millions of small fragments simultaneously in a targeted manner. Targeted sequencing enables the user to sequence a subset of genes across multiple samples or specific regions of interest in greater depth, providing a cost-effective high throughput option that can produce sequences in a shorter time frame than the previously described Sanger sequencing method. The Illumina MiSeq, Roche 454, Ion torrent and Ion proton sequencing platforms are commonly used NGS platforms. Both illumina and Roche 454 make use of optical signals for sequencing, whereas the Ion torrent and Ion proton sequencing utilise the H^+ ion released during the addition of a dNTP to a DNA polymer for sequence identification. By sequencing multiple individuals at the same time, NGS provides a powerful snapshot of complex community dynamics. Current understandings of bacterial diversity within aquaculture systems are primarily based on 16S rRNA functional gene specific probes and 16S rRNA gene libraries as culture-based techniques are becoming insufficient (Schreier, et al., 2010).

1.8 Concluding remarks

Microbial management strategies in intensive aquaculture operations and their interactions with abalone are important and have received little attention. Moreover, process control of onshore aquaculture production systems requires quality control to minimise risks associated with the bio-technical aspects of operating a Recirculating Aquaculture Systems. Blancheton (2013), outlined the key biological mechanisms involved in RAS and reviewed the role of bacterial community compositions in various parts of RAS as well as bacterial-fish interactions in RAS. A key characteristic of RAS is the ability to control characteristics of water quality, which is dependent on the bacterial populations of the biofilters (in this case the *Ulva*) and of the recirculating water. In marine environments, microbes play important roles in nutrient fluxes (Asmala, et al., 2017; Soares, et al., 2017; Vadstein, et al., 2012), in turn impacting water quality and host health, which is increasingly important in RAS compared to more open systems (Beardsley, et al., 2011; Martins, et al., 2013). Aquaculture operations that practice either direct circulation, such as in RAS, or indirect circulation,

such as utilisation of effluent grown *Ulva* as feed, are complex environments which are affected by (1) feed type and feed regime , (2) management operations, (3) abalone-associated microflora, (4) water parameters, and (5) selective pressures within the system (Schneider, et al., 2006; Attramadal, et al., 2012; Blancheton, et al., 2013). Moreover, land-based abalone aquaculture operations have intensive feeding regimes which release a high amount of organic matter and nutrients into the water column, impacting the microbial communities which process the matter. The two primary bacterial groups identified in aquaculture systems include autotrophic nitrifiers, which oxidise ammonia, and heterotrophs, which degrade organic matter (Hagopian & Riley, 1998; Kindaichi & Okabe, 2004).

Currently, there is little to no published information on microbial communities associated with the seawater and *Ulva* obtained from intensive land-based mariculture systems supplied with abalone (*Haliotis midae*) effluent water or fertilized natural seawater. Knowledge of how *Ulva* and its associated bacterial populations, especially effluent grown *Ulva*, impacts abalone health in an intensive mariculture system could have important implications for the growth and sustainability of the industry as the perceived threat of disease is a major constraint to the on-going growth and development of integrated abalone/*Ulva* systems in South Africa (Bolton, et al., 2009). Improved knowledge of the microbiome of *Ulva* and surrounding seawater in systems maintained under different physiochemical conditions could help farmers optimise and improve existing recirculating aquaculture technologies and gain a better understanding of the potential benefits and disease risks associated with growing *Ulva* in integrated systems with abalone. Disentangling the closely-knit relationship between bacteria, their hosts (*Ulva*), and the water column in which they reside is crucial if we seek to manage effluent water bioremediation in an environmentally friendly manner whereby the waste may be turned into a product that can be utilised or incorporated into feed without the fear of a biosecurity threat. Furthermore, understanding the integration of *Ulva* into aquatic systems will additionally aid in the promotion of other activities that could integrate *Ulva* (such as urchins and shrimp farms), thereby improving the sustainability, productivity and profitability of the aquaculture sector as a whole.

1.9 Objectives of this study

The principal aim of this study was to investigate the biosecurity risks of incorporating effluent grown *Ulva* into abalone feed, on a Cape south coast abalone farm (Irvin and Johnson - Abalone Farm, Danger Point). The farm was chosen to gain a better understanding on the microbial communities associated with seawater and *Ulva* obtained from *Ulva* production systems supplied with either abalone effluent water or natural seawater that had been fertilized. Samples were taken at the inlets and outlets of several raceways to accommodate for differences within as well as between the

raceways. Samples were additionally taken from the inlets and outlets of two seawater tanks that are occasionally fertilised and contain no abalone effluent to serve as a control. It is hypothesized that the presence of *Ulva* within the raceways does not act as a sink for harmful bacteria, such as *Vibrio,* and hence is not of biosecurity concern to utilise as a feed for aquacultured abalone. It is further hypothesised that either the *Ulva* itself, or its core microbiota, are capable of positively impacting the water column in terms of managing harmful bacteria which are known to induce disease responses. The primary objectives of this study formulated to test these hypotheses were as follows: (1) To evaluate the genetic diversity and community structure of the bacteria present on the *Ulva* as well as the bacteria present within a fertilised seawater and an abalone effluent water system, (2) to examine whether a core bacterial population exists, and (3) to investigate the microbial relationships between the bacteria present on the *Ulva* as well as within the water systems. To assess these aims, a culture (traditional plate count method) and non-culture (NextGen sequencing of the 16S rRNA gene) based approach were adopted. The culture-based approach utilised three media types for the isolation and quantification of bacteria; namely tryptic toy agar (TSA), thiosulfate-citrate-bile-sucrose (TCBS) agar (Vibrio selective), and Ulvan agar which contains the primary polysaccharide of *Ulva* as its main carbohydrate source. Post isolation, bacterial cultures were identified by sequencing the 16S rRNA gene and stored for future analysis if required. The NextGen sequencing approach was used to categorize the bacterial microbiome by sequencing the hyper-variable V3-4 of the 16S rRNA gene which enabling distinction between closely related bacteria. Downstream sequence analyses of the 16S rRNA gene sequences were performed using the Mothur (v1.35.1; Schloss, et al., 2009) pipeline, and analysed using the online program Microbiome Analyst (Dhariwal, et al., 2017). In addition to bacterial sequencing, the identity of the *Ulva* was determined using the *rbcL* gene.

SECTION 2. MATERIALS AND METHODS

2.1 Collection of water and Ulva samples

2.1.1 Study site

Water and *Ulva* samples were collected from Irvine & Johnson (I&J) Cape Abalone Farm at Danger Point, Gansbaai (34°34'60 S; 19°21'0 E) on the 17th of August 2017.

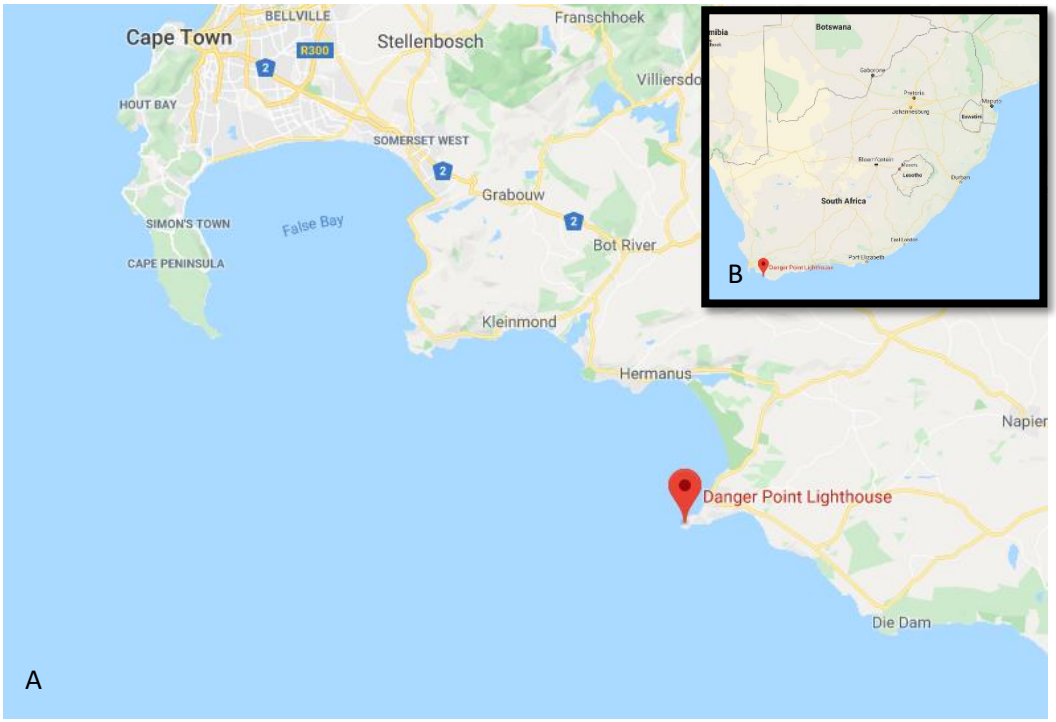

Figure 3. Map depicting the location of the Irvin and Johnson abalone farm at Danger Point (a), along the western coast of South Africa (b).

Both *Ulva* and water samples were collected from two separate *Ulva* production systems on the farm. One system consisted of tanks that received seawater (FSW Fig. 4), supplemented with nitrogen and phosphorus at the farmers discretion to ensure optimal growth of the *Ulva,* whereas the other system was comprised of raceways receiving abalone effluent water (AEW Fig. 5). *Ulva* samples were collected from within each system, whereas the water samples were collected at the inlet and outlet of each system. In total, 2 FSW tanks and 5 AEW raceway systems (Raceways 1,3,5,9, and 11) were sampled.

2.1.2 Ulva collection

Healthy vegetative *Ulva* thalli (approximately 15grams fresh weight) were collected from the FSW and AEW systems and immediately transferred into separate pre-labelled sterile 50mL conical centrifuge tubes that had been placed in a cooler box with ice. Thereafter, samples were transferred within 3 hours to the Department of Agriculture, Forestry and Fisheries (DAFF) Marine Research Aquarium (MRA) in Cape Town, South Africa, where they were processed further. Only healthy blades of *Ulva* with no necrotic white tissue were used for genetic and microbiological analyses. Little to no necrotic lesions were observed on the sampled *Ulva.* At the research facility, the freshly collected *Ulva* was rinsed with 10 mL of 0.22µm filtered autoclaved seawater (ASW) via inversion for 20 seconds, to remove any loosely attached organisms and debris. Directly after rinsing, a portion of the *Ulva* (approximately 20mg) was used for quantifying culturable bacteria (described 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 next generation sequencing (NGS) analysis (described below).

2.1.3 Water collection

Inlet and outlet water samples (500 mL) were collected in triplicate from the FSW and AEW systems in sterile 500 mL Schott bottles. Following collection, each bottle was placed in a biosecurity bag, to prevent spillage and possible contamination of other samples, and immediately stored on ice in a large polystyrene cooler. Samples were transferred to the MRA in Cape Town as described above. At the MRA, water samples were processed for enumeration of culturable bacteria and NGS analysis as described below.

2.1.4 Description of tanks and integrated raceway systems sampled

Fertilized seawater (FSW) Tanks

The FSW systems serve as algal “seeding tanks” for the larger raceways and are based on the system previously described by Robertson-Anderson (2003). These systems are characterised as two semi-circular, PVC lined containers with an approximate surface area of 3 m x 1 m and a depth of 0.5 m supported on a frame. The tanks water source consisted of fresh seawater that had been pumped directly from the adjacent surf zone into the tank via a sub-surface filter system, which left the tank passively via an outlet 17cm from the top. The outlets contained mesh grids welded to the tanks to prevent *Ulva* washout. The tanks were actively aerated by a 30-mm PVC pipe (containing 3mm holes spaced 250 mm along the pipe), that ran along the bottom centre of the tanks to promote algal growth. The tanks are pulse fertilised with nitrogen and phosphorus weekly, on Tuesdays and Fridays, to prevent algal degradation, especially during the summer months when algal growth is most vigorous. During algal degradation, the *Ulva* becomes bleached, fragments and dies. The tanks are occasionally drained for cleaning.

Figure 4. Fertilised seawater tank 1 and 2 at I & J Danger Point abalone farm.

Abalone effluent water (AEW) raceways

The *Ulva* raceway systems investigated on I & J Abalone Farm were first built in 2005, and function in a manner whereby nutrient rich effluent water from abalone tanks flows through several macroalgal raceways, whereby some of the nutrients are consumed by *Ulva* (the primary alga in the system), before flowing into the southern point of Walker Bay, South Africa (Jonell, 2008). The abalone wastewater raceways contain the *Ulva* that is utilised as a supplementary feed for abalone and were initially built to recirculate water using *Ulva* for bioremediation. In total, five raceways were sampled, i.e. raceway 1,3,5,9 and 11, to get a broad representation of the bacterial populations within the raceways (Figure 5,6).

The raceways sampled in this study had dimensions of 27.7 m long and 8.23 m wide, with an approximate depth of 0.45 m (Figure 5, Browne 2009). The effluent raceways are usually filled with abalone effluent (~0.39 m deep), that is gravity fed through the abalone culture tanks before being gravity fed into the raceways. The water coming from the abalone culture tanks enters through a central canal, after which it passively flows through each of the 11 parallel raceways via openings along the channel. No mixing of water occurs between the raceways. The water entry points of the raceways are fitted with steel grids to prevent algal washout. The effluent water exits the raceways passively via shallow overflows on the opposite end of the raceway, where

it re-enters a central canal and runs either into a partial recirculation system or Walker Bay. A paddlewheel is used to circulate and agitate the algae within each raceway, as well as to oxygenate the water. Furthermore, a white plastic is used to line the inside of the raceways to maximise light reflection and to aid cleaning. The system was initially designed as a re-circulating system and ran on re-circulation for many years. Nonetheless, the farm was not running re-circulation at the time, due to bio-security concerns, however, do have the facilities to do so in dire instances, such as during a HAB event.

Harvesting of raceways occurs once a month on a rotational basis, whereby the tanks are fully drained through large pipes at one end of the raceway. The *Ulva* flows through these pipes and is collected in 50 kg plastic baskets. During harvesting, the remaining *Ulva* is swept to the side of the raceway, closest to the outflow and placed into baskets, after which it is weighed out to determine the month's harvest. On average, 500 kg of *Ulva* is replaced after harvesting to re-seed the raceways after harvesting. The effluent raceways are pulse fertilised with nitrogen and phosphorus every second week to reduce nutrient stress on the *Ulva*, as the nutrients provided by the abalone effluent wastewater alone is insufficient.

Figure 5. *Ulva* raceways at I & J abalone farm containing abalone effluent water.

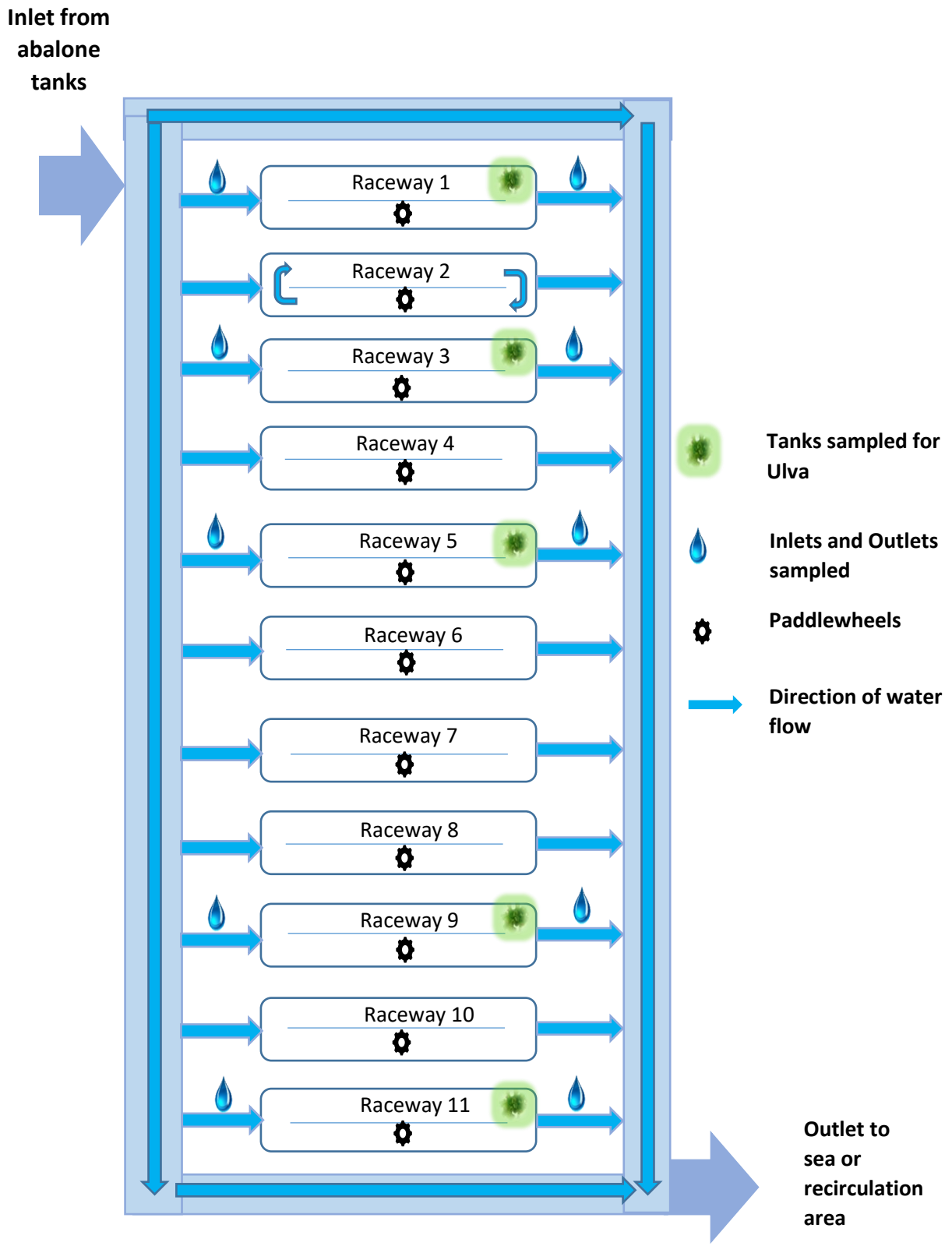

Figure 6. Schematic of the abalone effluent wastewater (AEW) systems at I&J showing the general outlay of the effluent water tanks and the location of the sample points.

2.2 Molecular analysis of Ulva

Partial sequences coding for the *rbcL* gene region were used to determine the phylogenetic position of the *Ulva spp.* collected in this study among other documented *Ulva* species. The *rbc*L gene is a highly stable sequence positioned within the chloroplast genome of photosynthetic eukaryotes as well as numerous prokaryotes and encodes for the large subunit of ribulose-1,5-bisphosphate carboxylase/ oxygenase, which has commonly been used for phylogenetic analyses of *Ulva* species (Goloubinoff, et al., 1989; Morden & Golden, 1991; Morden, et al., 1992; Hanyuda, et al., 2000; Hughey, et al., 2001; Hayden 2003). The *rbc*L gene was amplified using the universal primers 1385r and RH1 (Table 1) described by Manhart (1994), which amplify a 1,400 bp region of the *rbc*L gene, covering 95% of the gene. The rbcL gene region is frequently used for phylogenetic analyses of green plant groups and forms the basis of several taxonomic studies on green marine macroalgae (Hayden & Waaland, 2002; Hayden 2003; Group, et al., 2009; Okelly, Mahendran 2014; Mahendran & Saravanan, 2017).

2.2.1 DNA extraction and purification

Genomic DNA was extracted from frozen *Ulva* (in duplicate) using a modified protocol previously described by Hughey et al. (2001) and used an extraction buffer defined by Dellaporta et al. (1983). Frozen liquid nitrogen ground *Ulva*, collected as described above from FSW tank 1 (Fig. 4), was used for the genomic DNA extraction. Approximately 20mg of ground *Ulva* was placed into two separate pre-labelled 1.5mL microcentrifuge tubes, containing 700µL of extraction buffer (Dellaporta, et al., 1983). The extraction buffer consisted of 100mM Tris (pH 8.0), 50mM EDTA, 500mM NaCl, 10mM 2-mercaptoethanol (added just before use, 7 µL/mL^{-1} extraction buffer), 50µL of 20% SDS, 10µL of 0.1 M DTT, 4mg of lyophilized Proteinase K. The samples were ground for 30sec using a held pellet pestle (Sigma Aldrich, cat.#Z359971) and subsequently lysed at 65ºC on a heating block overnight. The polysaccharides were removed from the lysed cell suspension by adding 250µL of potassium acetate (5M), incubating on ice for 30min, and centrifuging at 12,000 x g for 30min (Dellaporta, et al., 1983). The supernatant (800µL) was extracted with an equal volume of chloroform and centrifuged at 12,000 x g for 20min. DNA was precipitated from the aqueous phase using two-thirds volume isopropanol for one hour at -20ºC before being centrifuged at 12,000 x g for 30 min to create a pellet. After carefully removing the supernatant, the pellet was washed with 500µL of 70% ethanol, spun at 12,000 x g for 2-3min, air dried, and resuspended in 100µL of Millipore water and stored at -80 ºC for further analyses.

2.2.2 Polymerase chain reaction (PCR) amplification and DNA sequencing

A 1,400-bp fragment of the *Ulva rbcL* gene region was amplified using 400nM of the forward primer RH1 and 400nM of the reverse primer 1385r (Table 1; Manhart, 1994). The PCR reaction mixtures (25µL; performed in triplicate) were prepared using 1µL of diluted DNA [~25ng/µL], 12.5µL Kapa*Taq* ReadyMix (Kapa Biosystems; Catalog #kk1006), 10.5µL Millipore water, and 0.5µL of each primer (400nM). Amplification was conducted using a Labnet Multigene Thermal Cycler (Labnet International™) and consisted of an initial denaturation at 94ºC for 6min, followed by 35 cycles of 1min at 94 ºC, 1min at 45ºC, and 2min at 72ºC, with a final extension of 72ºC for 10min (Hayden et al., 2003). A non-template control (PCR-grade H_2O) was analysed to ensure that the reagents were not contaminated and that here was no amplification except for the *Ulva* DNA. The amplified PCR products, including the negative controls, were assessed spectrophotometrically by determining the 260/280 ratio (a ratio of 1.8 indicates that the DNA prep is pure and free of protein contaminants; protein absorbs maximally at 280nm) and analysed by 0.8% gel electrophoresis to verify reaction specificity and fragment size (1,400-bp fragment expected) before being purified using a PCR purification kit (Roche). The purified PCR products were sequenced in both directions using a BigDYE Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI3730xl Genetic Analyzer (Applied Biosystems) according to the sequence manufacturer's instructions.

2.2.3 Sequence assembly and phylogenetic analysis

Each *rbcL* gene sequence, from both the forward and reverse reads were manually edited and assembled to create a consensus sequence using CLC Main Workbench version 6.8.4 (CLC bio, www.clcbio.com). Homology searches were carried out using the BLASTN algorithm (Altschul, et al., 1990) provided by the Internet service of the National Centre for Biotechnology Information (NCBI; http://www.ncbi.blast.nlm.nih.gov/BLAST/), which is an online search platform which enables matching of a given query sequence to a public database of sequences, based on percentage similarity. The *rbcL* gene sequence of the *Ulva* species obtained from the FSW tank at I&J Cape Abalone Farm was aligned with sequences from 38 known *Ulva* spp. and 5 outgroup species (2 *Umbraulva,* a *Percursaria,* a *Gemina* and an *Ulvaria*) downloaded from GenBank. Sequences were aligned and edited with CLC Main Workbench version 6.8.4 and exported in Phylip format for construction of maximum likelihood (ML) trees using PHYML V.3.1 (Guindon & Gascuel, 2003). Best fit for ML analyses of the *rbcL* sequences were based on the Tamura-Nei substitution (HKY85) model and gamma parameters were estimated by the PHYML software. The reliability of the inferred phylogenetic tree was evaluated using the bootstrap test (Felsenstein, 1985) with 1000 bootstrap re-samplings. Tree files were viewed and edited using Mega V.7.0.26 (Kamur, et al., 2015). A single tree was constructed, displaying bootstrap values from maximum likelihood at each node. ML bootstrap values of 70 and above were indicated on the tree.

Table 1. Universal oligonucleotide primers used to amplify and sequence the rbcL gene of *Ulva* and the small subunit (SSU) rRNA gene of bacteria isolated from seawater and *Ulva*

	Primer	Oligonucleotide Sequence (5'-3')	Target	Fragment size	Annealing temperature	Reference
Ulva sequencing	Forward: RH1	ATGTCACCACAAACAGAAACTAAAGC	*rbc*L	1400bp	45ºC	(Manhart, 1994)
	Reverse: 1385r	AATTCAAATTTAATTTCTTTCC				
Bacterial sequencing (Sanger Sequencing)	Forward: 16S F-fD1	AGAGTTTGATCCTGGCTCAG	16S	1500bp	57ºC	(Lane, 1991)
	Reverse: 16SR-Rp2	ACGGCTACCTTGTTACGACTT				
Bacterial sequencing (Illumina)	Forward Primer: 16S_341F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	16S V3-V4	460bp	55ºC	(Klindworth, et al., 2013)
	Reverse Primer: 16S_805R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC				
	Forward Primer Overhang Adaptor Sequence:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG				
	Reverse Primer Overhang Adaptor Sequence:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG				

2.3 Enumeration of culturable bacteria in seawater and on *Ulva* (Selective Plating)

The culture-based approach utilised a standard plate-counting technique to quantify changes in the number of total bacteria (TB), total *Vibrio* species (TV) and *Ulva* specific bacteria (UB), growing on the *Ulva* and in the seawater from the FSW and AEW systems at I&J. Three media types 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). Ulvan Agar is a medium supplemented with Ulvan, the primary carbohydrate of *Ulva* (prepared according to Jaulneau, et al., 2010; described below). The main constituents of Ulvan are sulfated rhamnose residues that are linked to uronic acids, resulting in a repeated disaccharide unit (-D-glucuronosyl-(1,4)-L-rhamnose 3-sulfate) called aldobiouronic acid. The Ulvan serves as the sole carbon source of the media, enabling isolation and evaluation of *Ulva* specific bacteria. TSA is regarded as a general media and is routinely used for the isolation of marine bacteria, providing an indication of the total culturable bacteria (TCB) 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, allowing an overview of how *Vibrio* dynamics (TV) are changing within the systems (Kobayashi, et al., 1963).

2.3.1 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. The Ulvan plates consisted of 0.1% (w/v) yeast extract, 0.5% (w/v) Ulvan extract (described below), and 15g.L^{-1} of bacteriological agar in 1000 mL sterile autoclaved seawater (ASW). Ulvan was extracted from dry *Ulva*, obtained from I&J Cape Abalone Farm, according to the method described by Jaulneu at al. (2010). Briefly, 50g of dried *Ulva* was homogenized into a coarse powder (<1cm) in a food processor before diluting the *Ulva* powder with 450mL Millipore water and autoclaving at 120ºC for 15min. After allowing partial cooling, the algal suspension was filtered through a sieve (0.25mm) and centrifuged at 5,000 x g for 5min. The pellet was discarded and 2.5 volumes (1,125mL) of 70% ethanol was used 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 x g for 5min, washed with 96.9% ethanol, and vacuum dried in a centrifuge (1,200rpm) at 30ºC for 4 hours. The dry Ulvan was ground into a fine powder using a mortar and pestle and aliquots stored in Eppendorf tubes in a -20ºC freezer until needed. On average, 50g of dried *Ulva* yielded 4.612g of Ulvan (9.2% dw).

2.3.2 Enumeration of bacteria in seawater

For the isolation and enumeration of bacteria from seawater, the 500mL water samples collected from the FSW 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, the filter membranes were gently scraped with a sterile scalpel blade to loosen the bacterial cells, before transferring each filter and its accompanying cells into separate sterile hawk tubes containing 10mL sterile ASW. Tubes were vortexed for 5 minutes to remove any remaining bacteria attached to the membrane before re-suspending the cells in the ASW. Post vortexing, the membranes were discarded and 0.1mL aliquots of the re-suspended cells were serially diluted (10^{-2} - 10^{-5}) and plated in triplicate (100µL aliquots) on TSA, TCBS and Ulvan plates. . Immediately following plating, petri dishes were sealed with Parafilm, and the TSA and TCBS plates were incubated at room temperature for two days, whereas Ulvan plates were incubated at the latter temperature 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 9.9mL of ASW containing bacteria (from each sample) was centrifuged for 10min at 11,000 rpm to concentrate the bacterial cells. Following centrifugation, the supernatants were carefully decanted and the tubes containing the cell pellet were stored at – 80°C until required for DNA extractions and NGS analysis.

2.3.3 Enumeration of bacteria on Ulva

Bacteria were isolated from fresh vegetative thalli of *Ulva* sp. using a modified version of the method described by Nakanishi *et al.* (1996). Algal thalli were gently washed in autoclaved seawater (ASW) to detach any loosely associated debris, after which approximately 0.5g of thallus was vortexed vigorously in 10mL ASW for 10 minutes to detach the associated epibacteria. The thallus was then removed, and the remaining supernatant was serially diluted (10^{0} - 10^{-3}) and 100µL aliquots were plated in triplicate onto the three media types: Ulvan, TSA, and TCBS. Plates were incubated and enumerated as described above.

2.3.4 Isolation, identification and storage of culturable bacteria

Following 2-7 days of incubation, bacterial colonies of interest were selected from each media type (TSA, TCBS or Ulvan agar) and transferred to fresh plates of the corresponding media type for the isolation of pure colonies. Bacterial colonies that appeared unique, based on colony form (shape, colour, size & texture), elevation (flat, raised, umbonate, convex or pulvinate) and margin (entire, undulated, lobate or filiform), or occurred in high abundance on the different media types were selected for further analysis. Colonies were sub-cultured until pure/ monocultures were obtained, which were used for long term storage.

For long term storage, glycerol socks were prepared for each isolate and stored in a – 80ºC ultra-freezer (Hollander & Nell, 1954; Hubalek, 2003). Glycerol freezing stocks were prepared by adding 5mL (20%) sterile glycerol to 20mL pre-autoclaved Tryptic

Soy Broth solution (TSB; 0.625 g NaCl, 0.6 g TSB powder in 20ml Millipore water). Bacterial colonies from a pure culture of each isolate were aseptically transferred to the glycerol freezing solution, vortexed thoroughly for several minutes to ensure a homogenous solution with no cell clumps and 0.5mL aliquots of the resuspended cells were then transferred to 1mL pre-labelled cryovials and immediately stored at -80ºC.

To identify the isolated bacteria, genomic DNA was extracted from each of the chosen bacterial isolates and subjected to 16S rRNA gene amplification and sequencing. Genomic DNA was isolated using a modified version of the Chelax extraction method described by Greeff *et al.* (2012). Briefly, cells from a single bacterial colony were picked and suspended in 300µL of sterile Millipore water in a 1.5mL micro-centrifuge tube. Each tube was vortexed vigorously and centrifuged at 16,000 × g for 5 min. The supernatants were carefully removed, and the remaining pellets re-suspended in 300µL of sterile water. Bacterial pellets were homogenised for 1min using a hand-held pellet pestle and transferred to new sterile 1.5mL microcentrifuge tubes containing 0.04±0.05g Chelax-100 beads. Chelex-100 is comprised of negatively charged microscopic beads that chelate metal ions which are required as catalysts or cofactors in enzymatic reactions. The samples containing Chelax-100 beads were briefly vortexed and incubated for 20min at 56ºC on a heating block. Post incubation, samples were briefly vortexed and incubated for a further 30min at 95ºC to lyse bacterial cells. The lysed samples were placed on ice to cool for 5min, vortexed briefly and centrifuged at 16,000 × g for 5min. The resulting supernatants (20µL), containing genomic bacterial DNA, were analysed by PCR.

The universal broad-spectrum bacterial primers 16S F-fD1 and 16SR-Rp2 (Table 1; Lane 1991) were used to amplify a 1.5kb fragment of the 16S rRNA gene region of each isolate. PCR reaction mixtures (25µL; performed in triplicate) consisted of 1µL of genomic DNA (~25ng), 12.5µL KapaTaq ReadyMix (Kapa Biosystems; Cat#KK1006), 10.5µL Millipore water, and 0.5µL of each primer (400nM). Amplification was conducted using a Labnet Multigene™ Thermal Cycler (Labnet International Inc.) and consisted of an initial denaturation at 96ºC for 3min, followed by 40 cycles of 45secs at 96ºC, 30secs at 57ºC, and 1min at 72ºC, with a final extension of 72ºC for 5min. Known amounts of bacterial genomic DNA, previously analysed by PCR and subjected to sequencing, were analysed as positive controls during the PCR and non-template controls (PCR-grade H_2O) were analysed as negative controls to ensure that there was no amplification except for the bacterial DNA. The amplified PCR products and the positive and negative controls were analysed by 0.8% agarose gel electrophoresis to assess reaction specificity and fragment size (~ 1.5kb fragment expected).

The PCR product obtained from each bacterial colony was purified and sequenced as described above. The universal 16S forward primer F-fF1 was used for cycle sequencing. The sequences were edited and assembled using CLC Main Workbench and homology searches were carried out using the BLASTN algorithm (Altschul *et al.,* 1989) provided by the NCBI (as described above).

2.3.5 Statistical analyses for colony forming unit (CFU) data

SigmaPlot 12.0 software was used to perform all statistical analysis. To determine whether the number of culturable bacteria differed between the inlet (incoming) and outlet (outgoing) water within and between each system (abalone effluent waste water (AEW) and fertilized seawater (FSW) systems), one-way analysis of variance (ANOVA) was performed. Data for the different media types (TCBS, TSA and Ulvan) were treated separately. Similarly, to determine whether the number of culturable bacteria differed on *Ulva* (CFU.g^{-1} of *Ulva*) obtained from the AEW and FSW systems, a one-way ANOVA was conducted. Data for CFU.mL^{-1} seawater and CFU.g^{-1} *Ulva* were square root transformed prior to statistical analysis. All data were tested for normality (Kolmogorov-Smirnov test) and equal variance. The Holm-Sidak method was used for all post-hoc comparisons between individual samples. Significance was assigned to *p*-values less than 0.05 for one-way ANOVA and Holm-Sidak analysis. Data values on all figures refer to means ± standard error (SE) of the mean.

2.4 Bacterial community profiling by 16S rRNA Illumina MiSeq sequencing

2.4.1 DNA Extraction and library preparation

Microbial DNA extraction from seawater and Ulva

Microbial genomic DNA was isolated from the frozen ground *Ulva* samples (prepared as described in 2.1.1 above) and bacterial pellets, obtained from the seawater samples (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 seawater sample was used for genomic DNA isolation, whereas approximately 5mg of each frozen ground *Ulva* sample collected from the FSW and AEW systems at I&J was used for DNA extraction. A total of 14 frozen bacterial pellets and 7 ground *Ulva* samples were processed. DNA extractions were performed in duplicate and a negative control, containing only Millipore water, was included to account for possible contamination. Post DNA extraction, DNA concentration, integrity and fragment size were determined via spectrophotometry and 0.8% agarose gel electrophoresis, as described above.

Generation of the 16S rRNA amplicon library

The 16S_341F and 16S_805R primer pair (Klindworth, et al., 2013), with added Illumina adapter overhang nucleotide sequences (table 1), were used to amplify the 16S V3-V4 hypervariable gene region of each sample for NGS analysis. PCR reaction mixtures (25µL; performed in triplicate) consisted of 1-5µL of genomic DNA (+/- 25ng total), 12.5µL 2×KAPA HiFi HotStart ReadyMix, for hot-start PCR (Kapa Biosystems; Catalog #KM2605), 5.5-10.5µL ddH_2O, and 0.5µL of each primer (200nM). Touchdown PCR amplifications were carried out in a Bio-Rad CFX96 Real-Time PCR

Detection System Instrument (Bio-Rad Laboratories™) using a modified version of the cycling conditions outlined by the Illumina 16S metagenomics sequencing library preparation manual (Illumina, 2013). PCR amplification cycling consisted of an initial denaturation at 95ºC for 5min, 10 cycles of touchdown PCR (30 secs at 95ºC, 30secs at 65ºC, with a 1ºC per cycle decrement, and 30secs at 72 ºC), followed by an additional 25 cycles of PCR (30 secs at 95ºC, 30secs at 55ºC, and 30secs at 72ºC), and a final extension step for 10min at 72ºC. Negative controls, containing all components excluding DNA templates, were run in parallel. Amplified PCR products and negative controls were electrophoresed as above to confirm successful amplification, reaction specificity and fragment size (~ 460 bp fragment expected).

DNA purification, sequencing and library preparation

Post validation PCR purification, indexing and library preparation were performed at the Next Generation Sequencing Facility (NGSF) at the University of the Western Cape (UWC), where the PCR products from each sample were used to create libraries with unique barcodes (n=21 samples/libraries). Briefly, PCR products were purified away from free primers and primer dimer species using AMPure XP beads (Beckman Coulter), as per manufacturer's instructions. Thereafter, dual indices and Illumina sequencing adaptors were attached to the purified PCR products using the Nextera XT Index Kit, followed by a second PCR purification step using AMPure XP beads, final library quantification and normalization. Normalized libraries were pooled prior to sequencing on the Miseq Illumina system, as per manufacturer protocols.

2.4.2 Raw data processing, data filtration and normalisation.

Downstream sequence analyses of the 16S rRNA gene sequences generated using Illumina's MiSeq platform were performed using the Mothur (v1.35.1; Schloss, et al., 2009) pipeline, as per the 16S metagenomics standard operating procedure (SOP) provided by Kozich et al. (2013) at Schloss labs (MiSeq SOP at http://www.mothur.org/wiki/MiSeq_SOP). Contigs were formed from forward and reverse fastq. files, duplicate reads and reads with ambiguous base calls were removed, and sequences were trimmed to 460 bp using screen.seqs for the V3-V4 gene region. Cleaned reads were aligned with the recreated SILVA database (Silva.seed_v132; provided by Mothur) and cleaned using screen.seqs. with a maximum homopolymer length of 8. Noise filtering and overhang removal were performed using the vertical = T and trump = . options. Any redundancies created were removed using the unique.seqs command, and any pyrosequencing errors were omitted by pre-clustering (pre.cluster.), which splits sequences by group and sorts them by abundance, favouring 1 difference for every 100bp. Chimeras were detected using chimera.vsearch and removed using remove.seqs commands. Undesirables were checked using Bayesian classification, whereby they were classified according to the taxonomy corresponding to the reference database trainset16_022016 (provided by Mothur) and removed using the remove.lineage command. Sequences

were clustered into OTUs using cluster.split (for larger datasets), whereby the sequences were split by classification into bins, and clustered within each bin at a taxa level of 4 (for genus level), with a cut-off of 0.03. Finally, a consensus taxonomy of OTUs was created using the make.shared. command, and defined using the classify.otu command.

Following quality assessment, chimeric trimming and other filtering, the OTU consensus taxonomy was filtered and normalised using the program MicrobiomeAnalyst (Dhariwal, et al., 2017) (http://www.microbiomeanalyst.ca/faces/home.xhtml). Data was filtered for low abundance reads (minimum count of 2 with 20% prevalence in samples), whereby OTUs containing mostly or only zeros were removed to account for potential sequencing errors and low-level contaminations (Dhariwal, et al., 2017). Low variance reads (10% based on standard deviation), unlikely to be associated with study conditions, were removed to reduce the effects of multiple testing to optimise downstream comparative analyses. Filtered data was used to quantify OTU abundance and calculate alpha diversity (within sample) statistics (Chao1 index, Simpson index, Shannon index and observed OUT prevalence).

Filtered data was normalised through relative log expression (RLE) transformation. RLE is optimal for datasets with a small sample size as it can reveal unwanted variation and removes batch effects by scaling a samples raw read count through a sample-specific factor that depends on the median and on the mean of each sample, accounting for sparsity, under-sampling and uneven sequencing depth in downstream analyses (Gandolfo & Speed, 2018). Normalised data was used for all successive multivariate beta diversity (between sample) statistics (principle coordinate analyses (PCoA) and non-metric multidimensional scaling (NMDS)), clustering (cluster analysis), differential abundance (univariate analysis) and linear discriminate analysis effect size (LEfSe).

2.4.3 Overview of sequencing and microbial diversity at a higher level

For a broad comparison of phylogenetic groups, bacterial community alpha diversity was evaluated using the R phyloseq (McMurdie & Holmes, 2013) and vegan (Dixon, 2003) packages implemented in Microbiome Analyst. The Chao1 (Chou & Talalay, 1984), Shannon (Shannon, 1948) and Simpson (Simpson, 1949) non-parametric diversity indexes were evaluated to assess species richness, evenness and uniqueness, respectively. The Chao1 index assesses species diversity based on the minimum richness (number of rare OTU's, either singlets or doublets) within each sample, and corrects for variance assuming the number of observations has a poisson distribution (Oikonomou, et al., 2012). The Shannon diversity index combines richness (total number of OTUs) and evenness (relative number of OTUs) estimates to assess within sample diversity. Generally, low indexes correlate with communities with a single dominant species, whereas a high index represents a more evenly distributed community (Johnson & Burnet, 2016). The Simpson index estimates sample diversity by describing the probability of drawing the same OTU from two randomly selected

reads, whereby less evenly distributed communities have a lower index. Statistical significance between cohorts was analysed by means of an analysis of variance (ANOVA) for each index.

2.4.4 Comparison of bacterial community structure among *Ulva* and water libraries.

Ordination analysis

Beta diversity analyses were evaluated in MicrobiomeAnalyst, which is based on the R phyloseq (McMurdie & Holmes, 2013) and vegan packages (Dixon, 2003; Dhariwal, et al., 2017), whereby two multivariate ordination evaluations, a between-sample distance based principal co-ordinate (PCoA) analysis and a between-sample similarity based non-metric multidimensional scaling (NMDS) analysis, were conducted at the genus level to provide an overview of and measure the distance or dissimilarity between the microbiota present within the fertilised seawater and abalone waste water systems, as well as the microbiota on the *Ulva* within each system. Both evaluates are based on calculations for the Bray-Curtis dissimilarity index as samples were not collected linearly (Legendre & De Cáceres, 2013). 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). Statistical significance ($P < 0.001$) was assessed using a permutational multivariate analysis of variance (PERMANOVA), which tests for homogeneity across data points and supports the degree of dissimilarity between the samples. Hierarchical relationships between cohorts were assessed at 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.

2.4.5 Comparison of bacterial community structure among sample types

OTU Abundance profiling

OTU abundance was evaluated by constructing abundance tables ($n < 2500$ counts merged and denoted as “Others”) based on the relative abundance (%) of the 21 samples at family and genus level. Relative OTU abundance is a useful tool to analyse and interpret large microbiome data sets (Ong, et al., 2013; Gloor, et al., 2016). The four most prevalent OTUs in each cohort at genus level were identified.

Linear discriminate analysis effect size (LEfSe) analysis

A linear discriminate analysis (LDA) was used to determine the effect size (LEfSe) to test for significant associations between cohorts and their associated microbiota, whereby the 25 OTU's ($P<0.05$; LDA score > 2) most likely to explain the differences between the cohorts were identified. LEfSe is a biomarker discovery and explanation method which incorporates statistical significance with effect size (biological consistency) estimation. Firstly, a factorial Kruskal-Wallis rank sum test was applied to each OTU relative to the class factor and cohort of each sample, followed by LDA to estimate the effect size of each differential feature (Segata, et al., 2011). LDA attempts to maximise the separability between the OTU's to improve decision making.

Differential abundance (Univariate statistical comparisons)

Differential abundance across the water and *Ulva* samples was assessed using the DESeq2 univariate analysis method (Love, et al., 2014). DESeq2 uses negative binomial generalised models to estimate dispersion and fold changes, whereby a normalised dataset is used to calculate the mean, variance and mean dispersion estimates. The mean, variance and mean dispersion estimates were calculated for each OTU using a normalised dataset. The Mann-Whitney/Kruskal-Wallis statistical method was used to normalise the dataset and to identify OTU's with means exceeding the threshold for the OTU ($P < 0.05$) (Hawinkel, 2015). Moreover, OTU's were considered significantly different when its mean exceeds the calculated threshold for that OTU. Furthermore, the false discovery rate (FDR) was calculated to adjust P-values for multiple comparisons, mitigating the possibility of type I errors (Benjamini & Hochberg, 1995).

SECTION 3. RESULTS

3.1 Molecular analysis of Ulva

In order to identify the *Ulva* cultivated at Irvine & Johnson (I&J) Cape Abalone Farm, the *rbcL* gene sequence of the isolate collected from this facility was determined. The edited and assembled nucleotide sequence of the *rbcL* gene, had a total length of 867 bp. Homology searches performed using BLASTN algorithm indicate that the *rbcL* sequence showed 100% similarity with both *Ulva armoricana* (AB097632) and *Ulva scandinavica* (AY255870) in the GenBank database. The phylogenetic analysis of the *Ulva* chloroplast *rbcL* gene sequences confirmed the BLAST search results of the GenBank database with the *Ulva* samples collected from I&J clustering within the monophyletic subclade consisting of *U. scandinavica, U. armoricana* and *U. rigida, U. fasciata* and *U. clathrata* (Fig. 7). The clustering is well supported with a high bootstrap value of 96% in the Maximum Likelihood analysis. Nodal support values correspond to ML bootstrap values.

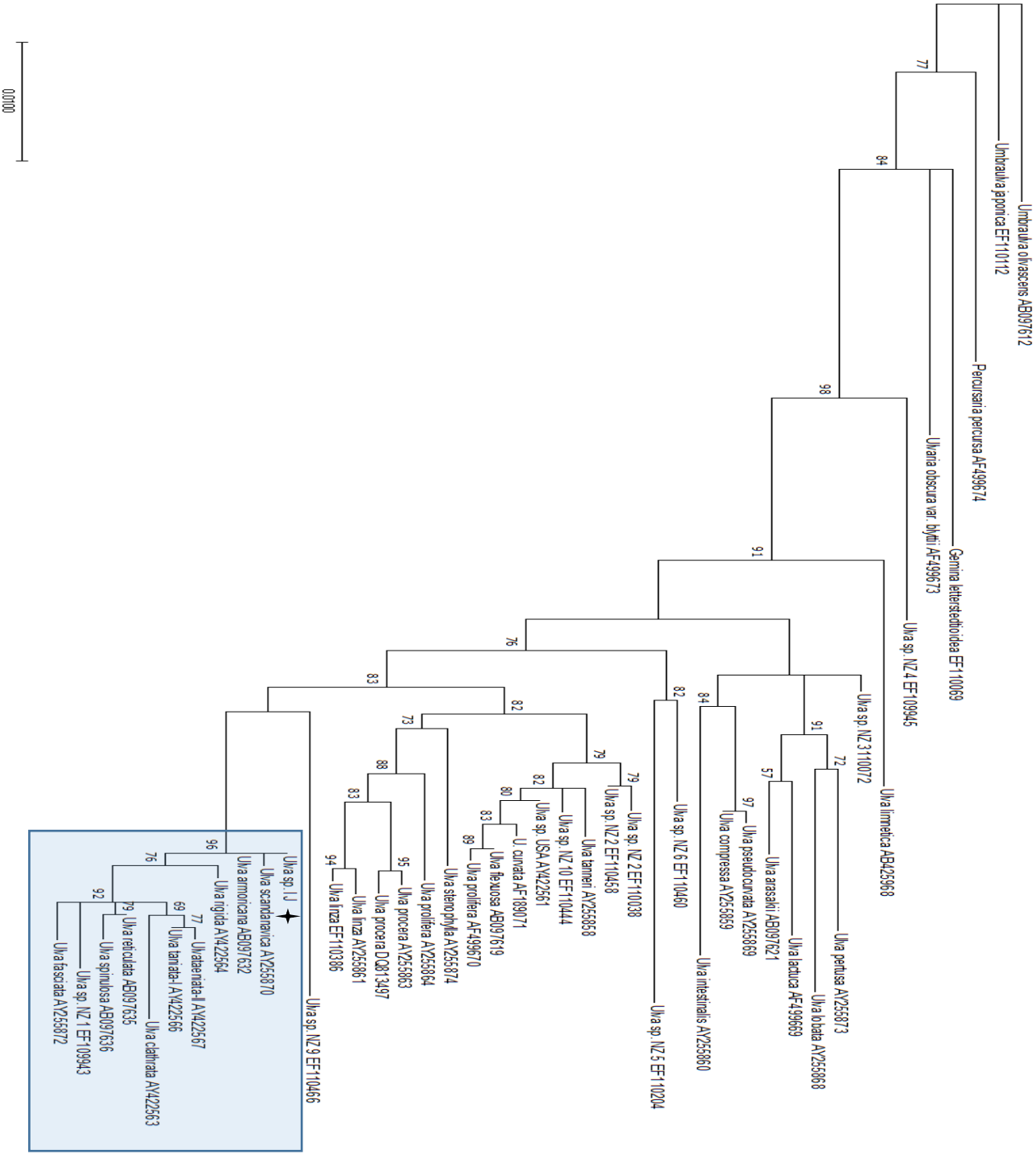

Figure 7. Maximum likelihood tree based on the chloroplast *rbcL* gene sequence of the *Ulva* sp. collected from I&J Cape Abalone Farm, 37 organisms within the family *Ulvaceae* and 5 out-group species (2 *Umbraulva*, a *Percursaria*, a *Gemina* and an *Ulvaria*). The reliability of the inferred phylogenetic tree was assessed using the bootstrap test with 100 bootstrap re-samplings. The numbers on the branches show the bootstrap values; with values below 70% omitted from the tree. Numbers next to specific names correspond to the accession numbers of the chloroplast rbcL gene sequences. The bar indicates 1 base substitution per 100 nucleotides.

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

Bacterial numbers were significantly higher in the system receiving effluent water from the abalone raceways when compared with the *Ulva* tanks receiving seawater that was fertilized (Fig. 8). The bacterial abundance of the *Ulva* cultured in the effluent system was also higher, on all three selective media types, than the *Ulva* cultured in the fertilized system (Fig 9). In both water 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 (150 x 10^3 to 37 x 10^3 cells. mL^{-1} and 6000 x 10^3 to 392 x 10^3 cells. mL^{-1} for the FSW and AEW systems, respectively; Fig. 8). The reduction in *Vibrio* species between the inlet and outlet water samples tended to be larger in the effluent water system than the fertilised seawater system. In contrast, there was no significant decline in the number of general marine bacteria between the inlets and outlets in both systems; as indicated by growth on TSA. Moreover, the bacterial species cultivated on TSA appear to be dominant when compared to both TCBS and Ulvan in the inlet and outlet water samples of the fertilised water systems. Interestingly, the Ulvan specific bacteria have a higher abundance than the TSA abundance in the incoming effluent water, after which the *Ulvan* specific abundance decreases significantly between the inlet and the outlet for the effluent water systems (1,565,168 x 10^3 to 390,000 x 10^3 cells. mL^{-1}; Fig. 8).

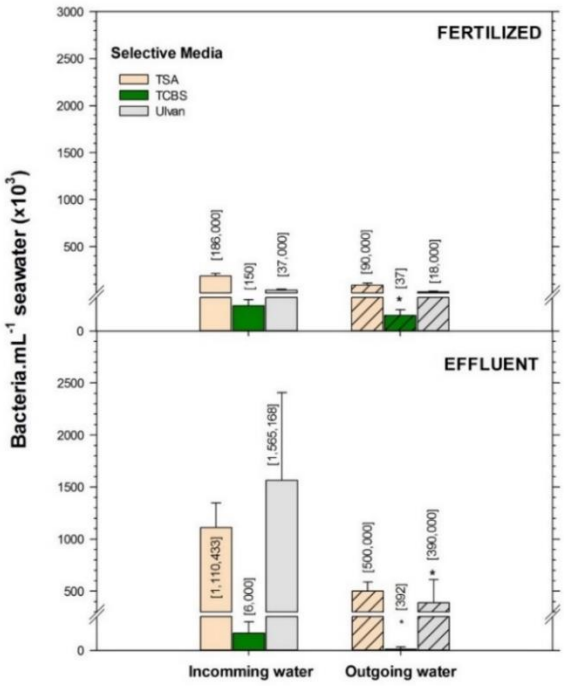

Figure 8. Average Number of culturable bacteria in effluent and fertilized seawater systems growing on three selective media; Tryptone soy agar (TSA), Thiosulfate Citrate Bile Sucrose Agar (TCBS) and Ulvan. Significant figures are marked with an asterisks.

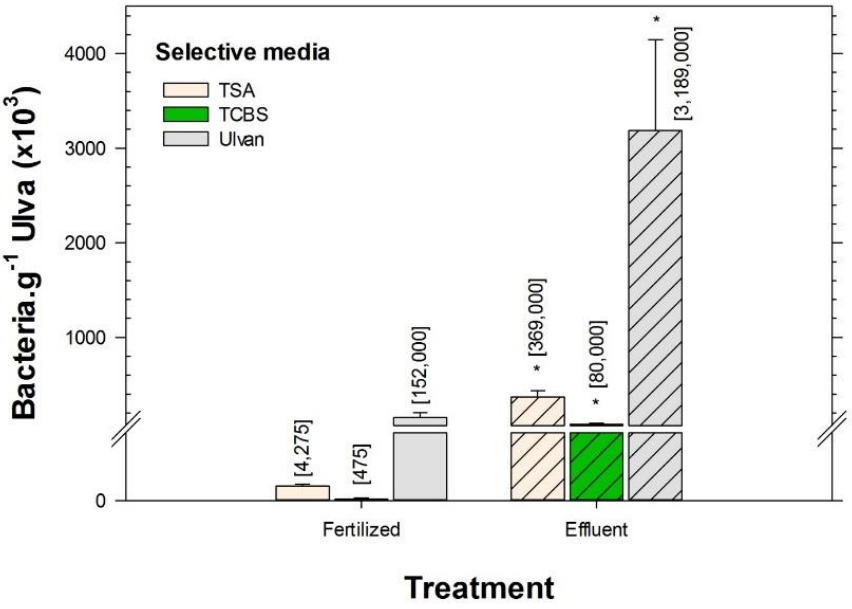

Figure 9. Average Number of culturable bacteria on *Ulva* growing on three selective media; Tryptone soy agar (TSA), Thiosulfate Citrate Bile Sucrose Agar (TCBS) and Ulvan. Significant figures are marked with an asterisks.

3.3 Identification of culturable bacteria

Of the 11 bacterial isolates evaluated in this study, 3 were isolated from Ulvan (U2, U10 and U14), 5 from TSA (T3, T5, T6, T8, and T10) and 3 from TCBS (V1, V2, and V3). The edited and assembled nucleotide sequences obtained following PCR amplification of the 16S rRNA gene region of each of the isolated bacteria of interest resulted in sequences of approximately 1,500bp. A BLAST search of the GENBANK database revealed that most of the sequences showed high similarity to organisms that are frequently isolated and identified from the marine environment including several *Vibrio* and *Pseudoalteromonas* species. Furthermore, some of the isolated species, such as *Pseudoalteromonas*, *Roseobacter* and *Agrivorans*, have previously been isolated and identified from seaweeds, including *Ulva*, with some shown to be important in development and morphogenesis (Table 2).

Table 2. The top three sequence similarities as obtained from the BLAST search of the GENBANK database with the ~460 bp 16S rRNA gene sequence of the 11 bacterial isolates. Sample sources are indicated, whereby; FSW In = fertilised seawater inlet, FSW Out = fertilised seawater outlet, EW In = effluent water inlet

16S rRNA gene sequences			
Sample ID (Source)	Closest 3 matches	% Sequence similarity	GENBANK Accession number
U2 (FSW In)	*Celeribacter* sp. str. 70067	97	MF045112.1
	Celeribacter sp. R-52665	97	KT185135.1
	Tropicibacter napthalenivorans	97	KJ004586.1
U10 (EW In)	Uncultured bac. clone IWNB040	99	FR744587.1
	Marine bac. IVA013	99	KJ814253.1
	Roseobacter sp. pp_D2A 2	99	KC250895.1
U14 (FSW Out)	*Modestobacter* sp. KNN46-8	99	KY510682.1
	Modestobacter sp. KNN46-3	99	KY510681.1
	Modestobacter caceresii str. KNN 45-2b	99	NR_137398.1
T3 (EW In)	*Agrivorans* sp. hydD622	99	KM203871.1
	Agrivorans sp. VibC-Oc-061	99	KF577090.1
	Mucus bac. 63	98	AY654801.1
T5 (EW In)	*Pseudoalteromonas* sp. str. 70375	99	MF061293.1
	Pseudoalteromonas sp. str. 70325	99	MF061291.1
	Pseudoalteromonas sp. str. 70320	99	MF061290.1
T6 (EW In)	*Vibrio splendidus* str. HZN-26	99	KT023540.1
	Vibrio cyclitrophycus str. HQN-48	99	KT023534.1
	Vibrio cyclitrophycus str. HHN-28	99	KT023526.1
T8 (EW In)	Uncultured bac. AND_GV0309	100	JQ032521.1
	Halamonas ventusa str. NBRC101992	100	AB681651.1
	Halamonas sp. MSSRF DN61	99	KU131286.1
T10 (EW In)	*Vibrio splendidus* str. CHN-30	100	KR347199.1
	Vibrio atlanticus str. HZN-50	100	KR270149.1
	Vibrio cyclitrophycus str. HZN-43	100	KR270142.1
V1 (FSW In)	*Vibrio cyclitrophycus* str. Mj149	99	GQ454943.1
	Vibrio cyclitrophycus str. P6-1-1a	99	KY382786.1
	Vibrio splendidis str. 5Zx	99	KX108987.1
V2 (FSW In)	*Vibrio splendidus* str. HQN-37	100	KR270317.1
	Vibrio cyclitrophycus str. HQN-3	100	KR270285.1
	Vibrio cyclitrophycus str. FA97	100	JQ083317.1
V3 (FSW In)	*Rhodobacteriaceae* sp.	96	AM990859.1
	Celeribacter sp. R-52665	96	KT185135.1
	Celeribacter sp. R-52661	96	KT185131.1

3.4 Bacterial community profiling by 16S rRNA Illumina MiSeq sequencing

3.4.1 Overview of OTU sequence clustering and richness estimation

Microbial diversity within the abalone effluent water, fertilised seawater and on the *Ulva* grown in both systems was assessed via NextGen sequencing of the V3-V4 region of the 16S rRNA gene using the Illumina MiSeq platform. A total of 1,488,157 quality raw sequence reads were generated from the 21 samples analysed from seawater and *Ulva* collected from I&J Cape Abalone Farm, which averaged on 70864 reads per sample (Fig. 10).

Following quality assessment, chimeric trimming and other data filtering, a total of 237 low abundance features were removed based on prevalence and a total of 31 low variance features were removed based on standard deviation, resulting in the total removal of 270 features. A combined total of 707 bacterial OTUs were identified to the genus level, from the 21 samples analysed, following mapping to the SILVA database (Fig. 10). The obtained Library size was compared at genus level to check data integrity, where a large difference between maximum and minimum library size was noted (19405 – 216789 – Fig. 11).

Data type:	OTU abundance table
File format:	mothur
OTU annotation:	SILVA
OTU number:	707
OTU with ≥ 2 counts:	538
Sample number:	21
Number of experimental factors:	1
Total read counts:	1488157
Average counts per sample:	70864
Maximum counts per sample:	216694
Minimum counts per sample:	19405

Figure 10. Text summary overview of the OTU abundances over all metagenome samples.

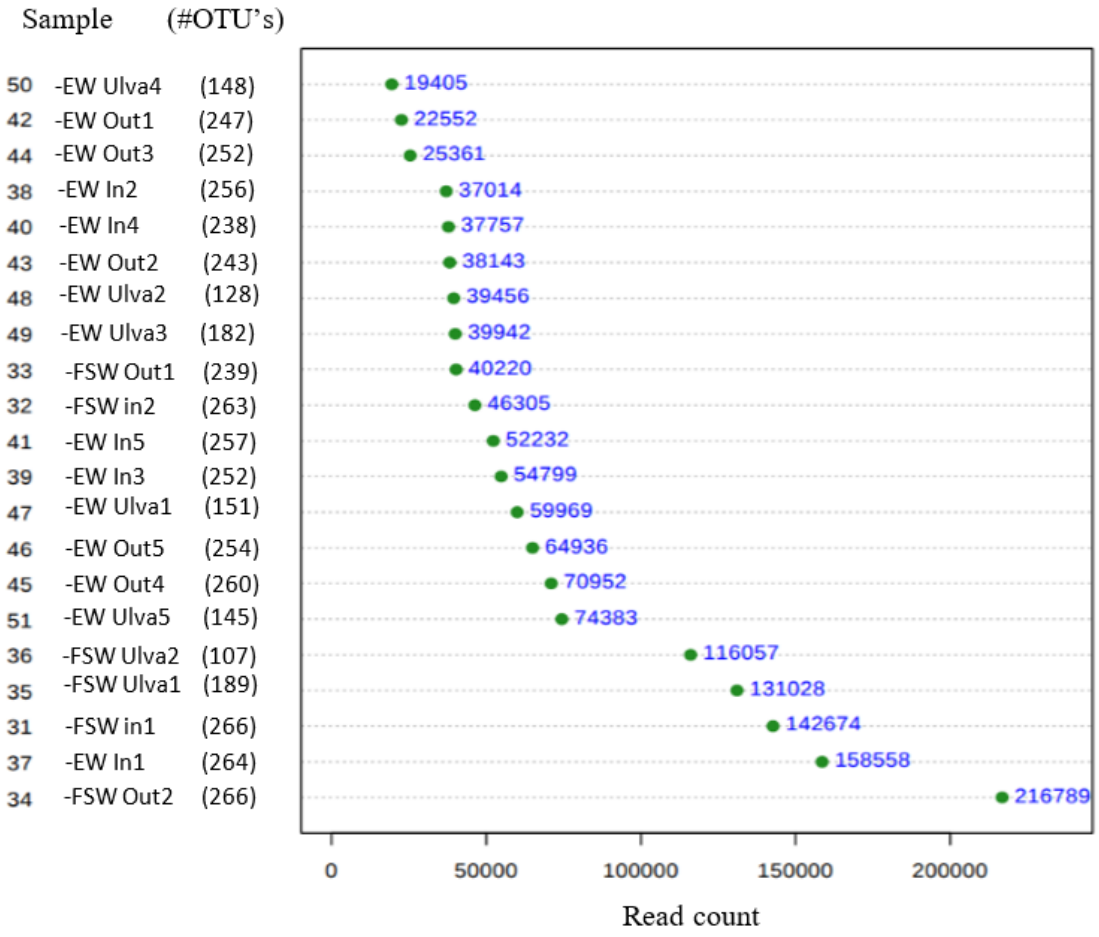

Figure 11. Library size overview based on the total number of reads for each sample at genus level. Number of OTU's is indicated in brackets.

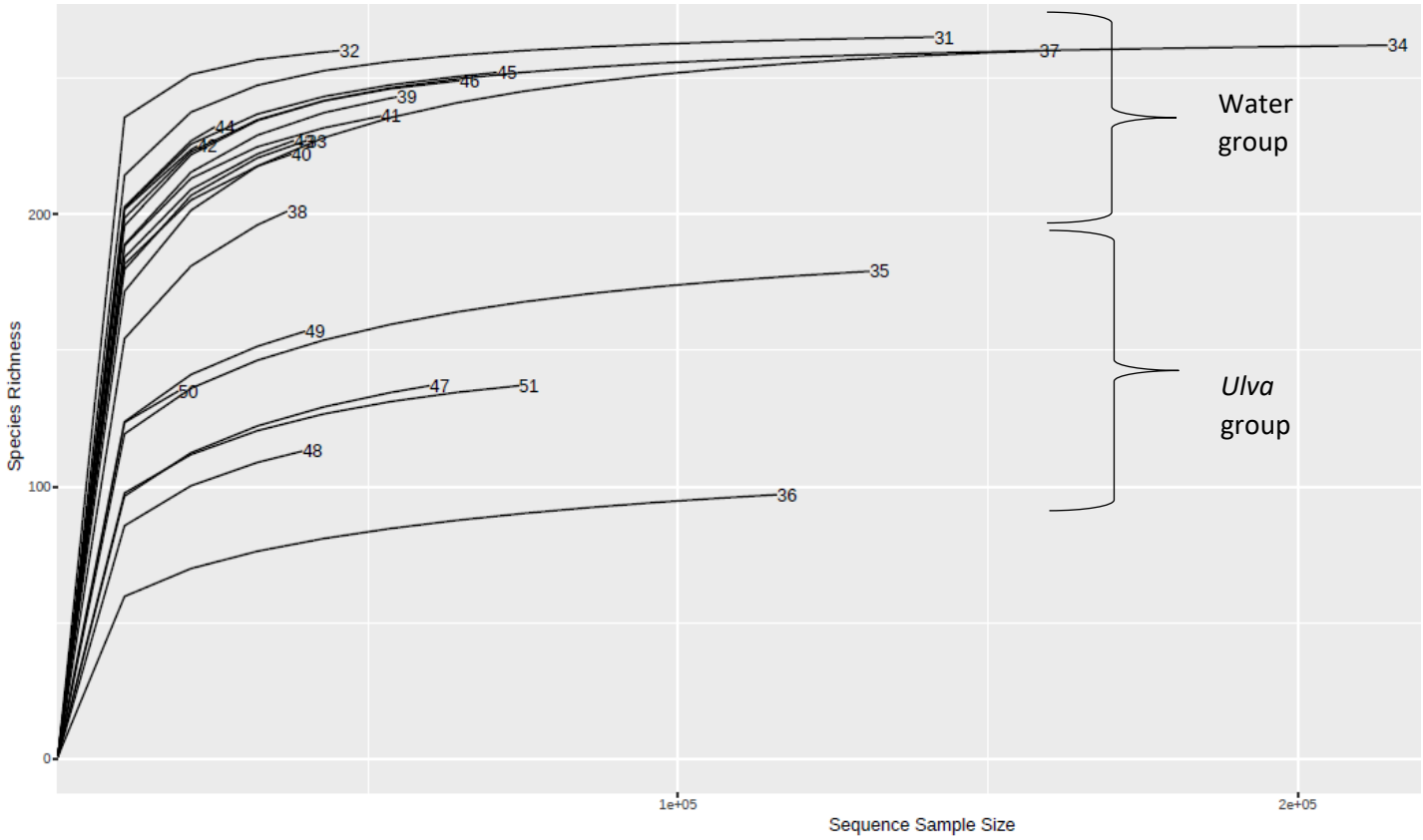

Figure 12. Rarefaction curves of filtered OTU's, whereby OTU's were grouped into an *Ulva* group and a water group. Numbers associated with each curve depict the sample number.

All rarefaction curves approached the saturation plateau, indicating that there was sufficient sequence reads within each of the 21 samples (Fig. 12). The rarefaction curve indicated a large variation in the total number of OTU's between the different samples, which ranged from 263 to 107 OTU's. The samples collected from the *Ulva* in both the fertilised seawater and abalone wastewater systems had significantly lower bacterial alpha diversity than the water samples collected. Overall, the fertilised seawater tanks (e.g. sample 31 and 32) had the highest OTU density (Fig. 12). Interestingly, two groups seem to form, i.e. a water group (e.g. sample 31-34 and 37-46) and a *Ulva* group (sample 35, 36 and 47-51). Compared with the fertilised seawater tanks (sample 31-34) and the abalone effluent raceways (sample 37-46), both the effluent *Ulva* (sample 47-51) and FSW *Ulva* (35 and 36) have lower OTU density.

Moreover, bacterial diversity did not vary significantly across the samples collected from effluent sea water, fertilised seawater and Ulvan, as no significant differences were observed for both the Shannon and the Simpson diversity indexes, with F statistics ranging from 0.97 to 2.13; indicating a high degree of similarity (Fig. 13). Conversely, the richness estimator Chao1 was significantly higher for the water samples (both FSW and AEW) than for the *Ulva* samples ($F = 22.7$, $P = 1.6 \times 10^{-6}$), indicating a high degree of unique OTUs in the water column.

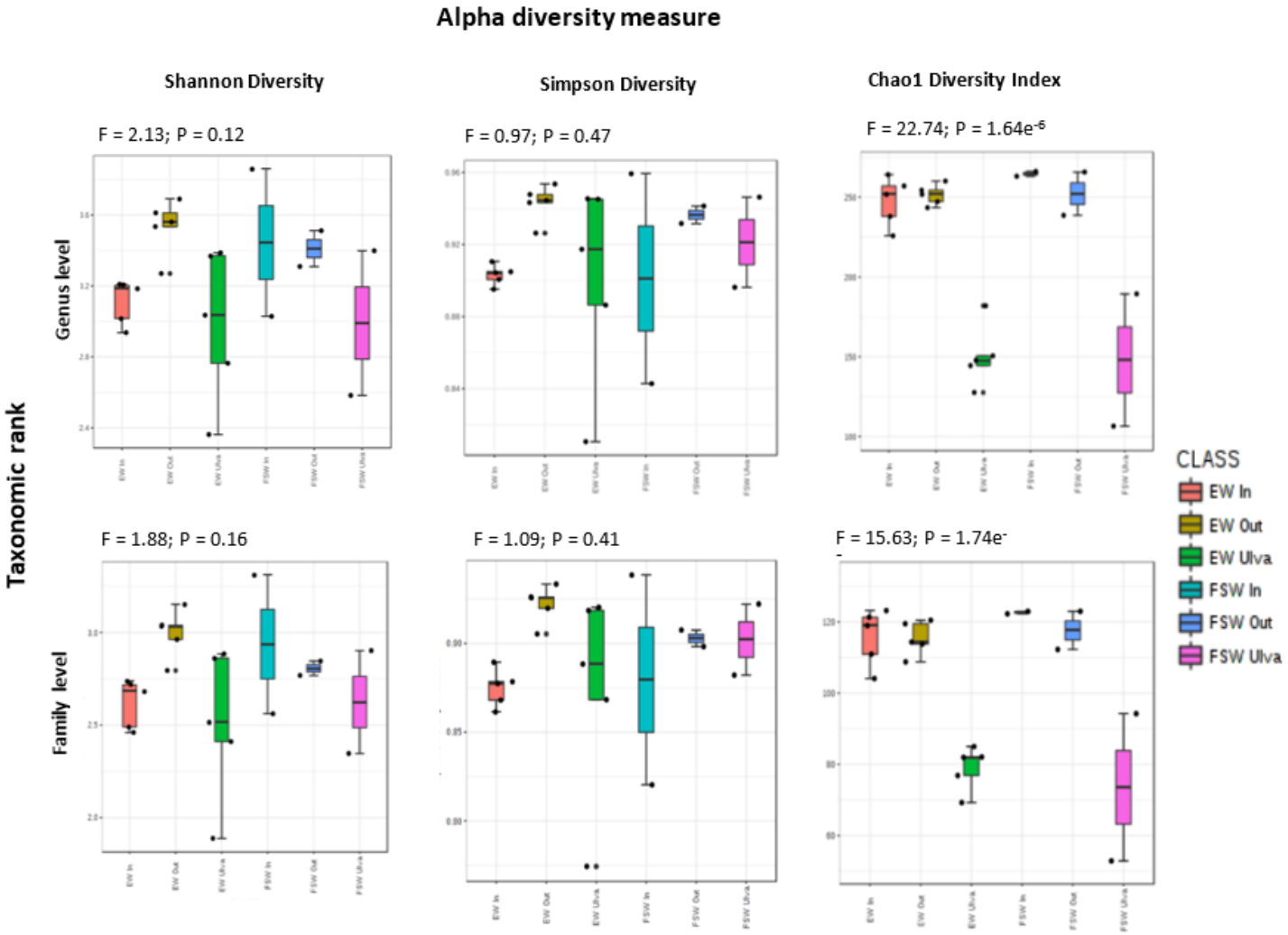

Figure 13. Average diversity measures (Chao1, Shannon and Simpson) for each cohort at family and genus level.

3.4.2 Comparison of bacterial community structure among *Ulva* and water libraries.

The resolutions of the principal co-ordinate analysis (PCoA) between phylum and genus level were significantly different (Fig. 14). At phylum level, there was partial overlap between the samples, whereas at genus level, the PCoA indicates three distinct clusters, namely EW In, EW Out, and EW *Ulva*. The overlap at phylum level indicates that the samples had similar microbial communities when assessed at a higher taxonomic level, however the clustering at genus level implies that the taxa within each sample differed at a lower taxonomic level. In addition, the PCoA conducted at genus level showed reasonable distance along the PC1 axis (with a high variation of 45.7% explained). The partial overlap between some of the samples in the PCoA at phylum level indicates that the communities in most of the locations shared similar phyla diversity. Nonetheless, the distinct boundaries present at the genus level indicate that clear differences exist between the inlet, outlet and *Ulva* within the effluent water system. An important observation is the clear separation seen between samples obtained from the *Ulva* (EW *Ulva* and FSW *Ulva*) and those obtained from the inlets and outlets of the effluent and fertilised seawater systems. The non-metric multidimensional scaling analysis (NMDS) conducted at genus level supports the separation between the *Ulva* and water sample types. The permutational multivariate analysis of variance (PERMANOVA), which tests for homogeneity across data points, further supports the high degree of dissimilarity between the samples (F-value: 11.379; R-squared: 0.791; p-value < 0.001). The plotted scores indicate a shift in the overall structure of the microbiota from the inlets to the outlets of the waterways in both the fertilised seawater tanks and abalone wastewater raceways.

In contrast to the PCoA, the NMDS analysis indicates partial overlap between the EW In and EW Out samples at genus level. The NMDS stress of 0.058461 implies a good fit, allowing confident inferences on the points. In both the PCoA and the NMDS the FSW In, FSW Out and FSW *Ulva* samples were not clustered, implying they have insufficient variation, which is likely due to their small sample size (2 samples per sample type) relative to the effluent samples (5 samples per sample type). Moreover, in the PCoA it is seen that the FSW In and FSW Out samples overlap with the EW In and EW Out cluster, whereas in the NMDS analysis, the FSW In and FSW Out samples seem to form an independent cluster, which may be bolstered given a greater sampling size. This is of significance as the lack of overlap between the bacterial communities from the fertilised seawater and abalone effluent water indicates that the farmed abalone, as well as their effluent within the raceways, has a definitive impact on the bacterial communities within the water column, despite the impact of *Ulva* and its holobiont.

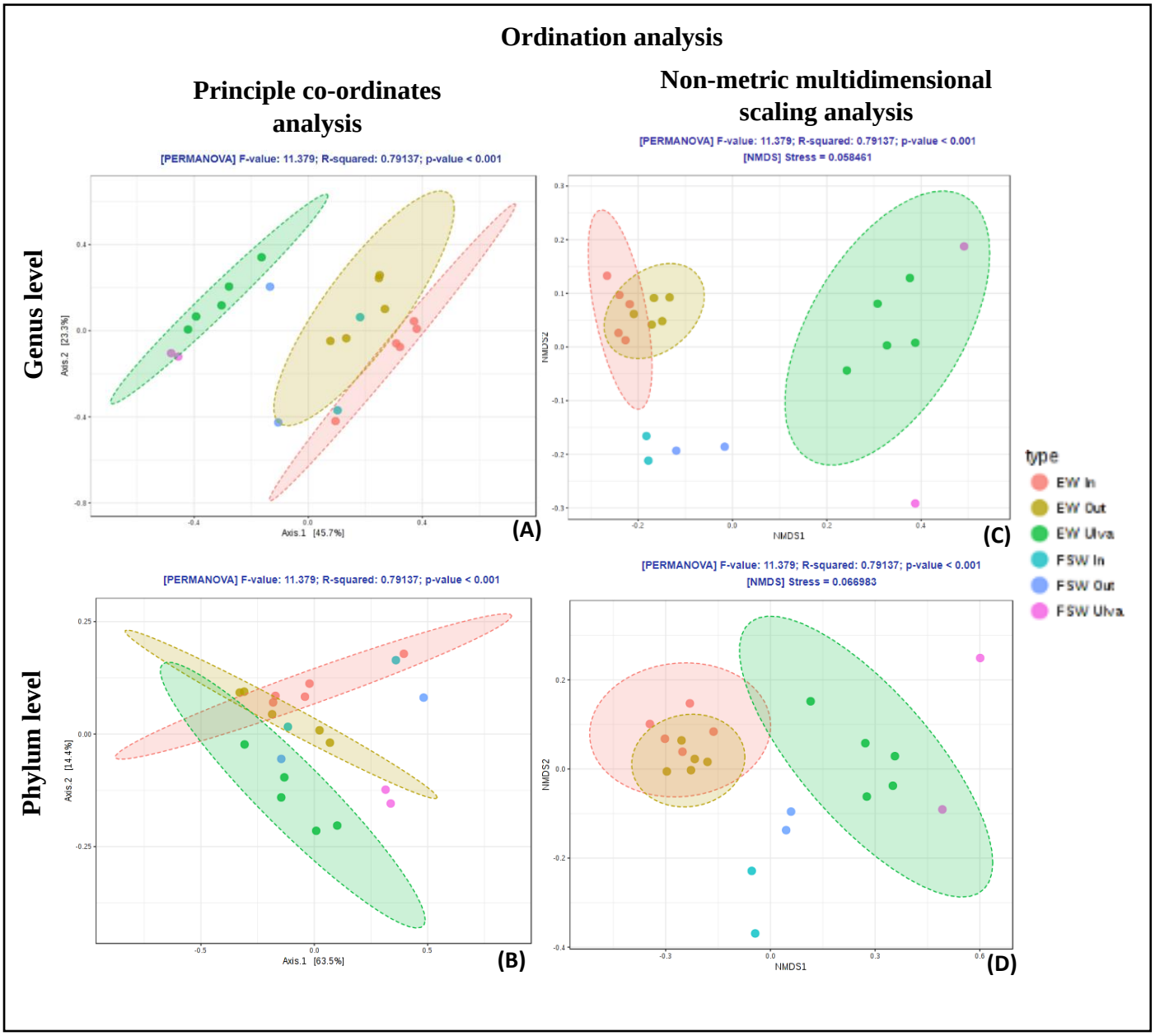

Figure 14. Principal co-ordinate analysis (PCoA; **A,B**); and Non-metric multi-dimensional scaling analysis (NMDS; **C,D**) and at phylum and genus level showing the difference in bacterial communities of the 7 *Ulva* samples and 14 water samples according to Bray-Curtis distance.

Cluster Analysis

According to the cluster analysis, the 21 samples are broadly arranged into two clades (Fig. 15). The first clade is principally composed of water samples from both the effluent and fertilised seawater systems, whereas the second clade is primarily composed of *Ulva* samples from both systems. Interestingly, the *Ulva* clade contains a single water sample, belonging to the outlet of a fertilised seawater system. In contrast, the FSW inlets and outlets seem to be more randomly distributed, which may partly be due to their small sample size. Furthermore, it appears as if several subclades exist within the organisation. For instance, the inlets and outlets of the effluent water may be subdivided into two subclades. The *Ulva* samples are also grouped as such and may form their own subclades (i.e. *Ulva* growing in fertilised seawater and *Ulva* growing in effluent water).

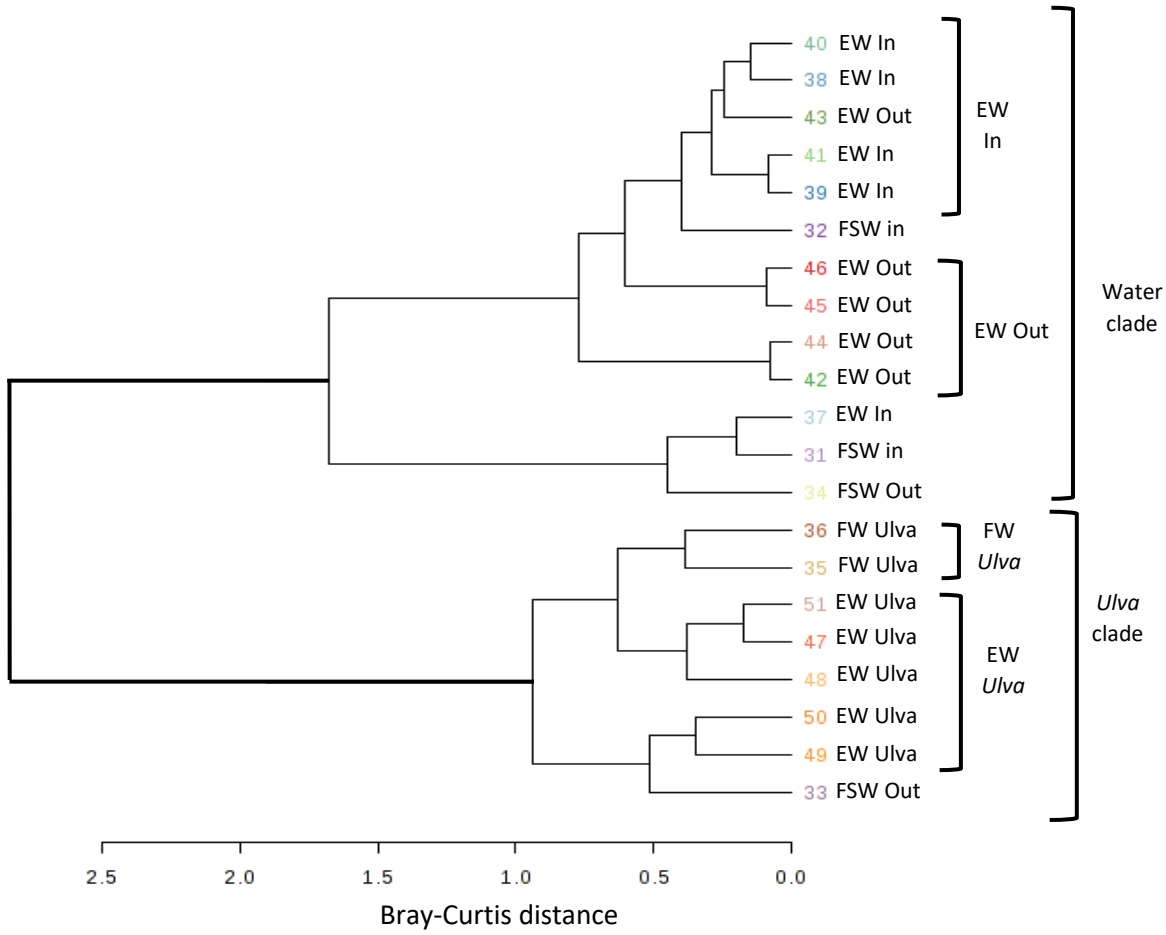

Figure 15. Cluster analysis using the Ward linkage method and Bray-Curtis distance measure, depicting the organisation of the 21 samples (31-51) taken from the effluent raceways, the fertilised seawater tanks and the *Ulva* within each of the systems at I&J Cape Abalone Farm. The samples broadly fit into two clades which have been denoted as the “Water clade” and the “*Ulva* clade”. Sample sub-clades are also indicated.

3.4.3 Comparison of bacterial community structure among sample types

Relative OTU abundance

Relative OTU abundance was used to compare bacterial community structure among sample types. The microbial community comprised 17 bacterial phyla, divided into 34 classes. Sequences that could not be classified into known groups were assigned as unclassified. Overall, when looking at the abundance of taxa within each sample, at the genus level, we can see there is a moderate degree of diversity, with some distinct differences between samples, especially for less abundant bacterial genera (Fig. 16). This is particularly evident for the seawater and *Ulva* samples, including the effluent water inlet & outlet versus the effluent water Ulva; as well as the FSW inlet & outlet versus the FSW *Ulva* samples. The four most prevalent OTU's at genus level in each sample consisted of bacteria which seemed to have a prevalence in either the seawater systems or on the *Ulva* itself (Table 3). For instance; *Pseudoalteromonas* (10-27%)*, Psychromonas* (6-13%)*, Psychrilyobacter* (7-9%)*,* various *Vibrio* sp. (6-8%) and *Polaribacter* (5-8%) are more prevalent in the water systems*,* whereas *Rhodobacteriaceae* (8%)*,* and *Granulosicoccus* (6%) are more prevalent on *Ulva.* General taxa, such as *Gammaproteobacteria* (7-22%), *Cocleimonas* (14-16%)*, Bacteriodetes* (11-17%) and *Hellea* (8%) are present in high abundance in both the seawater as well as on the *Ulva*. The *Gammaproteobacteria* (10-38%)*, Bacteriodetes* (8-17%) and *Saprospiraceae* (11-19%) appear to be the dominant taxa on the *Ulva* grown in both fertilised and effluent systems, whereas *Cocleimonas* and *Hellea* were more prevalent in the *Ulva* that had been grown in fertilised seawater. Interestingly, the *Polaribacter* only occurred in high abundance on the effluent grown *Ulva* sample (6%) and in the Abalone wastewater outlet sample (5%).

It is noted that bacterial abundance shifts occur in both the effluent and the fertilised seawater systems from their inlets to their outlets. The fertilised system tends to adopt some of the features present on the *Ulva.* For instance, the abundance of *Cocleimonas* and *Hellea* is higher in the FSW Outlet than the Inlet, implying that the presence of *Ulva* within the water system can induce a consortium shift as the dominant taxa observed in the Outlet water are prevalent in high abundance on the *Ulva.* The average abundance of *Pseudoalteromonas* and *Vibrio* decreased significantly between the inlet and the outlet in the effluent water system from 24.8% to 12.6% and from 6% to 3.9%. The *Psychromonas* and *Psychrilyobacter* also tend to decrease*.* In contrast, the average abundance of the *Gammaproteobacteriaceae* increased significantly (6% to 11%) from the Inlets to the Outlets within the effluent raceways. Interesting, the majority of the bacterial genera isolated and sequenced in the culture-based technique were in low abundance in the Illumina based assessment. The *Celeribacter* (U2), *Roseobacter* (U10), *Modestobacter* (U14), *Agrivorans* (T3) and *Halomonas* (T8) were present in low abundance, whereas the Pseudoalteromonas (T5) and *Vibrio* (T6, T10, V1 and V2) were present in high abundance.

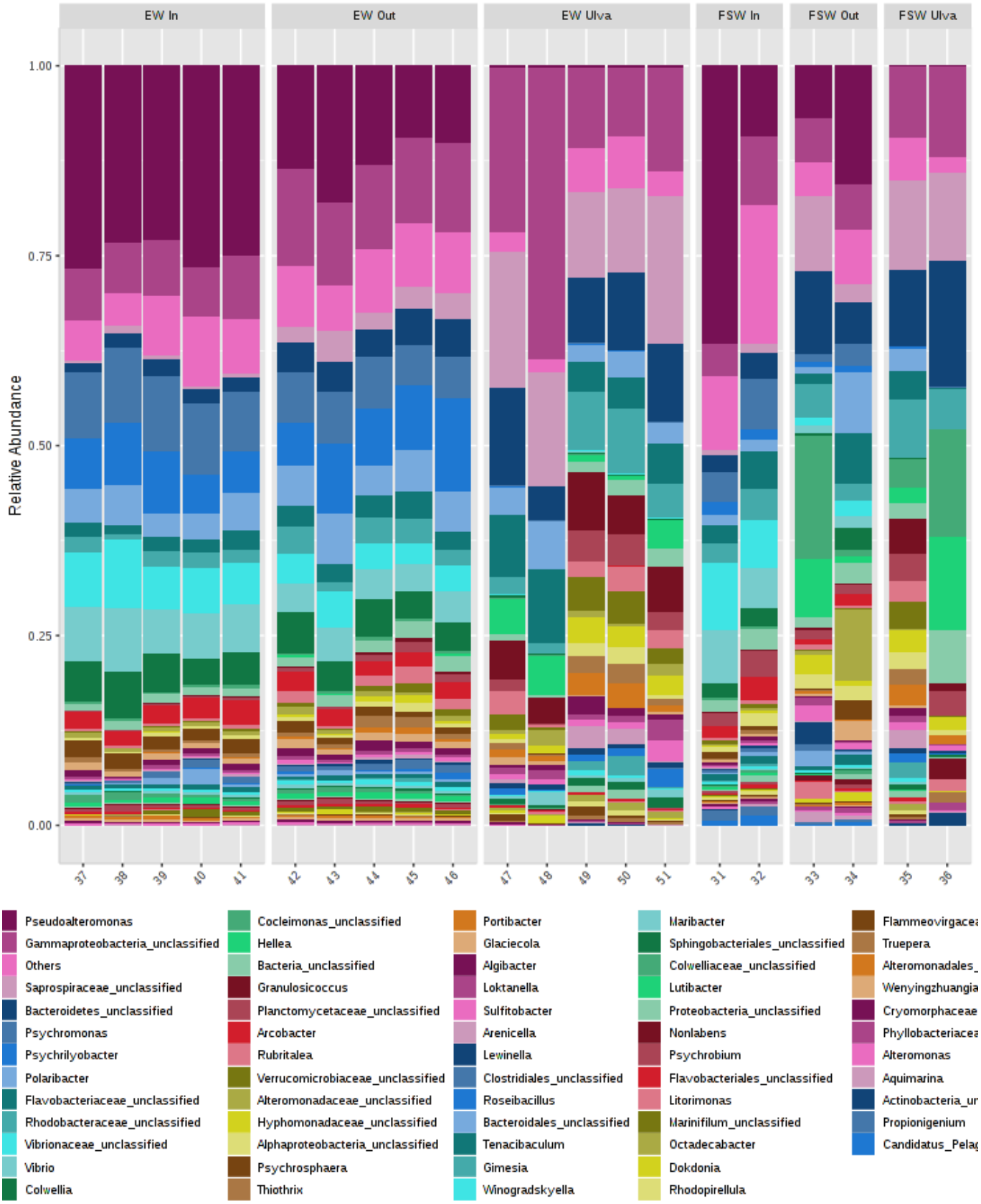

Figure 16. Relative OTU abundance (percentage abundance) across 21 samples at genus level. Less prominent OTU's (counts < 2500) were merged and denoted as "Others".

Table 3. Top four most prevalent OTU's at family and genus level, with percentage abundance indicated in brackets.

Classification	Source		Most prevalent OTUs			
Family	FSV in	Tank 1	Pseudoalteromonas (37%)	Vibrionaceae (16%)	Flavobacteriaceae (7%)	Gammaproteobacteria uncl. (4%
		Tank 2	Vibrionaceae (12%)	Flavobacteriaceae (11%)	Pseudoalteromonas (10%)	Gammaproteobacteria uncl. (9%
	FSV Out	Tank 1	Cocleimonas (16%)	Saprospiraceae (13%)	Hyphomonadaceae (13%)	Bacteriodetes uncl. (11%)
		Tank 2	Flavobacteriaceae (20%)	Pseudoalteromonas (18%)	Alteromonadaceae (12%)	Gammaproteobacteria uncl. (6%
	FSV Ulva	Tank 1	Saprospiraceae (15%)	Rhodobacteriaceae (11%)	Bacteriodetes uncl. (10%)	Gammaproteobacteria uncl. (9%
		Tank 2	Bacteriodetes uncl. (17%)	Hyphomonadaceae (16%)	Cocleimonas (14%)	Saprospiraceae (13%)
	AVV In	Raceway 1	Pseudoalteromonadaceae (29%)	Vibrionaceae (15%)	Flavobacteriaceae (10%)	Psychromonadaceae (9%)
		Raceway 2	Pseudoalteromonadaceae (25%)	Vibrionaceae (18%)	Psychromonadaceae (10%)	Flavobacteriaceae (9%)
		Raceway 3	Pseudoalteromonadaceae (25%)	Vibrionaceae (12%)	Psychromonadaceae (10%)	Flavobacteriaceae (9%)
		Raceway 4	Pseudoalteromonadaceae (28%)	Vibrionaceae (12%)	Psychromonadaceae (9%)	Flavobacteriaceae (9%)
		Raceway 5	Pseudoalteromonadaceae (27%)	Vibrionaceae (12%)	Flavobacteriaceae (12%)	Gammaproteobacteria uncl. (8%
	AVV Out	Raceway 1	Pseudoalteromonadaceae (15%)	Flavobacteriaceae (13%)	Gammaproteobacteria uncl. (13%	Vibrionaceae (8%)
		Raceway 2	Pseudoalteromonadaceae (19%)	Flavobacteriaceae (13%)	Gammaproteobacteria uncl. (11%	Vibrionaceae (9%)
		Raceway 3	Pseudoalteromonadaceae (14%)	Flavobacteriaceae (12%)	Gammaproteobacteria uncl. (11%	Fusobacteriacaea (8%)
		Raceway 4	Flavobacteriaceae (13%)	Gammaproteobacteria uncl. (11%)	Pseudoalteromonadaceae (10%	Fusobacteriacaea (9%)
		Raceway 5	Fusobacteriacaea (12%)	Flavobacteriaceae (12%)	Gammaproteobacteria uncl. (12%	Pseudoalteromonadaceae (11%)
	AVV Ulva	Raceway 1	Gammaproteobacteria uncl. (22%	Saprospiraceae (20%)	Flavobacteriaceae (13%)	Bacteriodetes uncl. (13%)
		Raceway 2	Gammaproteobacteria uncl. (38%	Flavobacteriaceae (20%)	Saprospiraceae (17%)	Hyphomonadaceae (7%)
		Raceway 3	Saprospiraceae (15%)	Rhodobacteraceae (11%)	Gammaproteobacteria uncl. (10%	Flavobacteriaceae (10%)
		Raceway 4	Saprospiraceae (15%)	Rhodobacteraceae (12%)	Bacteriodetes uncl. (10%)	Flavobacteriaceae (10%)
		Raceway 5	Saprospiraceae (21%)	Gammaproteobacteria uncl. (14%)	Rhodobacteraceae (12%)	Bacteriodetes uncl. (10%)
Genus	FSV in	Tank 1	Pseudoalteromonas (37%)	Vibrionaceae uncl. (9%)	Vibrio (7%)	Gammaproteobacteria uncl. (4%
		Tank 2	Pseudoalteromonas (9%)	Gammaproteobacteria uncl. (9%)	Psychromonas (7%)	Vibrionaceae uncl. (6%)
	FSV Out	Tank 1	Cocleimonas uncl. (16%)	Bacteriodetes uncl. (11%)	Saprospiraceae uncl. (10%)	Hellea (8%)
		Tank 2	Pseudoalteromonas (16%)	Alteromonadaceae uncl. (9%)	Polaribacter (8%)	Flavobacteriaceae uncl. (7%)
	FSV Ulva	Tank 1	Saprospiraceae uncl. (12%)	Bacteriodetes uncl. (12%)	Gammaproteobacteria uncl. (10%	Rhodobacteriaceae (8%)
		Tank 2	Bacteriodetes uncl. (17%)	Cocleimonas uncl. (14%)	Hellea (12%)	Gammaproteobacteria uncl. (12%
	AVV In	Raceway 1	Pseudoalteromonas (27%)	Psychromonas (13%)	Vibrio (7%)	Vibrionaceae uncl. (7%)
		Raceway 2	Pseudoalteromonas (23%)	Psychromonas (10%)	Vibrionaceae uncl. (9%)	Vibrio (8%)
		Raceway 3	Pseudoalteromonas (23%)	Psychromonas (10%)	Psychrilyobacter (8%)	Gammaproteobacteria uncl. (7%
		Raceway 4	Pseudoalteromonas (26%)	Psychromonas (9%)	Gammaproteobacteria uncl. (6%	Vibrio (6%)
		Raceway 5	Pseudoalteromonas (25%)	Gammaproteobacteria uncl. (8%)	Psychromonas (8%)	Vibrio (6%)
	AVV Out	Raceway 1	Pseudoalteromonas (13%)	Gammaproteobacteria uncl. (13%)	Psychromonas (7%)	Psychrilyobacter (6%)
		Raceway 2	Pseudoalteromonas (18%)	Gammaproteobacteria uncl. (11%)	Psychrilyobacter (9%)	Psychromonas (7%)
		Raceway 3	Pseudoalteromonas (13%)	Gammaproteobacteria uncl. (11%)	Psychrilyobacter (8%)	Psychromonas (7%)
		Raceway 4	Gammaproteobacteria uncl. (11%)	Pseudoalteromonas (9%)	Psychrilyobacter (9%)	Polaribacter (5%)
		Raceway 5	Psychrilyobacter (12%)	Gammaproteobacteria uncl. (12%)	Pseudoalteromonas (10%)	Psychromonas (6%)
	AVV Ulva	Raceway 1	Gammaproteobacteria uncl. (22%	Saprospiraceae uncl. (18%)	Bacteriodetes uncl. (13%)	Flavobacteriaceae uncl. (8%)
		Raceway 2	Gammaproteobacteria uncl. (38%	Saprospiraceae uncl. (15%)	Flavobacteriaceae uncl. (10%)	Polaribacter (6%)
		Raceway 3	Saprospiraceae uncl. (11%)	Gammaproteobacteria uncl. (10%)	Bacteriodetes uncl. (8%)	Rhodobacteriaceae (8%)
		Raceway 4	Saprospiraceae uncl. (11%)	Bacteriodetes uncl. (10%)	Gammaproteobacteria uncl. (9%	Rhodobacteriaceae (9%)
		Raceway 5	Saprospiraceae uncl. (19%)	Gammaproteobacteria uncl. (14%)	Bacteriodetes uncl. (10%)	Granulosicoccus (6%)

Linear discriminate analysis effect size (LEfSe) analysis

The LEfSe of bacterial core OTUs revealed that some of the identified OTU's were more abundant in certain groups compared to others (Fig. 17). The LEfSe analysis also incorporated and addressed low abundance samples that were grouped into the "Others" category in the differential abundance. According to the LEfSe analysis, 110 OTUs were identified that can discriminate between the 133 significant differentially abundant OTU's which were observed at genus level. The LEfSe analysis revealed that the fertilized seawater inlet (FSW In) had the largest number of differentially abundant OTUs associated with it at genus level, followed by the FSW *Ulva* and the FSW outlet. Conversely, the effluent water seemed to have less differentially abundant bacteria. The inlet samples of the fertilised seawater tanks had the greatest proportion of differentially abundant bacteria, which predominantly belonging to genera common in the marine environment, such as *Marinomonas, Lutimonas, Psychrobacter, Oceanospirillales, Paramoritella, Photobacterium, Amphritea* and *Planktomarina.* The FSW inlet samples additionally include differentially abundant OTU's corresponding to the genera *Vibrionaceae* and *Propionigenium,* as well as the sulfate reducing genera, *Desulfovibrio*, and the sulfur-oxidising genera, *Thioalkalispiraceae* uncl. Alternatively, the genera *Spongiispira, Oleispira* and *Kordia* were most commonly associated with samples collected from the fertilised seawater outlet. For the effluent water inlet, the sulfate reducing bacteria *Desulforhopalus* was most frequently observed, whereas facultative anaerobes such as *Draconibacterium* and the green sulfate reducing bacteria *Desulfobacter* was frequently observed in the effluent water outlets. The four differential genera most likely to explain the uniqueness of the *Ulva* obtained from the fertilised seawater system include the common marine bacteria *Saprospiraceae* and *Portibacter*), as well as algal specific bacteria (*Granulosicocus* and *Hellea*), whereas the differences between the *Ulva* samples belonging to the effluent system are most likely explained by the sulfur-oxidising genera *Thiohalophilus*. Unclassified taxa within the *Vibrio* clade appear to be less present on the *Ulva* and are more prominent in the seawater, especially in the incoming water systems.

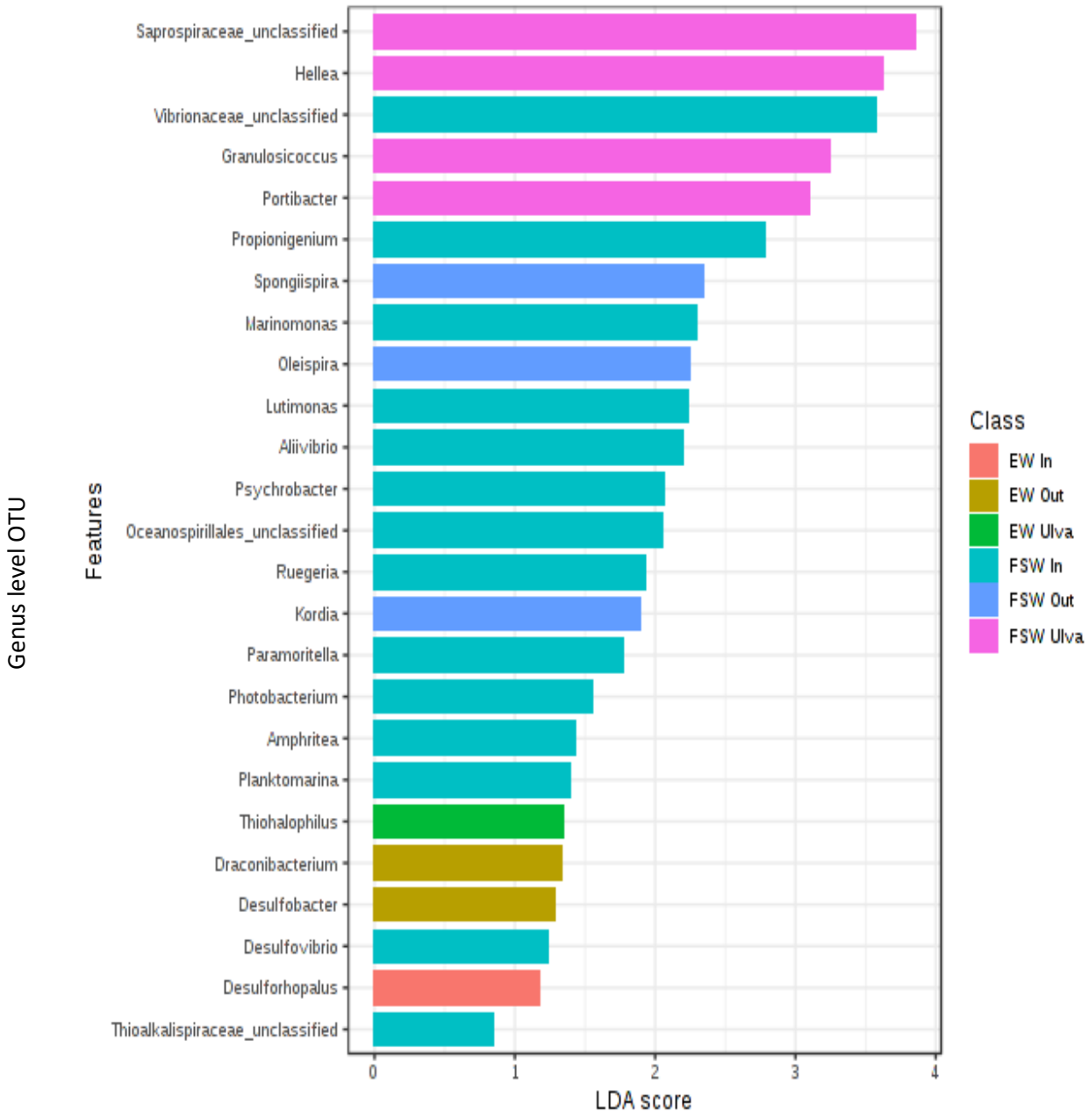

Figure 17. Linear discriminate analysis effect size (LEfSe) of bacterial OTUs, with the 25 OTUs most likely to explain differences between classes (effluent water inlet, effluent water outlet, effluent water *Ulva*, fertilised seawater Inlet, fertilised seawater outlet and fertilised *Ulva*) at genus level. Different colours represent different classes. The LDA score represents the relevance or effect size of differential abundant features.

Differential abundance (Univariate statistical comparisons)

In totality, 133 significant differentially abundant OTU's were observed at genus level, 66 were found at family level and 36 at the order level that passed the OTU ($P<0.05$) and false discovery rate ($FDR<0.04$) cut-off thresholds. The top 25 most significant taxa at genus level, along with the *Vibrio*, are displayed (Fig.18). The results show that OTUs assigned to the genera*, Amphritea, Desulfobacter, Oleispira Photobacterium, Marinomas, Draconibacterium,* and *Ruegeria* have an increased relative abundance in samples from the effluent and fertilised seawater, while OTUs assigned to *Hellea, Saprospiraceae, Granulosicoccus* and *Thiohalophilus* have an increased relative abundance on the *Ulva.* Conversely, OTUs assigned to *Amphritea, Marinomas, Desulforhopalus, Propionigenium, Vibrionaceae, Aliivibrio, Desulfovibrio* and *Ruegeria* decrease in abundance and are not dominant on the *Ulva.* Moreover, OTUs assigned to *Desulfobacter, Desulforhopalus* and *Draconibacterium* have an increased relative abundance in samples from effluent water, while OTUs assigned to *Marinomas, Oleispira, Propionigenium, Kordia, Planktomarina, Psychrobacter, Photobacterium, Spongiispira, Paramoritella* and *Oceanospirillales* have an increased relative abundance in the fertilised seawater systems. OTUs assigned to unclassified *Vibrionaceae* and *Vibrio* have an increased relative abundance in samples from the effluent and fertilised seawater relative to the *Ulva.*

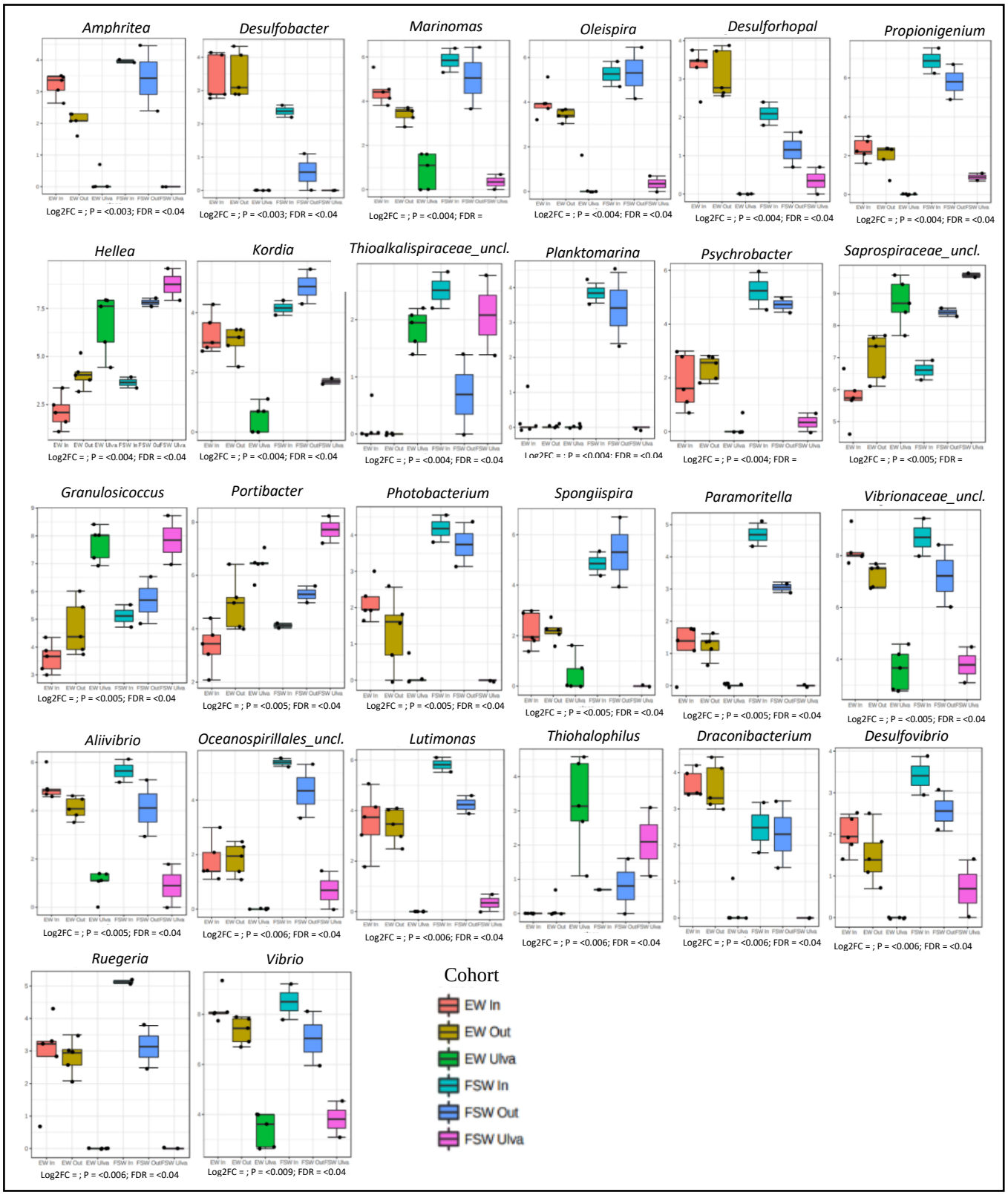

Figure 18. Differentially abundant OTU's at genus level, where the P-value and false discovery rate (FDR) are indicted. Each box corresponds to the distribution of one of 26 differentially abundant bacteria across the cohort. Cohorts from left to right are as follows: effluent water in, effluent water out, effluent *Ulva*, fertilised seawater in, fertilised seawater out, fertilised *Ulva*.

SECTION 4. DISCUSSION

4.1 Molecular analysis of Ulva

Post *rbcL* gene amplification and editing, a total length of 867bp that was used for construction of the maximum likelihood (ML) tree, covering approximately 60% of the *rbcL* gene, and should be sufficient for inferring broad classifications (Fig. 7). The ML tree constructed for the *rbcL* gene indicated that the *Ulva* sample clustered within the *U. rigida* clade. Loughnane et al. (2008) noted seven lineages which he considered to represent the following species*; Ulva rigida, U. scandinavica, U. lactuca, U. gigantea, U. rotundata, U. californica* and *Umbraulva olivascens.* Authors have suggested that *U. scandinavica, U. armoricana* and *U. rigida* are taxonomically synonymous (Bliding, 1968; Kirkendale, et al., 2013), and that *U. rigida* should take nomenclature priority as it is the oldest name. The *Ulva* sampled from I&J concurs with this statement as its two closest matches on the GenBank database are *Ulva rigida* (KP233772) and *Ulva scandinavica* (EU484416) and is henceforth referred to with the name of its corresponding molecular synonym, i.e. *U. rigida.* Moreover, the *Ulva* obtained from I&J was confirmed to be *U.* rigida (Bachoo, 2018), who utilised both the *rbcL* and ITS genes for genetic analyses, which further correlates with Robertson-Anderson's (2003) remark that *U. rigida* was originally used to stock the tanks at I&J. Interestingly, the *Ulva* clustered closely to *U. clathrata* (AY422563) (Hayden & Waaland, 2004), which appears beneficial in impacting disease management (including several *Vibrio* sp.) and metabolic turnover rate of the Pacific white shrimp *Litopenaeus vannamei* (da Silva Copertino, et al., 2009; Gamboa-Delgado, et al., 2011).

The rbcL plastid and 18S rDNA subunit have been widely used as established DNA barcodes for several plant groups and have formed the basis of resolving numerous phylogenetic and taxonomic matters (Hayden & Waaland, 2002; Hayden, et al., 2003; Lewis & McCourt, 2004; Group, et al., 2009; O'kelly, Mahendran 2014; Mahendran & Saravanan, 2017). Despite this, uncertainty remains surrounding the use of the rbcL gene due to the presence and variability of introns which complicate the ability to amplify and sequence reads due to the large fragment sizes processed within a single bidirectional read (Hanyuda, et al., 2000). Moreover, green algae are prone to acquiring intron sequences, of which their abundance within the rbcL gene is poorly understood and requires further evaluation (Hanyuda, et al., 2000), which has been known to negatively affect the universality of rbcL barcode markers for green marine macroalgae classification (Hayden & Waaland, 2004). More recently, studies have indicated that the *tufA* gene has a higher resolving power at species level in comparison to both the *rbcL* and ITS genes (Saunders & Kucera, 2010), and has successfully been used for *Ulva* phylogenetics in Australia (Kirkendale, et al., 2013; Lawton, et al., 2013) and in the United States of America (Mao, et al., 2014). Hence,

the *tufA* gene may prove useful for future investigations regarding structuring within the inner clades of *Ulva'*s phylogeny.

4.2 Enumeration of culturable bacteria in seawater and on Ulva

Marine surfaces are a host to a suit of microbial communities, several of which have been shown to impact the health and normal function of the host both positively and negatively (Nakanishi, et al., 1996; Marshall, et al., 2006; Rosenberg, et al., 2007). Increased evidence suggests that microbial consortia are specific to their host (Taylor, et al., 2004; Taylor, et al., 2005; Reis, et al., 2009), and numerous studies, dating back to the 1970s, demonstrate host specific differences between microbial communities present on macroalgae and in the surrounding seawater, between alga of different species and even on a single species from different locations (Bolinches, 1988; Tujula, 2006). Host specificity may be defined as the occurrence of a set of given bacterial communities on an algal host, which are unique to the host and absent (or of low abundance) on other algal hosts (Egan, et al., 2012). Evidence provided by 16S rRNA gene sequencing and denaturing gradient gel electrophoresis (DGGE) fingerprinting have emphasised the uniqueness of bacterial communities inhabiting algae, with respect to the surrounding seawater (Staufenberger, et al., 2008) and other biotic surfaces (Lachnit, et al., 2009). Rusche et al., (2007) noted on their global ocean sampling expedition that different niches (tropical, marine and non-marine environments) had distinguishable bacterial ribotypes, and identified the *Alpha* and *Gamma Proteobacteria*, *Bacteroidetes, Actinobacteria, Acidimicrobidae* and *Plantomycetes* as widespread bacteria. Since then, it has been observed that bacterial communities may vary temporally, seasonally as well as from species to species (Singh & Reddy, 2014).

The significantly higher numbers of bacteria growing on the *Ulva* (Fig. 8) and in the water column, within the effluent water system compared to the fertilised seawater tanks (Fig. 9) is not surprising, given the higher nutrient load within the effluent system (Robertson-Andersson, 2003; Probyn, et al., 2016), as bacteria are known to proliferate in nutrient rich environments (Matsushita & Fujikawa, 1990; Walker, 2000). The increase in observed abundance within the effluent raceways coincide with results published by Cho & Kim (2000) which display an increase in both bacterial diversity and abundance in subsurface aquifers receiving livestock wastewater input. In both fertilised seawater tanks and abalone effluent raceways, the macroalga *Ulva* had a strong inhibitory effect on *Vibrio* species within the systems, as indicated by the significant decrease in bacteria growing on TCBS (*Vibrio* selective media) between the inlets and the outlets of both fertilised and effluent water systems. Moreover, the inhibitory effect seems to be nutrient dependent, with a more substantial decrease in *Vibrio* numbers observed in the effluent water system. These findings support 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 phosphorus. The decrease in *Vibrio* could be due to a host of factors including competition and inhibition, however whether it is bacterially driven (Lemos, et al., 1985; Egan, et al., 2000) or driven by the *Ulva* (Paulert, et al., 2007; Lu, et al., 2008) remains unknown. Total *Vibrio* counts on the *Ulva* were higher in the effluent system than in the fertilised seawater system, but reflect values typically found in seawater and on seaweeds in the natural environment (values range from 10^2 – 10^4 CFU.g^{-1} seaweed; Muhamud, et al., 2008). Overall, the decline in *Vibrio* between the inlets and the outlets of the *Ulva* raceways, as well as the low abundance of *Vibrio* within the raceways suggests that the *Ulva* cultivated within the nutrient rich abalone effluent waters has a beneficial influence, with respect to industry and health standards, on the biota within the water column.

The abundance of general marine bacteria cultured on TSA did not vary significantly between the inlets and the outlets of the water systems (Fig. 8). This may be a positive indicator, as there was no spike in bacterial abundance and the clear majority of these marine bacteria identified from the plates are frequently isolated from the marine environment, including *Pseudomonas, Alteromonas,* and *Vibrio* (Rengpipat, et al., 2003; Romanenko, et al., 2005; Vandecandelaere, et al., 2008). Nonetheless, one must consider that both probiotic and pathogenic marine bacteria may be cultured on TSA (Rengpipat, et al., 2003), and that their relative abundances within the system may shift. The greater abundance of marine bacteria cultivated on TSA within the water column, relative to Ulvan, is likely due to the host specific niche provided by the *Ulva,* as the *Ulva,* and its associated constituents, may be associated with their own unique holobiont, which is known to possess inhibitory properties (Egan, et al., 2000; Rao, et al., 2007; Tan, et al., 2012). The high number of bacteria that grew on the selective media containing Ulvan, the main carbohydrate of *Ulva* (Ray & Lahaye, 1995), suggests that there are a high number of bacteria capable of growing on *Ulva* within the water column. Nonetheless, the bacterial density on the Ulvan plates of the fertilised and effluent grown *Ulva* (152 x 10^6 and 3,189 x 10^6 bacteria.g^{-1} ; Fig. 9) is not unusual as marine macroalgal bacterial densities are known to range from 10^2 to 10^7 cells cm^{-2} depending on thallus section, season and species (Tujula., 2006; Bengtsson, et al., 2010). The tips of red seaweeds like Gracilaria, which display apical growth apices (Gargiulo, et al., 1992), are often devoid of bacteria (Weinberger, 1999), and have been shown to exhibit antimicrobial activity (Sasidharan, et al., 2009). Several species of *Ulva* are known to display antimicrobial activity against a suit of marine bacteria and fungi (Alang, et al., 2009; Kolanjinathan & Stella, 2011; Dhanya, et al., 2016). Additionally, Long *et al.* (2005) noted that certain bacteria can impede the growth of pathogenic bacteria via the production of the antibiotics such as andrimid, which he linked to the increased competitiveness of *Vibrio cholerae.* Similarly, bacteria inhabiting the *Ulva,* or the *Ulva* itself, may release antibiotics capable of inhibiting *Vibrio* sp. The production of antimicrobial compounds may protect

the *Ulva* and its holobiont from pathogens, however a deeper insight into the functionality of *Ulva's* metabolome and microbiome is required in order to disentangle the protective associations between *Ulva* and its holobiont.

In contrast, several bacteria colonising algal surfaces are known to benefit from the organic constituents produced by the alga, such as polysaccharides, which the bacteria utilise for reproduction and biofilm formation (Steinberg, et al., 2002; Lachnit, et al., 2009). These observations are supported in the current study, where we observed a higher abundance of bacteria capable of growing on *Ulva*, whereby a significant increase in bacteria capable of utilising Ulvan as its carbohydrate source was observed in both the effluent and fertilised systems respectfully (Fig. 9). Moreover, the significantly high abundance of Ulvan specific bacteria in the effluent inlet may be explained by the fact that abalone are herbivores that naturally feed on various wild seaweeds (Simpson & Cook, 1998; Naidoo, et al., 2006), hence it is logical to assume that their faeces are capable of inoculating the water system with bacteria able to degrade the polysaccharides found within their gut (Erasmus, et al., 1997). For instance, it is known that I&J uses *Ulva* as a feed (Robertson-Andersson, 2003; Bolton, et al., 2009), hence the abalone's excrement would be assumed to have a high abundance of bacteria capable of degrading sugars in *Ulva* such as Ulvan. Nonetheless, the persistence of abalone gut bacteria within the water column post excretion is yet to be tested. Erasmus et al., (1997) for instance listed several bacteria isolated from the digestive system of *H. midae* fed *Ecklonia maxima* and *Gracilaria verrucosa* that were able to degrade the polysaccharides laminarin, alginate and agarose.

The significant decrease in bacteria capable of growing on Ulvan between inlet and outlet of the effluent water systems may also be nutrient dependant, with a substantial decrease in Ulvan specific bacterial numbers in the effluent water system. Nonetheless, the decrease of Ulvan specific bacteria within the water column may indicate that the *Ulva,* it's associated microbiota, and or the bacteria within the water column may inhibit the abundance of bacteria capable of utilising Ulvan within the water column. Alternatively, more of the bacteria within the effluent water column may be colonising the *Ulva,* as it is highly possible that these bacteria may come from the abalone in the systems being fed with *Ulva,* and hence originated from *Ulva*. This seems to be supported by the data from the *Ulva* (Fig 9). Nonetheless, if this is the case, the isolated bacteria do not appear to be causing any direct biosecurity concerns, as indicated by the status of the *Ulva* cultivated within the raceways on the farm which seemed disease free at the time of sampling (pers. comm.). In 2006, Macey and Coyne tested the probiotic potential of three bacteria that had been previously isolated from the gastrointestinal tract of *H. midae and* reported a positive relationship between amylase and protease activity within the intestine of *H. midae* when fed probiotic-supplemented feed that had been inoculated with the probionts *Cryptococcus* sp. SS1 and *Vibrio midae* SY9 respectively. Macey (2006) went on to

note that the abalone should be fed probiotic-supplemented feed at least every second day for maximum benefit, as the number of probiotic cells colonising the abalone digestive tract decrease after a 2-day period. This observation is of great interest, as the abalone on the I&J abalone farm defecate into the water system, the effluent then inoculates the *Ulva,* after which the *Ulva* is fed back to the abalone. Hence, the abalone faeces may be inoculating the *Ulva* with probiotic bacteria, capable of aiding in the digestion of the *Ulva.* At present, the biotic impacts on enzyme activity within the gut of abalone fed effluent grown *Ulva* is unknown and could serve as a further indicator of the biosecurity risks/ benefits of feeding abalone effluent grown *Ulva.*

The culture-based results indicate that utilising effluent grown *Ulva* as abalone feed is not of significant concern as most bacteria within the inlet of the effluent water system seem to be more specific to the Ulvan, with low numbers of *Vibrio* detected. Moreover, the *Ulva* and its associated microbiota seem to inhibit the *Vibrio* within the water column, emphasising the antipathogenic activity of *Ulva* and its holobiont. Even so, the question remains as to what these bacteria are and how their species abundance shifts within the water systems once introduced to *Ulva* and its associated microbiota.

4.3 Identification of culturable bacteria

The BLAST search of the bacteria that were isolated on the Ulvan, U2 and U10, showed high sequence similarity to the *Celeribacter* (Ivanova, et al., 2010) which is a member of the *Roseobacter* (Table 2; Buchan, et al., 2005). Both *Celeribacter* and *Roseobacter* are members of the family *Rhodobacteraceae,* which is a major chemo-organotrophic phylogenetic assemblage found within marine surface waters (Giovannoni, 2000; Baek, et al., 2014b). Interestingly, the *Roseobacter* are known to occur as free living, associated with particulates or found in commensal relationships with marine phytoplankton, vertebrates and invertebrates (Buchan, et al., 2005). Moreover, 16S rRNA gene analysis has shown that it is not uncommon for the *Roseobacter* to represent 20-30% of the bacterial community in the upper mixed layer of the ocean (Acinas, et al., 1999; González, et al., 2000), whereby they are often associated with algal cultures, marine algae and phytoplankton blooms (González, et al., 2000; Riemann, et al., 2000; Geng & Belas, 2010; Hahnke, et al., 2013). *Roseobacter* are also capable of inducing host-specific adaptions which may be commensal or parasitic (Sison-Mangus, et al., 2014). Despite this, pathogenic *Roseobacter* have been identified and are a known causative agent of juvenile oyster disease (JOD) in cultured Eastern oysters (Boettcher, et al., 2005). Interestingly, U14 showed high sequence similarity to the genus *Modestobacter,* which have been commonly isolated from Antarctic and Atacama Desert soils samples (Mevs, et al., 2000; Busarakam, et al., 2016) calcareous stone surfaces (Reddy, et al., 2007) and deep sea sediments (Xiao, et al., 2011). Nonetheless, this observation is not surprising

as in bacterial taxonomy, the clear discrepancy between marine and non-marine fauna and flora is not applicable (Jensen & Fenical, 1996) and representatives of most culturable bacterial taxa can be isolated from both marine and terrestrial environments (Bowman & Nichols, 2002).

The bacterial isolate T3, isolated on TSA from effluent inlet water, showed high sequence similarity to the genus *Agrivorans,* which may be characterised by its agarolytic activity. The genus was first isolated by Kuruhashi & Yokota in 2004 from a healthy marine mollusc (*Omphalius pfeifferi*), and has since been isolated from several marine environments including the marine water column (Park, et al., 2014), tidal flats (Kim, et al., 2016) and macroalgae (Du, et al., 2011), whereby it has been suggested to form part of the indigenous flora. The widespread nature of agar degrading bacteria has been attributed to their role in carbon cycling whereby they break down agar and other polysaccharides (Armisen & Galatas, 1987), which forms a significant component of the cell walls of marine macroalgae. In contrast, the T8 isolate obtained from the incoming effluent water, showed a high sequence similarity to the genus *Halomonas* (Arahal, et al., 2002), a cosmopolitan euryhaline halophile (Romano, et al., 2006), which has been well investigated for its ability to produce molecules of biotechnological interest (Sánchez-Porro, et al., 2003; Llamas, et al., 2006). *Halomonas* have been identified in a diverse array of habitats, ranging from shrimp associated with hydrothermal vent plumes (Simon-Colin, et al., 2008) to deep-sea sediment, estuaries and saline lakes (Poli, et al., 2007; Xu, et al., 2010; da Silva, et al., 2013). Certain species of *Halomonas* are however known to display pathogenic potential in molluscs and humans. Mass mortalities of larval cultures in a commercial hatchery of the Chilean scallop *Argopecten purpuratus* was attributed to the CAM2 *Halomonas* sp. (Accession number DQ885389.1), which was the first report of a pathogenic outbreak due to *Halomonas* species, prompting further assessment of *Halomonas* on hatchery management (Rojas, et al., 2009). In another study, *H. stevensii* sp. nov. was isolated from patients suffering from bacteraemia in a dialysis centre (Stevens, et al., 2009). Moreover, *H. ventusa,* which had a high level of similarity to the T8 isolate, has been isolated from a patient suffering from a fish bite (von Graevenitz, et al., 2000), indicating partial pathogenic activity within the *Halomonas* genus.

Bacterial isolate T5 shared a high level of similarity with the genus *Pseudoalteromonas*, which belongs to the family *Pseudomonadaceae,* containing over 191 species (Euzéby, 1997). Culturable heterotrophic, Gram-negative bacteria isolated from marine waters can be broadly divided into two subgroups, defined by their ability to ferment carbohydrates, i.e. a fermentative or non-fermentative subgroup (Baumann, et al., 1972). In 1995, Gauthier et al. revised the *Alteromonas* within the non-fermentative group and suggested that it should be divided into two genera i.e. *Alteromonas* and *Pseudoalteromonas* are frequently associated with higher organisms and is used to describe eukaryotic host-microbe interactions (Rao, et al.,

2005; Gardiner, et al., 2014) as numerous species within the genus produce biologically active metabolites capable of impacting a range of biota (Holmström & Kjelleberg, 1999). Interestingly, unlike the *Modestobacter* and *Vibrio* which can be isolated from both terrestrial and marine habitats, the genus *Pseudoalteromonas* is restrictive to marine waters and has been isolated from several marine environments around the globe including sea urchins and macroalgae, including *Ulva* (Enger, et al., 1987; Holmström & Kjelleberg, 1999; Rao et al. 2007). Moreover, Rao et al., (2007) noted that *Pseudoalteromonas* associated with *Ulva australis* inhibit the attachment of fouling organisms, and several members including *P. auranitia* (Gauthier & Breittmayer, 1979), *P. luteoviolacea* (Gauthier & Flatau, 1976) and *P. rubra* (Gauthier, 1976) release high molecular weight antibiotics. Nonetheless, several members are known to induce disease in macroalgae including *Laminaria japonica* (Sawabe, et al., 1998) and *Gracilaria gracilis* due to their agarolytic activity (Schroeder, et al., 2003). In general, it seems as though the impact of the *Pseudoalteromonas* is dependent on the host-bacterium interaction, whereby species that are likely to naturally associate with their host seem to play a probiotic role, whereas if they are foreign to the host, they seem to induce disease. This observation may be re-enforced when observing the competitive interactions in mixed-species biofilms containing *Pseudoalteromonas tunicata*, whereby it is known to competitively exclude several bacteria when incorporated into a biofilm, however is less effective at inhibition when exposed to pre-established biofilms (Rao, et al., 2005).

On the other hand, the bacterial isolates T6, T10, V1 and V2 (all isolated from the incoming seawater in both systems) shared a high sequence similarity with the genus *Vibrio,* whereby they all seemed to match closely within the *V. splendidis, V. cyclitrophicus* and *V. atlanticus* subclade reviewed by Sawabe et al., (2014). It is interesting to note that the vibrio were all isolated from the incoming water, and that the *Ulva* reduces their numbers within the system. Nonetheless, it is not surprising as several species of *Ulva* have been known to display anti-*Vibrio* activity (Lu, et al., 2008). The genus *Vibrio* falls within the family *Vibrionaceae* which contains several marine pathogens that are known to infect a wide range of marine organisms, including abalone (Dixon, et al., 1991; Nicolas, et al., 2002; Cheng, et al., 2004), marine bivalves (Paillard, et al., 2004), corals (Ben-Haim & Rosenberg, 2002), macroalgae (Largo et al. 1995b), finfish (Kraxberger-Beatty, et al., 1990), prawns (Jiravanichpaisal, et al., 1994) and even captive bred seahorses (Balcázar, et al., 2010). Vibriosis due to *V. splendidis* infection has not been previously described in abalone, however their non-specific pathology does not exclude them from becoming opportunistically pathogenic. In contrast, mass mortalities have been previously attributed to *V. harveyi* which has been known to induce white foot lesions on abalone in Japan (Nishimori, et al., 1998) and France (Nicolas, et al., 2002). *V. alginolyticus* has also been shown to induce mass mortality in larvae of the abalone *Haliotis rufescens* within 24 h at concentrations above 10^3 cells. ml^{-1} (Anguiano-Beltran, et al., 1998). Nonetheless, the isolated *Vibrio* sp. may be pathogenic, probiotic, opportunistic, or represent normal bacterial

presence as low concentrations of diverse *Vibrio* species have been isolated from both healthy and diseased marine organisms (Nakanishi et al., 1996; Gomez-Gil, et al., 1998; Nishimore et al., 1998; Macey & Coyne., 2006; Joint et al., 2007). *V. gallaecicus, V. xuii, V. ichthyoenteri* and *V. parahaemolyticus* for instance have been isolated from marine aquaculture environments, of which only the latter two are believed to be potentially pathogenic and are often associated with disease (Zorrilla, et al., 2003; Gauger, et al., 2006; Martins, et al., 2013) . Assessing species-specific roles within the *Vibrio* genus is essential if one wishes to make inferences on the impacts of *Vibrio* abundance on host functionality as the genus is exceptionally dynamic.

4.4 Bacterial community profiling by 16S rRNA Illumina MiSeq sequencing

4.4.1 Overview of sequencing and microbial diversity at a higher level

The total reads per sample often differ for metagenomic sequence data sets, and sufficient coverage is required to capture the bacterial diversity as random variations within sampling environments may be taken as true variation if there are large differences in sample coverage (Rodriguez-r & Konstantinidis, 2013; Rodriguez-r & Konstantinidis, 2014).The microbial diversity within the abalone effluent water, fresh seawater and *Ulva* grown in both systems seemed to have sufficient coverage (1,488,157 quality raw sequence reads and 707 OTUs; Fig. 10), when compared to results published by Ma et al., (2014) who obtained at least 15,930 effective sequences and 805 OTUs from 12 wastewater samples from the steel industry. Furthermore, The sequence depth of the microbial diversity within the seawater being pumped into the fertilised tanks seem adequate, when compared with the 16S rRNA gene libraries constructed by Burke et al., (2011) who identified 5293 sequences from *U. australis* and 10884 sequences from seawater. More recently, Parrot et al., (2019) analysed the surface of *F. vesiculosus* by amplicon sequencing of the V3-V4 hypervariable region, and identified 4,060 sequences for whole seaweed, and 1,965 sequences for seawater, post sequence filtering.

Moreover, the primary bacterial diversity remained stable within the samples from effluent water, fertilized seawater and Ulvan as no significant differences were observed for both the Shannon and the Simpson diversity indexes, with F statistics ranging from 0.97 to 2.13, emphasising the high degree of similarity within the samples (Fig. 13). The observed similarity is a good indicator that the systems were adequately sampled, as one would expect both replicate systems (i.e. fertilised seawater tanks), and more importantly joined systems (i.e. effluent raceways) to contain similar microbial communities. Interestingly, the richness estimator Chao1 was significantly higher for the water samples (Both FW and AEW) than for the *Ulva* samples ($F = 22.7$, $P = 1.6$), indicating a high degree of unique OTU's in the water column. The increased presence of unique OTUs in the water column may be explained by the

interchangeable nature of the water with the environment, whereby fresh seawater is constantly pumped in from the ocean and over the abalone, after which it is pumped into the effluent raceways. Naturally, one would expect the incoming seawater to possess a greater bacterial abundance and diversity relative to the *Ulva* which has not left or entered the system post its initial seeding.

4.4.2 Broad comparison of bacterial community structures between *Ulva* and water libraries.

As observed in the ordination analysis and the cluster analysis, the 21 samples are broadly arranged into two clades, I.e. a water clade, and an *Ulva* clade (Fig. 14). The clear separation observed in the PCoA and NMDS plots at genus level between the microbial communities present on the *Ulva* as well as those present within the fertilised seawater and abalone wastewater systems correlates with several molecular studies which indicate that bacterial communities may differ between a host and its environment, and that limited core communities (~15%) specific to the host at lower taxonomic ranks are present (Burke, et al., 2011a; Bengtsson, et al., 2012). The higher level of separation between cohorts on the NMDS, relative to the PCoA is likely due to the higher power possessed by the NMDS when assessing non-linear relationships between cohorts (Ramette, 2007), as the samples are scaled according to rank and not linearly. The two primary clades seen on the cluster analysis support the separation between the *Ulva* and the seawater and outlines the separation between the FW *Ulva* and the AEW *Ulva,* suggesting they may form their own subclades (i.e. *Ulva* growing in fertilised seawater and *Ulva* growing in effluent water; Fig. 15). Cluster analyses comparing the similarity between *Ulva australis* and seawater, based on 16S gene libraries published by Burke. et al., 2011, support the clear separation between the *Ulva* and the seawater.

In both the PCoA and NMDS the FSW In, FSW Out and FSW *Ulva* samples were not clustered, likely due to the small sample size, however the points still lie within close proximity to each other and may cluster given more samples. The partial overlap of microbial communities observed in the PCoA is not peculiar as bacterial epiphytes common to most marine environments have been frequently identified at phylum level (Burke, et al., 2011a, b). General marine bacteria such as *Pseudoalteromonas, Alteromonas* and *Vibrio* have been frequently identified from several hosts such as *U. lactuca, Ulvaria fusca* and the tunicate *Ciona intestinalis* (Skovhus, et al., 2007; JP, 2007; Thomas, et al., 2008) and are known to additionally occur as floating free within the water column (Brettar, et al., 2001; Labrenz, et al., 2007). In the PCoA it is seen that the FSW In and FSW Out samples overlap with the EW In and EW Out cluster, implying a level of commonality. In contrast the NMDS analysis indicates that the inlet and outlet of the fertilised seawater tanks seem to form an independent cluster, which is expected as the water column has undergone dramatic change in terms of bacteria and nutrient load from the farmed abalone. Anthropogenic perturbations such as

nutrient increases associated with high density aquaculture and recirculating water within aquaculture systems are known to greatly impact bacterial communities (Schneider, et al., 2007).

The three distinct clusters (EW In, EW Out and EW *Ulva*), observed in the microbial community structures highlight the differences that exist between the inlet, outlet and *Ulva* within the effluent water system, and indicate that *Ulva* and/or its associated holobiont is capable of shifting the microbiota within the water column. Moreover, the effluent water inlet and effluent water outlet subclades present on the cluster analysis enforces the impact of the *Ulva* on the water column. The FSW outlet sample contained in the *Ulva clade* (on the cluster analysis) was interesting as it exacerbates the impact of *Ulva's* holobiont on the surrounding seawater. These observations are supported by Paver et al., (2013) who noted that phytoplankton are capable of impacting bacterial community succession in a lake. Paver et al., (2013) documented that fourteen out of 101 bacterial OTU's positively correlated with algal populations and one out of 59 negatively correlated with algal populations. Moreover, several authors have documented algal host specificity in a set of bacterial epiphytes, including the red alga *Laurencia dendroidea*, where transcriptomic profiling indicated little differences in taxonomic composition across different sample sites (Lachnit, et al., 2009). The *Alphaproteobacteria, Gammaproteobacteria, Bacteriodetes and Cyanobacteria* for instance are common epiphytic bacteria that associate with multiple algal groups (green, red and brown; Hollants. et al., Egan. et al., 2012). Interestingly, Egan. et al., (2012) noted that similarities at higher taxonomic ranks (i.e. phylum) are not observed at lower ranks (i.e. genera), which is apparent in both the PCoA and NMDS analyses. Moreover, continental scale variation in algal host-associated bacterial communities is suggested to be a function of host condition, not geography, and 'core' microbial communities characteristic of healthy alga appears to be lost when the host is stressed (Lachnit, et al., 2009; de Oliveira, et al., 2012; Marzinelli, et al., 2015). The distinct differences observed between the systems is a positive indicator for biosecurity concerns as it implies that the *Ulva* has its own unique microbiota, which are separate from the water column containing the abalone effluent. Interestingly, Marzinelli, et al., (2015) noted that the microbial communities on stressed individuals had a higher degree of similarity than among those on healthy hosts, further enforcing the importance of the uniqueness of the bacterial community associated with the *Ulva.* The Interactions between hosts and their associated holobionts can fundamentally alter the ecology of the holobiont, and in marine systems, microbial communities can fluidly interact within the systems. Due to the close associations in marine systems and the implications of these associations and interactions, understanding the complexities underlying the differences between the *Ulva* and the water systems is essential.

4.4.3 Assessment of bacterial community structure within fertilised seawater and abalone effluent water

The inter-individual variations observed between the replicates within the relative OTU abundance analysis is not surprising as each individual and species within aquaculture systems are known to introduce their own unique microbial flora, impacting the composition of the water (Fjellheim, et al., 2012; Ghanbari, et al., 2015). Moreover, the composition of the water within aquaculture systems is known to vary on a regional basis (Williamson, et al., 2008) as well as from one system to another (Auffret, et al., 2013; Bartelme, et al., 2017). Differences within bacterial communities are known to occur from variations within system designs, such as methods of filtration, physical parameters of the system as well as cleaning and disinfection techniques, which are known to impact free-living bacterial communities but have less impact on deeper biofilm layers (Schneider, et al., 2006; Wietz, et al., 2009; Schreier, et al., 2010). In support of studies of epibacterial communities associated with *U. australis*, the sequenced seawater communities isolated at I&J abalone farm showed similarities at genus level with libraries from seawater samples containing *Ulva* isolated from two different rock pools at Bare Island, La Perouse, which both displayed sequences of *Gammaproteobacteria*, *Flavobacteriaceae, Alphaproteobacteria* uncl. and *Rhodobacteriaceae* (Burke, et al., 2011). Interestingly the *Ulva,* fertilised seawater and particularly the abalone wastewater were dominated by sequences of the heterotrophic bacterial genus *Pseudoalteromonas* that were not previously reported by Burke et al., (2011a), however, have been associated with healthy recirculating aquaculture systems (Schreier, et al., 2010). The majority of the bacterial genera isolated and sequenced in the culture-based technique were in low abundance in the Illumina based assessment. The *Celeribacter* (U2), *Roseobacter* (U10), *Modestobacter* (U14), *Agrivorans* (T3) and *Halomonas* (T8) were present in low abundance, whereas the *Pseudoalteromonas* (T5) and *Vibrio* (T6, T10, V1 and V2) were present in high abundance. This observation acknowledges the fact that not all bacteria are culturable (Barer & Harwood, 1999; Eilers, et al., 2000), stressing that the culture-based techniques are not a true representation of the bacteria consortia present within the water column (Hilton, et al., 2016; Kraková, et al., 2018).

Bacterial communities within the fertilised seawater tanks

The water inlet that enters the fertilised seawater tanks (i.e. FSW In), contains no fertiliser as it is before the system (i.e. the entry point) and is a representation of the seawater being pumped through the abalone tanks prior entry into the AEW raceways. Additionally, the FSW Inlet is a representation of the same water that is used to settle juvenile abalone and serves as a baseline to compare the effluent bacterial populations against. In the FSW, the majority of the bacteria originate from the sea, which is close to a large kelp forest. The filtration system and pipes supplying the water may impact the microbial consortia in the water as sloughing of microorganisms in aquaculture systems is known to occur (King, et al., 2004; Wietz, et al., 2009).

Several bacterial sequences from the fertilised seawater libraries correspond with studies published by Burke 2011a, who constructed seawater libraries which were clearly distinct from *U. australis* libraries. The FSW libraries additionally correlated with libraries from the Global Ocean Sampling Expedition, which collected 7.7 million sequence reads from the planktonic marine niche across a transect from the North Atlantic to the South Pacific (Williamson, et al., 2008). The seawater library similarities include sequences from the *Alphaproteobacteria, Gammaproteobacteria, Flavobacteriales* and *Rhodobacteriaceae* although in this study, *Pseudomonas, Psychromonas,* and *Psychrilyobacter* are in notably higher abundance. The seawater entering the fertilised seawater tanks is exceptionally dynamic as it has the greatest proportion of differentially abundant bacteria that are likely to explain the differences between the systems. The bacteria most likely to explain the uniqueness of the FSW inlet predominantly belong to genera common in the marine environment, such as; *Marinomonas, Lutimonas, Psychrobacter, Oceanospirillales, Paramoritella, Photobacterium, Amphritea* and *Planktomarina.* The genus *Marinomonas* are cosmopolitan marine bacteria that have been isolated from seawater, sediment as well as cultured oysters along the Mediterranean coast (Romanenko, et al., 2003; Macián, et al., 2005; Arahal, et al., 2016). *Lutimonas* are members of the family Flavobacteriaceae and have been isolated from the abalone *H. tuberculata*, polychaetes, tidal flat sediments and are believed exclusively inhabit marine invertebrates and seawater, and have not been associated with algae (Yang, et al., 2007; Kim, et al., 2011; Kim, et al., 2016; Gobet, et al., 2018), although associations with marine sponges have been observed (Haber, et al., 2013). Additionally, *Psychrobacter* are common marine bacteria that have been isolated from seawater, sediment and crustacea (Romanenko, et al., 2004; Romanenko, et al., 2009) and have been studied for their bioremediatory potential (Abd-Elnaby, et al., 2016). The high proportion of differentially abundant bacteria common to the marine environment sequenced in the fertilised seawater inlet environment is not surprising as Sogin et al., (2006) indicated that rare phylotypes establish the majority of the diversity in marine environments (Sogin, et al., 2006). The differential abundance of *Propionigenium* is interesting, as this genus is strictly anaerobic, with a metabolism based entirely on sodium ion coupling, and are assumed to typically inhabit anoxic marine sediment (Schink & Pfennig, 1982; Schink, 1992). The presence of both aerobic and anaerobic microorganisms is not unlikely, as environmental complexity is known to promote micro niches capable of housing highly diverse consortia (Eisenhauer, et al., 2013).

The differential abundance of bacteria present within the phosphorus metabolising group (*Ruegeria*) and the "Sulfur Bacteria" group (*Desulfovibrio* and *Thioalkalispiraceae*) are of significance as these groups play vital roles in nutrient cycling (Thorseth, et al., 1996; Hao, et al., 1996; Vadstein, 2000; Sebastián & Ammerman, 2011). The "Sulfur Bacteria" include sulfur and sulfate reducing bacteria as well as sulfur oxidising bacteria (McCallan & Weedon, 1940). These bacteria may be considered an irritation in aquaculture operations due to their release of sulfurous

compounds such as hydrogen sulfide which are toxic and have been shown to have adverse reactions in bivalve aquaculture in excessive concentrations (Newell, 2007). *Ruegeria* is a model marine bacterium that fall under the Roseobacter clade which aids in the breakdown of phosphorus, carbon monoxide and DMSP (Sebastián & Ammerman, 2011; Todd, et al., 2012; Cunliffe, 2013). Sulfate reducing bacteria, such as *Desulfovibrio* are a cosmopolitan group of anaerobic organisms that range from marine sediment to human intestines (Beijerinck, 1895; Sahrani, et al., 2008), whereas sulfur oxidising bacteria, such as *Thioalkalispiraceae* generally associated with marine and saline environments (Mori, et al., 2011). Bacterial transformation of sulfur rich compounds such as DMSP (Moran, et al., 2012) play important roles in aquaculture operations as they limit the presence of noxious agents responsible for the bad smells reported during abalone processing (Robertson-Andersson, et al., 2006b).

The fertilised seawater entering the abalone farm is a vector for both opportunistic bacterial pathogens as well as naturally occurring bacteria that may be neutral or probiotic. The potentially pathogenic bacteria identified in the LEfSe analysis that are of concern in the FSW inlet include the genera *Aliivibrio* and *Vibrionaceae*. *Aliivibrio* is a newly re-classified genus that was originally described as *Vibrio fischeri* (Urbanczyk, et al., 2007; Ast, et al., 2009). Species within the genus are predominantly pathogenic, and are known inducers of several diseases including the Hitra disease (Egidius, et al., 1981), cold water vibriosis (Egidius, et al., 1986) and winter ulcer disease (Lunder, et al., 2000) which impact Atlantic salmon. In contrast, *Aliivibrio* have also been associated with bioluminescence and are known to for symbiotic relationships with squid (Verma & Miyashiro, 2013) and have not been associated with disease in abalone aquaculture. Moreover, the high differential abundance of unclassified *Vibrionaceae* in the incoming seawater raises alarm due to the pathogenic associations made with several members of this genus (Rodrick, 1991).

The water flowing out of the FSW tanks is a representation of the interactions between the bacteria present within the water columns and that of *Ulva*'s holobiont and serves as a negative control to document the impacts of *Ulva* and its holobiont on the bacterial community composition of the water in the absence of abalone effluent. The bacterial communities present within the fertilised seawater tanks are influenced by the *Ulva* and its associated holobiont, the fresh seawater that flows into the tanks, the fertiliser used in the system as well as any additional microbiota that may inhabit the FSW tanks such as biofilms and planktonic biota. The nutrients in the fertilised system originate from the sea and are supplemented on a weekly basis with nitrogen and phosphorus (pers. comm.). Bentzon-Tilia & Gram (2016), suggested that moderate levels of fertilisation, either chemical or via the addition of agricultural waste, is capable of positively impacting recirculating aquaculture systems by acting as a feed for microbiotic communities, promoting a balanced ecosystem in nutrient deprived RAS, preventing shifts from neutral bacterial consortia to potentially pathogenic ones. The primary differential bacteria present within the FSW tanks do not appear to be pathogenic, and correlate with bacteria previously found in marine water and on algae.

Kordia for instance, is a common marine bacterial genus that has been associated with both marine and freshwater sources, green marine alga such as *Ulva,* polychaetes as well as diatoms and is known to display algicidal activity (Choi, et al., 2011; Park, et al., 2014; Qi, et al., 2016; Pinder, et al., 2019). Likewise, *Spongiispira,* has been isolated from marine sponges and has a high level of sequence similarity with other common marine bacteria (Kaesler, et al., 2008; Pike, et al., 2013), although little is known on the genus. *Oleispira* is a novel genus that has been noted for its oil-degrading potential (Yakimov, et al., 2007; Kube, et al., 2013), prefers colder waters, and is frequently associated with Antarctic regions (Yakimov, et al., 2003).

Bacterial communities within the abalone wastewater raceways

The abalone effluent water originates from the nearby shallow coastal seawater, passes through the abalone rearing tanks and then flows into the raceways. The bacterial communities present within the inlet of the abalone effluent raceways are additionally influenced by the abalone and their associated holobionts (Schreier, et al., 2010), asides from the fresh seawater that flows into the rearing tanks as well as the feed used in the system. Moreover, the presence of macrobiota in abalone rearing tanks, such as polychaetes (Ruck & Cook, 1998), may further shift bacterial populations. The primary nutrients entering the abalone effluent raceways are in the form of organic matter that originates from digested feed and faeces from the abalone as well as any uneaten feed that may remain in the wastewater. Studies on fish gut microbiomes have indicated that gut microbes may colonise biofilms present within the aquaculture system, whereby the composition of the biofilm is dependent on the fish species being cultivated (Sugita, et al., 2005; Lahav, et al., 2009). Similarly, one would expect the faeces and unconsumed feed from abalone to inoculate and occupy niche space within the abalone wastewater raceways. Microbiota are known to have strong influence over carbon, nitrogen and phosphorus cycling in aquatic environments due to metabolic interactions with particulate (POM) and dissolved (DOM) organic matter (Pomeroy, et al., 2007). The proteins within the abalone feed and faeces are decomposed by heterotrophs to ammonia, after which nitrifying bacteria convert the ammonia from the excretion to nitrate via nitrite (Bender, et al., 2004; Itoi & Sugita, 2006). Heterotrophs are known for their roles in oxygen consumption, metabolic by-product production, and for competing for dominance with other autotrophic bacteria (Itoi & Sugita, 2006; Michaud, et al., 2006; Robinson, 2008). The majority of heterotrophs are suggested to be “neutral” and aid in maintaining healthy water systems by occupying space while preventing the proliferation of potentially pathogenic bacterial species (Attramadal, et al., 2012a; b). Several unidentified molluscs were present along the channel that brings the effluent water from the abalone cultivation tanks to the *Ulva* raceways (pers. comm.) which are additionally capable of influencing the microbiota within the water column (Kemp, et al., 1990).

The low abundance of nitrifying bacteria in the abalone wastewater raceways is noteworthy considering the raceways contain high levels of effluent, as nitrifying

bacteria have been frequently associated with effluent wastewater (Harms, et al., 2003; Lydmark, et al., 2007; Paungfoo, et al., 2007). Common nitrifying bacteria in aquaculture systems include species of the genus *Nitrosomonas, Nitrosococcus, Nitrobacter, Nitrosolobus, Nitrosovibrio* and *Nitrococcus* (Belser, 1979; Teske, et al., 1994). In conjunction with the effluent wastewater results, Itoi & Sugita., (2006) indicated that heterotrophic bacteria were the dominant bacteria present within RAS water columns. Opportunistic bacteria such as heterotrophs are known to proliferate in unstable environments containing high levels of organic matter in which growth promoting parameters are favoured (Painting, et al., 1989). Opportunists that are capable of proliferating rapidly (r-strategists) are the first to utilise available resources and are gradually outcompeted by the slower growing specialists, such as the nitrifying bacteria (k-strategists) (Salvesen, et al., 1999; Defoirdt, 2016). Thus it is not unlikely that the particulate organic matter released by the abalone in the effluent water is enhancing the heterotrophic bacterial growth which, in turn, outcompetes the slower growing nitrifying bacteria. It is however known that, due to their competitive nature, excessive levels of heterotrophic bacteria in integrated aquaculture systems may be considered problematic as they may hamper nitrification rates when oxygen levels are low (Michaud, et al., 2006). The bubbler systems within the fertilised seawater tanks and the paddlewheel systems utilised within the abalone effluent raceways at I&J aid in oxygenating the water column (Robertson-Andersson, 2003).

Co-existence of bacterial genera is often desirable, as seen in the symbiotic relationship between heterotrophic bacteria and nitrifiers allowing for simultaneous carbon and ammonia oxidation (Kindaichi, et al., 2004; Dong & Sun, 2007). Moreover, processes associated with dynamic biofilms such as niche and nutrient competition, as well as the creation of aerobic and anaerobic microenvironments may decrease the effectiveness of nitrifying bacteria (Fdz-Polanco, et al., 2000), impacting the spatial distribution of microorganisms. Easily biodegradable organic matter supports the growth of heterotrophic bacteria (Sharma & Ahlert, 1977), which outcompete the nitrifying bacteria and tend to grow towards the surface of mixed biofilms (Hagopian & Riley, 1998). Heterotrophic bacterial communities in aquaculture systems have been found to be dominated by *Alphaproteobacteria* and *Gammaproteobacteria* (Wietz, et al., 2009; Sugita, et al., 2005). Michaud et al., (2009) reported that sequences from *Alphaproteobacteria,* mainly *Rhodobacteriaceae,* accounted for 51% of all sequences in an experimental recirculation systems with sea bass (*Dicentrarchus labrax*). Moreover, the genus *Roseobacter,* which falls within the *Rhodobacteriaceae,* is exclusively marine or hypersaline and may represent up to 25% of coastal marine bacteria and 15% of mixed-layer ocean bacterioplankton communities (Buchan, et al., 2005). Non-pathogenic heterotrophic bacteria inhabiting RAS and IMTA systems have received little attention (Blancheton, et al., 2013; Natrah, et al., 2014). Interestingly, culture-based studies performed by Painting et al. (1989) documented that heterotrophs grew exponentially during phytoplankton growth, whereby *Pseudomonadaceae* dominated the water colony when maximum phytoplankton and

bacterial biomass was reached, after which *Flavobacteriaceae* became dominant after phytoplankton senescence. In a similar manner, the kelp forests that are near the seawater inlet could be influencing the proportions of heterotrophic bacteria in the water systems as heterotrophic bacteria are known to utilise the mucilage released by kelp (Linley, et al., 1981).

Several bacteria present in the relative OTU abundance analysis correlate with observations made by Schreier et al. (2010) who published an intensive list of bacteria present in aquaculture bio-filters. Asides from heterotrophic and nitrifying bacteria, they reported the following; (1) denitrifying heterotrophs, such as *Pseudomonas* sp., *α-Proteobacteria* and *Aquaspirillum* sp., (2) sulfide-dependent autotrophic denitrifiers, such as *Bacteriodetes, Protobacteria* and *Furmicutes,* as well as (3) dissimilatory nitrate producers such as *Desulfovibrio.* The presence of *Plantomycetes,* which are anaerobic oxidisers of ammonium and nitrate has been noted to bypass the denitrification reaction (Tal, et al., 2003). The differential presence of the bacterial genus *Desulforhopalus,* which are sulfate-reducing bacteria, is not alarming as these bacteria are naturally occurring and are associated with oxidising organic substrates to acetate and CO_2 (Widdel & Bak, 1992; Inagaki, et al., 2002).

Once the water has entered the effluent raceways, it passes through the "*Ulva* biofilter" before it exits back to the ocean through a canal. The water flowing out of the raceways is a representation of the health of the system post *Ulva* bioremediation and is a depiction of the impacts of *Ulva* and its associated holobiont on the bacterial community composition of the wastewater. The bacterial community changes that occur within the abalone effluent raceways are induced by *Ulva* and its associated holobiont, as well as by additional microbiota present within the raceways. The nutrients entering the abalone effluent raceways are primarily from abalone excrement and any uneaten feed that may remain in the water column which are decomposed by heterotrophs to ammonia, after which nitrifying bacteria convert the ammonia from the excretion to nitrate via nitrite (Bender, et al., 2004; Itoi & Sugita, 2006). Asides from the microbial reactions that take place in the system, the *Ulva* additionally utilises nutrients within the system. Rahman et al. (2012) utilised total count water testers (Millipore S.A.S. 67120 Molsheim, France) and an incubator to estimate the total bacterial abundance in effluent water in land-based recirculating abalone culture tanks along the coastline of Japan (Rahman, et al., 2012). His results indicated that bacterial abundance varied significantly over time and that incorporating a protein skimmer into the system significantly reduced the mean abundance of heterotrophic bacteria in the waters, and that mean bacterial abundance generally increased over time in the system in the absence of the protein skimmer. The waste removal potential of the *Ulva* coupled with the antibacterial activity associated with *Ulva* and it's holobiont may provide similar benefits and may aid in reducing the mean abundance of total bacteria in the waters. This may aid in explaining the decreases observed in the effluent system in the culture-based results discussed previously.

Literature on the bacterial presence within flow through abalone aquaculture systems is rare as the majority of authors have focused on recirculating aquaculture systems, the close associations between the organisms present within the systems and their implications for disease (King, et al., 2004; Pang, et al., 2006; Bolton, et al., 2009; Butterworth, 2010; Rahman, et al., 2012). Martins et al., (2013) utilised DGGE and barcoded 16S rRNA pyrosequencing to assess the bacterial composition in recirculating aquaculture systems for turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis*) to determine the potential pathogen abundance within the system. The study found considerable differences in the bacterial compositions between the turbot and sole RAS, implying strong species-specific effects on the bacterial communities in the water columns. Despite such species differences, Martins study shared commonalities with the sequences at I&J whereby bacterial OTUs from the genus *Pseudoalteromonas* were the most dominant in the sole RAS, which was linked to the low concentrations of dissolved inorganic nitrogen in the sole systems when compared to the turbot systems. Aranda et al. (2012) demonstrated that *Pseudoalteromonas* sp. strains have potential as *Vibrio* biocontrol agents and produce bacteriostatic compounds. Moreover, OTUs assigned to *Desulfobacter, Desulforhopalus* and *Draconibacterium* have an increased relative abundance in samples from effluent water. *Draconibacterium* are newly categorised facultative anaerobes that are most commonly associated with marine sediment (Du, et al., 2015; Li, et al., 2016) and have been identified in Mediterranean Sea water samples (Ruvira, et al., 2013), whereas *Desulfobacter* are green sulfate reducing bacteria that are abundant in anaerobic marine environments, are capable of metabolising small organic compounds, and have been used as a biomarker for oxic-anoxic treatment (Hiras, et al., 2016). Dissimilatory sulfate reducers have been known to promote denitrification in RAS systems (Schreier & Tal, 2008).

4.4.4 Assessment of bacterial community structure on *Ulva* cultivated in fertilised seawater tanks and in abalone effluent raceways.

Biotic surfaces in aquatic environments are hosts to diverse biofilm communities that are able to both positively and negatively impact the health of the hosts (Nakanishi, et al., 1996; Marshall, et al., 2006; Twigg, et al., 2014), as well as any organism that may associate with or predate on their hosts (Rico, et al., 2016; Gobet, et al., 2018). Numerous host-specific associations have been documented in the marine environment (Taylor, et al., 2005; Longford, et al., 2007; Reis, et al., 2009; Burke, et al., 2011a) which have proven beneficial in assessing microbial diversity in aquatic ecosystems (Taylor, et al., 2004; Egan, et al., 2008). Nonetheless, in depth analyses of microbial communities associated with macroalgae in aquaculture systems are scare, making it increasingly difficult to disentangle the complex interactions that promote pathogenic bacterial dominance.

The differences in abundance of several genera on the *Ulva* relative to the effluent and fertilised seawater concur with studies that distinguish the unique nature of *Ulva*'s holobiont from that of the surrounding water (Burke, et al., 2011a). This is evident when we consider the following: (1) the significantly lower number of differentially abundant OTU's of the genus *Amphritea, Desulfobacter, Marinomas, Oleispira Photobacterium, Draconibacterium, Ruegeria, Marinomas, Desulforhopalus, Propionigenium, Vibrionaceae, Aliivibrio, Desulfovibrio* and *Ruegeria* on the *Ulva* relative to both water systems; as well as (2) the increase in abundance of OTUs assigned to *Hellea, Saprospiraceae, Granulosicoccus* and *Thiohalophilus* specifically on the *Ulva*. The bacterial abundance shifts that occur in both the effluent and the fertilised seawater systems from the inlets to their outlets illustrate the influence of *Ulva* and its associated holobiont on the water columns. This is seen when assessing the bacteria within the genus *Cocleimonas*, *Hellea, Granulosicoccus, Portibacter* and *Saprospiraceae,* which increase in abundance in the effluent water systems and were prevalent in high abundance on the *Ulva.* Moreover, Bacteria within the genus *Amphritea, Marinomas, Photobacterium, Vibrionaceae uncl., Aliivibrio, Desulfovibrio, Ruegeria* and *Vibrio* decrease in abundance and were present in low abundance on the *Ulva.* The changes induced by the *Ulva* and its holobiont on the relative bacterial abundance within the water columns co-inside with observations made by Paver et al. (2013), who noted that algae and their associated holobiont are capable of impacting bacterial community succession in a lake. Nonetheless, a higher level of sequencing, perhaps down to species level, is required to disentangle how the bacteria relate to the lottery (Burke 2011, Ghaderiardakani 2017) and hologenome (Hollants 2013 and Spoener 2012) models of community succession.

Both *Ulva* libraries were characterized by sequences from common environmental bacteria such as *Saprospiraceae*, *Bacteriodet*es, *Rhodobacteriaceae* and unclassified *Flavobacteriaceae,* as well as algal specific bacteria including *Granulosicoccus* and *Hellea. Ulva* libraries additionally included sequences from unclassified *Planctomycetaceae* and unclassified *Gammaproteobacteria*, correlating with broad observations on epibacterial communities inhabiting both *Ulva* (Burke, et al., 2011a) and other marine macroalgae. 16S rRNA gene sequencing of surface bacteria associated with the green alga *Enteromorpha* sp. (now *Ulva* sp.) revealed a prevalence of *Gammaproteobacteria* (Patel, et al., 2003). Similarly, epibacterial communities present on the brown macroalga *Saccharina longissima* (as *Laminaria saccharina*) consisted of *Rhodobacteriaceae*, *Flavobacteriaceae* and unclassified *Gammaproteobacteria* (Staufenberger, et al., 2008), whereas the epibacteria on the red macroalga *Delisea pulchra* consisted of *Rhodobacteriaceae*, *Flavobacteriaceae, Plantomycetaceae* and unclassified *Gammaproteobacteria.* Additionally, both macrophytes in brackish lakes and seagrasses have been found to display similar associations (Uku, et al., 2007; Liu, et al., 2019), leading authors to suggest that bacteria within these taxonomic groups play important roles within marine plant microbiomes.

Bacterial communities on *Ulva* cultivated in fertilised seawater

The *Ulva* in the fertilised seawater tanks were most likely colonised by pioneer bacteria already present on the *Ulva* during the start-up period, and are likely to display subtle shifts in bacterial community dynamics on a frequent basis (Marrase, et al., 1992; Kirchman, et al., 2004). One must consider that the epibacteria associated with *Ulva* tend to be more sheltered within biofilms, whereas bacteria present within water columns are more subjective to stress (King, et al., 2004; Rurangwa & Verdegem, 2015), implying that fluctuations in algal holobionts would be less frequent than fluctuations within the water column. Post initial colonisation, the bacteria associated with *Ulva* in the FSW tanks may be influenced by the seawater flowing through the system as well as the microbiota present within biofilms inhabiting the tank (Blancheton, et al., 2013). The addition of inorganic phosphorus and nitrogen into the FSW tanks is likely to positively impact bacteria associated with *Ulva* as fertilisation is known to enhance feed conversion rates in aquaculture systems and promote beneficial interactions (Lara-Anguiano, et al., 2013; Cooper & Smith, 2015). Moreover, the addition of the inorganic nitrogen and phosphorus prevents bleaching and fragmentation of the *Ulva* (Robertson-Andersson, 2003), thus improving host health, which in turn would render it less susceptible to pathogenic microbiota.

In support of Burke's studies in 2011 on the epibacterial communities associated with *U. australis*, the sequenced *Ulva* showed similarities at genus level, sharing sequences of *Gammaproteobacteria*, *Flavobacteriaceae, Alphaproteobacteria* uncl. and *Rhodobacteriaceae* (Burke, et al., 2011). The bacteria most likely to explain the uniqueness of the FSW *Ulva* predominantly belong to genera that have been frequently isolated from recirculating aquaculture systems. For instance, *Saprospiraceae* are associated with biofilters in RAS systems (Li, et al., 2016), and have been incorporated into activating nitrifying bioreactors, whereby they consist of up to 3% of the consortium (Preena, et al., 2018). Moreover, *Saprospiraceae* have been found in the intestines of both healthy and unhealthy Atlantic salmon (*Salmosalar L.*), although had a higher abundance in the unhealthy fish (Wang, et al., 2018). In addition, *Portibacter* are members of the *Saprospiraceae* and have also been isolated from RAS biofilters (Yoon, et al., 2012; Li, et al., 2016), although little is known on the genus. Interestingly *Colwellia*, which are general marine bacteria that have been documented for their agar-digesting potential (Bowman, et al., 1998; Xu, et al., 2017), were present in significantly higher abundance in the water than on the *Ulva* and have also been isolated from RAS water systems (Ruan, et al., 2015; Zhu, et al., 2015).

Bacterial communities on *Ulva* cultivated in abalone effluent raceways

When we compare the dominant bacterial taxa present on the FSW *Ulva* with those found on the EW *Ulva*, it is apparent that certain bacterial taxa appear in similar abundance in both systems. This is not unlikely, as the farmers re-seed the effluent raceways with mature *Ulva* obtained from the raceways during harvesting, which originated from the fertilised seawater tanks prior introduction of the effluent water

(pers. comm.). The *Ulva* in the abalone wastewater raceways were most likely colonised by bacteria already present on the *Ulva* when it entered the system, which were initially altered by pioneer bacteria present in the water column during the start-up period (Watson, et al., 2015). One would expect the faeces and unconsumed feed from abalone to inoculate and occupy niche space on the *Ulva* within the wastewater raceways as faecal bacteria are known to persist in aquatic environments (Robertson-Anderson, et al., 2005; Beardsley, et al., 2011). Erasmus et al., (1997) identified several bacterial genera in the gut of the abalone *H. midae* which were also present in the effluent water and on the *Ulva* cultivated in the effluent raceways, including *Pseudoalteromonas, Flavobacteria* and *Vibrio*. Doeschate & Coyne., (2007) went on to note that *H. midae* fed kelp supplemented with *Pseudoalteromonas* sp. strain C4, isolated by Erasmus et al., (1997) , grew faster than those fed standard kelp. The colonisation of the intestinal tract with probiotic bacterial communities has numerous positive effects on host function and regulation (van Baarlen, et al., 2013; Bhatnagar & Lamba, 2015), and disturbances in mechanics induced by changes in bacterial abundance can induce disease (Cooney, et al., 2002). Moreover, Macey & Coyne (2006) indicated that abalone fed probiotic feed supplemented with *Vibrio midae* SY9 increased protease activity in the intestine of the abalone, and that probiotic-supplemented feed should be fed at least every second day to maintain the number of probiotic cells within the intestines. Macey and Coyne's observations of probiotic supplemented feed could be an additional advantage of effluent grown *Ulva* as the *Ulva* may be inoculated with probiotic bacteria from the faeces, which could in turn be re-inoculating the gut of the abalone in a microbial loop, preventing the requirement for continual probiotic supplementation. Nonetheless, associations between bacteria in the effluent water systems, on the effluent grown *Ulva* and within the gut of the abalone fed effluent grown *Ulva* have not been disentangled and require further assessment to determine the impacts of effluent grown *Ulva* on abalone gut microbiota and metabolic function.

If one considers *Ulva* as a living biofilter within effluent raceways, the bacteria present on the effluent grown *Ulva* shared similarities with biofilter studies in recirculating aquaculture systems (RAS) whereby bacterial communities shared similarities at genus level, including *Flavobacteria, Planctomycetes, Roseobacter, Pseudoalteromonas, Desulfovibrio* and *Vibrio* (Schrier et al., 2010). Similarly, as with the Ulva, differences exist between the dominant genera inhabiting the biofilters and the water columns (Cytryn, et al., 2003; Schreier, et al., 2010), even in instances where the microbial populations clearly impacted one another (Blancheton & Canaguier, 1995; Michaud, et al., 2006). The bacteria most likely to explain the uniqueness of the EW *Ulva* belong to genus associated with recirculating aquaculture systems and gut microbiota. For instance, *Granulosicocus* is gammaproteobacterium that comprises two species that have been documented exclusively in marine environments, whereby they have been found in surface seawater, brown algae and on leaves of seagrass (Kurilenko, et al., 2010; Baek, et al., 2014; Park, et al., 2014). Moreover, the genus *Hellea* are Alphaproteobacteria that contain only one species and have been isolated

from surface seawater and the gut of the seacucumber *Apostichopus Japonicus* (Alain, et al., 2008; Wang, et al., 2018) and were statistically significant on the healthy algae *Delisea pulchra* when compared to bleached individuals (Zozaya-Valdés, et al., 2017). *Thiohalophilus* are obligatory chemolithoautotrophic halophilic sulfur-oxidising bacteria that have been isolated from marine water systems, hydrothermal vents and hypersaline saline lakes (Sorokin, et al., 2010; Mori, et al., 2011) which are novel due to their denitrifying ability and thiocyanate metabolism (Sorokin, et al., 2007).

Several genera have been identified on the effluent grown *Ulva* that contain potential pathogenic bacterial species, including unclassified *Vibrionaceae, Vibrio, Aliivibrio* and *Flavobacterium* (Verschuere, et al., 2000; Balcázar, et al., 2006). The greater relative abundance of *Vibrionaceae* and *Vibrio* in the effluent system, compared to the fertilised seawater is reasonable as there is more particulate organic matter in the raceways compared to the fertilised tanks and *Vibrio* have commonly been associated with faecal matter (Park, et al., 2004; Butterworth, 2010; Okoh & Igbinosa, 2010; Igbinosa, et al., 2011). The greater abundance of *Vibrio* in the inlets relative to the outlets concurs with the culture-based results and is of significance as it implies the *Ulva* and/or its associated microbiota led to decreases in the levels of total *Vibrio* within the system. This observation correlates with studies on IMTA systems which indicate that integration of macroalgae such as *Ulva* and *Gracilaria* into abalone aquaculture systems lowers the vibrio composition in the water columns (Pang, et al., 2006; Lu & Liu, 2008). The decrease in abundance of potentially pathogenic bacteria such as *Vibrio* and *Aliivibrio* on the *Ulva*, relative to both water systems is a positive indicator that the *Ulva* does not harbour an excessive abundance of these pathogenic microbiota, supporting the reported inhibitory properties displayed by *Ulva* on potentially pathogenic bacteria such as *Vibrio* (Lu, et al., 2008; Sivakumar, et al., 2014). Despite the presence of potential pathogens, no diseased abalone were reported at the time the study was conducted.

Genera have also been identified on the effluent grown *Ulva* that contain probiotic bacterial species, including *Roseobacter, Vibrio, Alteromonas* and *Flavobacterium* (Verschuere, et al., 2000; Balcázar, et al., 2006). The impact of introducing probiotic bacteria into RAS and IMTA have been investigated (Taoka, et al., 2006; Martínez Cruz, et al., 2012), whereby the presence of probiotic bacteria within the water column has several benefits including nutrient and niche competition (Callaway, et al., 2008), antimicrobial and growth inhibitor production (Marty & Martin, 1992), and the release of quorum sensing quenchers (Balcázar, et al., 2006; Sahu, et al., 2008; Akhter, et al., 2015). Moreover, probionts can act as a nutrient source (Kawamura & Takami, 1995), aid in enzymatic digestive processes and improve growth rates (Erasmus, et al., 1997; Macey & Coyne, 2005), enhance pathogenic immune responses and have antipathogenic properties as discussed previously (Nogami & Maeda, 1992; Nakayama, et al., 2009). The advantages and probiotic potential of *Ulva* and its associated microbiota have also been documented (Egan, et al., 2013; Cyrus, et al., 2015; Rico, et al., 2016). Despite these observations, the probiotic potential of *Ulva* grown

in abalone effluent raceways and fed back to the cultured abalone is largely unexplored. For this reason, it is essential to evolve from disentangling bacterial communities to understanding the activity, roles and functionality of the individuals within the consortia if we seek to fully understand the risks associated with integrated multitrophic aquaculture.

4.5 Best management practices, future considerations and concluding remarks

4.5.1 Best management practices

A common concern with "biofilters" associated with integrated aquaculture systems (i.e. the *Ulva*) are their ability to house and protect bacterial pathogens that may occupy the biofilm, leading to either infection of the organisms within the system, or consumers after processing. Disease outbreaks often occur as a result of latent infections caused by opportunistic bacteria, and don't necessarily require the presence of identified pathogens (Vadstein, et al., 2004; King, et al., 2004; Rurangwa & Verdegem, 2015). Given the difficulty to treat disease in intensive aquaculture environments without hampering the microbiota within the system, microbial management of integrated aquaculture systems is essential from the start-up of the production process. Nonetheless, bacteria present on biological filters such as *Ulva* rely on interactions with the environment as a function of nutrient input and are hence not easily controlled (Burke, et al., 2011a, b). Both the culture dependent and culture independent techniques identified the presence of general marine bacteria as well as potentially pathogenic bacteria within the genus *Vibrio, Flavobacteria* and *Aliivibrio.* The sensitivity of abalone to different concentrations of marine consortia on their feed is largely unexplored and given the diversity of bacteria within the marine environment, requires more in-depth assessment to link the complex interactions between marine bacterial abundance associated with semi-recirculating systems and their synergistic impacts on host functionality. Disinfection of aquaculture systems routinely utilise both UV and ozone treatment to prevent bioaccumulation of heterotrophic and coliform bacteria in water systems (Gullian, et al., 2012; Mamane, et al., 2010; Gonçalves & Gagnon, 2011), thus reducing the spread of pathogenic organisms between systems. Despite this, Blancheton et al., (2013), noted that disinfection is unlikely to be useful in well managed systems as they are known to damage beneficial microbes, leaving space for opportunists to flourish, which may negatively impact the health of the cultured organism (Attramadal, et al., 2012b).

In general, incoming seawater is believed to have a lower proportion of opportunists if it has had sufficient time to mature (k-selection) and reach a stable carrying capacity (Blancheton, et al., 2013). Microbially mature intake water has been shown to increases ingestion, growth and survival of marine larvae (Skjermo, et al., 1997; Salvesen, et al., 1999). Well managed recirculating aquaculture systems (RAS) have been shown to aid microbial maturation, whereby the water within the recirculating

systems align with that of the rearing water if no strong disinfectants are used (Attramadal, et al., 2012a, b). It is likely that integrated multitrophic aquaculture systems are capable of promoting similar microbial maturation responses, whereby bacterial communities may establish at a stable carrying capacity within the system. Blancheton et al., 2013, suggested that k-selection contributes to the increased stability, robustness and resilience of microbial compositions within RAS compared to FTS (Attramadal, et al., 2012a). The principal co-ordinate analyses published by Attramadal et al., (2012 a, b), of a RAS and a Flow-through system (FTS) for Atlantic cod larvae revealed that with time, the RAS developed significantly different bacterial populations in the water system when compared to FTS, despite the influence of bacteria from the same feed and algae within the systems. Moreover, the results indicated that the bacterial populations in the RAS were more stable over time than those within the FTS. In mature, well managed RAS, bacterial growth potential is low as the microbial carrying capacity is composed of dominant organisms that compete well in the given conditions (Attramadal, et al., 2012a).

Similarly, *Ulva* that has remained in the system for sufficient time to enable microbial maturation may serve as a stable source of host specific microbes that interact with the water column as well as the growing *Ulva.* The continual re-stocking of *Ulva* in the AEW raceways may enable microbial maturity of the *Ulva*, whereby the bacteria present on the *Ulva* has sufficient time to adjust to the carrying capacity within the raceways. If so, microbially matured *Ulva* should exhibit less of a biosecurity concern as a stable carrying capacity on the host is reached, leaving less niche availability for opportunistic growth on *Ulva* given the robustness of mature biofilms. Bearing in mind that biofilms have a higher resistance toward stresses such as turbidity and antibiotic penetration than freestanding bacteria (Stewart & Costerton, 2001), it is not surprising that given time, potential pathogens may occupy the biofilms that form within integrated aquaculture systems. The fact that the *Ulva,* as well as its associated microbiota act as a natural disinfectant and antifouling agent (Egan, et al., 2000; Ravikumar, et al., 2016) lowers the risk of disease as it prevents proliferation of opportunistic bacteria present within biofilms on “biofilters”. Nonetheless, the extent of *Ulva’*s ability to act as a disinfection unit requires further investigation. The farmers at I&J practice good husbandry by draining the tanks and cleaning them between *Ulva* harvests (pers. comm), which is likely to aid in preventing the formation of excessive biofilms.

4.5.2 Future Considerations

In the past, limitations in molecular sequencing technologies have impeded the identification of genera, species and strain specific bacterial communities that are common or specific to marine environments. These limitations are seen when assessing the bacterial consortiums on the cosmopolitan green alga *Ulva australis* whereby DGGE-based evidence suggests that a spatially and temporally stable core community exists (Longford, et al., 2007; Tujula, et al., 2010), which contrasts against 16S rRNA gene sequencing studies which were unable to find a core community, and instead found only six bacterial species of a total of 528 to be common between six

alga (Burke, et al., 2011). Moreover, Martins et al., (2013) critically evaluated the advantages and disadvantages with using barcoded pyrosequencing of 16S rRNA gene fragments for monitoring fish pathogens and suggested that the 16S gene fails to provide sufficient resolution to resolve differences between the bacterial subspecies, *P. damselae* and *P. piscicida* (Osorio & Klose, 2000), which are known to induce different diseases in a variety of fish species (Toranzo, et al., 2005). Similar issues are known to exist for other bacterial species such as *Vibrio,* which have been classified at family and genus level, however are not accurately identified at higher resolutions such as species level (Thompson, et al., 2005). On another note, next generation sequencing technology does not produce long sequence reads of gene fragments (~460 bp used). Often complete nucleotide sequences (~1,500 bp) of the 16S rRNA gene are required to distinguish between closely related bacterial species (Gee, et al., 2003). Moreover, there are disputes regarding which region of the 16S rDNA gene should be sequenced, whereby the area sequenced may vary with experimental design, objectives and sample type (Martins, et al., 2013; Illumina, 2013). The utilisation of additional genes such as the *toxR* (Conejero & Hedreyda, 2003; Muñoz, et al., 2012), gyr*B* and *recA* (Mignard & Flandrois, 2006) may aid in discriminating between closely related bacterial subspecies. Recently, predictive functional profiling of bacterial consortiums using 16S rRNA data has served as a novel means to understand microbial interactions within aquaculture systems by providing insight into molecular pathways within the community interactions (Ortiz-Estrada, et al., 2019). The use of functional prediction analyses would greatly aid in assessing the complex interactions within effluent systems and enable a deeper insight into the biosecurity risks associated with using effluent grown *Ulva* as abalone feed.

Furthermore, the impacts of individual bacterial species on abalone production should be considered in greater detail as the bacteria present within the systems are known to metabolise waste compounds (organic matter, ammonia and nitrite) and interact with the abalone on various levels (potential pathogens, probiotics, growth stimulants, etc.) as discussed above. It would be interesting to disentangle potential differences in microbial communities present in the gut of abalone fed effluent grown *Ulva* and abalone fed *Ulva* grown in seawater to assess the impacts of effluent grown *Ulva* and its holobiont on the performance of the abalone. Flow through systems are highly dynamic and fluctuations in bacterial abundance may occur on a daily basis in response to changing environmental parameters (Gilbert, et al., 2010). Environmental stress and/or change (such as climate change and higher concentrations of faecal matter) can induce variation in the hologenome (Dethlefsen, et al., 2006; Zilber-Rosenberg & Rosenberg, 2008). *Vibrio* abundance for instance is known to vary seasonally, in which warmer months have a greater abundance (Pereira, et al., 2011a, b). Dietary change may additionally shift bacterial communities (Zhang, et al., 2018), and the impacts of artificial diets vs effluent-grown seaweed diets on the abalone as well as the effluent microbiome are unexplored. Precautions to reduce the influence of external bacterial

variance on the water channels could be taken to promote beneficial microbial maturation. One such precaution could be to remove the molluscs within the effluent canal, which may bioaccumulate potentially pathogenic microorganisms (Paul-Pont, et al., 2010). In such instances, farmers should take preference to physical means of cleaning over chemical agents (Delabbio, et al., 2004), as to maintain integrity of existing microbial communities.

4.5.3 Concluding remarks

In this study both a culture (traditional plate count method) and a non-culture (NextGen sequencing of the 16S rRNA gene) based approach were adopted to assess the biosecurity implications of utilising effluent grown *Ulva* as a feed/ feed supplement for the abalone *H. midae* on an integrated multitrophic abalone farm. The sequenced *Ulva* cultivated at I&J abalone farm shared close similarity with *Ulva rigida* (KP233772) and *Ulva scandinavica* (EU484416) on the GenBank database, and hence was referred to with the name of its corresponding molecular synonym, i.e. *U. rigida.* The results suggest a strong algal effect on bacterial communities from both fertilised seawater tanks and abalone effluent raceways. The culture-based approach revealed significant differences in bacterial abundance between the fertilised seawater tanks and abalone effluent wastewater raceways, whereby the he abalone effluent raceways had significantly more culturable bacteria in the inlets and outlets as well as on the *Ulva* when compared to the reciprocal samples in the fertilised seawater tanks. Moreover, it was observed that the *Ulva* has the potential to significantly reduce the bacterial load of abalone effluent water systems, with a decrease in CFU/mL^{-1} recorded on the TSA and TCBS in both the fertilised seawater tanks and abalone effluent raceways.

The Illumina Next Generation Sequencing approach provided fundamental information on bacterial compositions, enabling broad assessments of bacterial genera in the water systems and on the *Ulva*. The bacteria present on the effluent grown *Ulva* was found to share similarities at genus level with biofilter studies on healthy recirculating aquaculture systems (RAS), including *Flavobacteria, Planctomycetes, Roseobacter, Pseudoalteromonas, Desulfovibrio* and *Vibrio*. Both the culture-based and the non-culture based approaches highlight the presence of potentially pathogenic bacteria such as *Vibrio* within the water columns. Nonetheless, It appears as though the presence of *Ulva* in the raceways does not act as a sink for harmful bacteria such as *Vibrio*, and instead appears as though either the *Ulva* or its associated microbiota are capable of positively impacting the water column in terms of managing harmful bacteria known to induce disease. On another note, several probiotic bacterial genera including *Flavobacteriaceae, Roseobacter, Vibrio* and *Alteromonas* were documented on the *Ulva.* Asides from potential pathogenic and probiotic microbes, the Illumina results indicated the presence of several genera that are commonly found in the marine environment which have also been reported on *Ulva.* Moreover, the differentially abundant bacterial genera identified on the effluent grown *Ulva* were composed of general marine bacteria isolated from marine surfaces which are unlikely to pose a significant biosecurity threat.

In conclusion, It does not seem as though the *Ulva* acts as a sink for potentially pathogenic bacteria such as *Vibrio* and *Aliivibrio,* indicating that feeding effluent grown *Ulva* to abalone is not of significant biosecurity concern. Despite finding potential abalone pathogens in the water systems, no symptomatic abalone were observed during the study. As is generally accepted, higher levels of pathogen contamination would imply an increase in the numbers of diseased macrobiota (i.e. abalone, *Ulva* or potentially even consumers). Nonetheless, stress or disease symptoms appear absent in all the above, implying that the abalone is not of biosecurity concern according to Koch's postulate. Even though several commercial abalone farmers consider recirculation within aquaculture feed systems high-risk technology (pers. comm.), no papers have reported disease outbreaks due to the use of effluent grown *Ulva* as abalone feed. Moreover, a productive, bio-secure and disease-free integrated aquaculture system requires a comprehensive understanding of the microbial life present within the system and more in depth sequencing is required to disentangle the impacts of microbial consortiums associated with effluent grown *Ulva* on abalone health.

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