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RESEARCH

Bacterial microbiome dynamics in commercial integrated aquaculture systems growing *Ulva* in abalone effluent water

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Abstract

In South Africa, the green seaweed *Ulva lacinulata* is grown in land-based integrated multi-trophic aquaculture (IMTA) farms with the abalone *Haliotis midae*. The *Ulva* serves as a biofilter and the co-produced *Ulva* is often used as feed for the abalone. To better understand the potential benefits and risks associated with this practice, this study characterised the bacterial microbiome associated with the seawater and *Ulva* raceways receiving abalone effluent (IMTA system) and compared this to *Ulva* tanks supplied with fertilised seawater (non-IMTA; control). *Ulva* samples were collected from each *Ulva* system, and water samples were collected at the inlet and outlet of each system. Bacterial communities were assessed using a culture-based approach and next-generation sequencing (NGS) of the V3-V4 16S rDNA region. It was observed that *Ulva* has the potential to reduce the bacterial load of abalone effluent, with the total number of potential culturable *Vibrio* species declining from 150×10^3 cells mL^{-1} in the inlet to 37×10^3 cells mL^{-1} in the outlet of the *Ulva* system. The NGS dataset supported these findings, with a reduction observed in *Vibrio* and *Pseudoalteromonas* from the inlet to outlet samples. A lower number of genera ($p < 0.05$) were observed on *Ulva* when compared with water samples, indicating that *Ulva* has a beneficial, modulatory effect on bacteria. These findings contribute towards the growing body of evidence for the benefits of seaweeds in IMTA and addresses the biosecurity concerns of abalone farmers wishing to improve the circularity of their farming activities by incorporating seaweeds.

Keywords Bioremediation · 16S sequencing · Aquaculture · IMTA · Microbiome modulation · Seaweed holobiont

Introduction

The high stocking densities associated with most commercial aquaculture farms, including abalone farms, produces effluent water that often contains high concentrations of nitrogen and other dissolved and solid waste products. In partial and fully recirculating aquaculture systems (RAS), these waste products have been shown to impair growth, promote stress and/or disease, and can result in mortalities in more extreme cases (Primavera 2006). Additionally, opportunistic pathogen proliferation can be promoted by a nutrient-rich environment (Defoirdt 2016). Integrated aquaculture systems offer an environmentally friendly means to bioremediate nutrient-rich effluent water generated from monoculture (cultivation of single crop) facilities (Chopin et al. 2001). By using seaweeds to create an ecosystem-based/nature-based management approach to aquaculture, integrated multi-trophic aquaculture (IMTA) addresses some of the negative environmental impacts associated with high density monoculture (Soto et al. 2008), while allowing for

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the production of alternative/additional crops. For example, the green seaweed *Ulva* was identified as an ideal species for bioremediation in land-based aquaculture in the 1900s in Australia (Cotton 1910). In South Africa, *Ulva* spp. have been grown successfully in integrated systems since 2002; where *Ulva* is grown in abalone effluent and sometimes fed back to the abalone as a feed, thereby recycling nutrients sourced from feed and excreted by the animals (Bolton et al. 2009; 2016).

Extractive marine 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 can contribute towards improved sustainability of the aquaculture industry (Bolton et al. 2009). This biomass can be used for various applications, including their use as feeds or secondary crops, medicines (Mao et al. 2006; Hong et al. 2007), cosmetics (Farasat et al. 2014), fertilisers (Selvam and Sivakumar 2013) and other biorefined goods (Buchholz et al. 2012). The use of macroalgae as biofilters shows great promise as it reduces the impact of nutrient release into the environment (Vandermeulen and Gordin 1990; Chung et al. 2002; Nielsen et al. 2012; Lawton et al. 2013; Aníbal et al. 2014), resulting in improved nearby ecosystem health, social perception, and economic stability through crop diversification (Chopin et al. 2001; Soto et al. 2008). Furthermore, the use of *Ulva* as a biofilter in land-based aquaculture facilities has another advantage in that it can allow for partial wastewater recirculation in IMTA systems, substantially reducing pumping and energy costs on farms (Robertson-Andersson et al. 2008; De Prisco 2020) and contributing to the removal of common micropollutants (Hardegen et al. 2023).

Numerous facets of the land-based abalone-*Ulva* aquaculture systems in South Africa have been assessed, including nutrient recycling within the system, changes in water chemistry (De Prisco 2020; Geldart 2022), growth of farmed animals (abalone and urchins) fed diets supplemented with or without effluent grown macroalgae (Naidoo et al. 2006; Mulvaney et al. 2013; Cyrus et al. 2014, 2015a, 2015b; Kemp et al. 2015; Bansemer et al. 2016; Brink-Hull et al. 2022), and socio-economic evaluations (Troell et al. 2006). Nonetheless, very little attention has been devoted to assessing potential biosecurity risks associated with the recirculation of wastewater and use of effluent grown *Ulva* as a feed or feed additive. There has also been limited research on the potential health benefits of growing animals in land-based integrated systems and the effects of this seaweed integration on the microbiome. Managing aquatic environments is complex due to the intimate relationship(s) that exists between microorganisms and their hosts, especially in open flow-through or partially recirculating land-based (pump-ashore) aquaculture systems (Cripps and Bergheim 2000; Olafsen 2001). For example, water quality and disease control are directly impacted by the bacterial communities entering and/or 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 close association between bacteria and their microenvironment, it is vital to understand how communities assemble and function within integrated systems.

While a diverse array of microorganisms is capable of colonising marine macroalgal surfaces (Bolinches et al. 1988; Snoeijs 1994; Bengtsson et al. 2010; Godinho et al. 2013), bacteria are considered the primary colonisers of surfaces in marine environments and have the ability to impact subsequent colonisation by other organisms (Crisp and Ryland 1960; Bryers and Characklis 1982; Henschel and Cook 1990; Garrett et al. 2008). Bacterial colonisation of healthy hosts, as observed for algae (*Ulva*) and abalone on commercial IMTA farms in South Africa, suggests that a positive relationship exists between these hosts and the bacteria associated with them (collectively termed the "holobiont"). Interactions between macroalgae and bacteria have been widely documented and include both beneficial and pathogenic associations (Armstrong et al. 2001; Dale and Moran 2006; Goecke et al. 2010; Wichard 2015; Califano et al. 2020; Li et al. 2023). There are also possible communal relationships where host-associated bacteria do not impact host functionality (Macpherson and Harris 2004) or, in the case of aquaculture species, host productivity. However, *Ulva* performance/productivity (morphological development, growth and biochemical composition) has been previously linked to the seaweed beneficial microorganisms (SBMs) associated with them (Alsufyani et al. 2020; Polikovsky et al. 2020; Wichard 2015; 2023; van der Loos 2021; 2022; 2024; Li et al. 2023), releasing algal growth and morphogenesis promoting factors (AGMFs) (Ghaderiardakani et al. 2019). Microbial communities are thought to assemble and associate with hosts in one of two ways and these mechanisms are termed "the hologenome theory" and the "competitive lottery model". The hologenome theory proposes that a stable core microbial community is present and, collectively with its host, acts as a selective force for evolution (Zilber-Rosenberg and Rosenberg 2008). In contrast, the competitive lottery model suggests that a microbial community forms as a result of/in response to its surrounding environment, where individual taxa simply co-exist in a variable environment (Chesson and Warner 1981). Burke et al. (2011) suggested that the assemblages of *U. australis* are determined by a lottery model as opposed to symbiotic interactions. However, several specific bacteria that are essential for settlement, growth and morphogenesis have been consistently found in associated with *Ulva* spp. (Spoerner, et al. 2012; Wichard 2015; Ghaderiardakani

et al. 2019; Califano et al. 2020; van der Loos et al. 2021, 2022, 2024), suggesting that a holobiont exists but may be influenced by the environment (e.g., land-based vs open water aquaculture systems) and environmental parameters (e.g., water quality, seasons) to some extent.

In South Africa, five large (each producing 200 – 600 t of abalone per annum) commercial abalone farms collectively cultivate more than 2 000 t of *Ulva* in large D-shaped paddle-raceways (ca. 30 m long, 8 m wide, 0.5 – 1 m deep), mostly in abalone effluent (Bachoo et al. 2023). Despite this, no published information exists on the microbial communities associated with the seawater and *Ulva* in these intensive land-based pump-ashore mariculture systems. This information is particularly important given that some of these farms utilise the *Ulva* as feed for the abalone, which has been shown to have consumption, growth and health benefits for the abalone (Naidoo et al. 2006; Mulvaney et al. 2013; Kemp et al. 2015; Bansemer et al. 2016). However, the biosecurity concerns associated with feeding effluent-grown *Ulva* is preventing further IMTA uptake in South Africa and abroad (Bolton et al. 2009).

To better understand potential benefits and risks associated with the use of effluent-grown *Ulva* as a feed, as well as the role of *Ulva* in these IMTA systems, the bacterial microbiome associated with seawater and *Ulva* grown in non-recirculating (flow-through) raceways receiving either abalone effluent (IMTA) or fertilised seawater (non-IMTA; control) was assessed in this study. By improving the understanding of integrating *Ulva* into aquatic systems and its impact on the microbiome, this study could contribute to the improvement of existing recirculating aquaculture technologies and increase the uptake of the integration of *Ulva* in aquaculture practices, thereby improving the sustainability, productivity and profitability of the aquaculture sector.

Materials and methods

Study site and sampling

Water and *Ulva lacinulata* samples were collected from *Ulva* paddle-raceway systems at Irvine & Johnson (I&J) Cape Abalone Farm at Danger Point, Gansbaai (34°34'60 S; 19°21'0 E) in August 2017. Samples were collected from two separate *Ulva* production systems on the farm (Fig. 1). One system consisted of tanks that received seawater supplemented with inorganic fertiliser (non-IMTA/Seawater), whereas the other system was comprised of raceways receiving abalone effluent water (IMTA/Effluent). Triplicate *Ulva* samples were collected from within each system, whereas the water samples (in triplicate) were collected at the inlet and outlet of each system. In total, two seawater tanks (there were only two on the farm at the time of this study) and five effluent raceway systems were sampled (Fig. 1), resulting in n = 15 samples (five inlet, outlet and *Ulva*) in the IMTA systems and n = 6 samples in the non-IMTA systems (two inlet, outlet and *Ulva*), respectively.

The non-IMTA systems serve as algal "seeding tanks" for the larger *Ulva* raceways and are based on the system previously described by Robertson-Andersson (2003). These systems are comprised of two semi-circular, PVC lined containers with an approximate surface area of 3 m × 1 m and a depth of 0.5 m supported on a wooden frame. Fresh seawater is pumped directly from the adjacent surf zone into the tanks via a sub-surface filter system and exits the tanks passively. Tanks are actively aerated and pulse-fertilised with ammonium phosphate twice weekly to promote algal growth.

The effluent (IMTA) *Ulva* raceways systems receive nutrient rich effluent water from the abalone raceways. Water gravity fed from the abalone culture tanks enters through a central canal, after which it passively flows through each of the 13 parallel raceways (L × W × D; 27.7 m × 8.23 m × 0.45 m) via openings along the channel (Fig. 1). No mixing of water occurs between the raceways. A paddlewheel is used to circulate and agitate the *Ulva* within each raceway, as well as to oxygenate the water. 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. Each effluent *Ulva* raceway consistently produces 1.5 to 1.75 t wet biomass per month (Shuuluka 2011; Bolton et al. 2016) and is utilised as a feed for abalone on the farm.

Healthy vegetative *Ulva* thalli (approximately 15 g fresh weight), with no necrotic white tissue or noticeable epiphytes, were collected from the seawater and effluent systems and immediately transferred into separate pre-labelled sterile 50 mL conical centrifuge tubes that had been placed in a cooler box with ice. Thereafter, samples were transferred to the Department of Forestry, Fisheries and the Environment (DFFE) Marine Research Aquarium (MRA) in Cape Town, South Africa, within three hours for further processing. At the research facility, the freshly collected *Ulva* was rinsed with 10 mL of 0.22 µm filtered autoclaved seawater via inversion for 20 s, to remove any loosely attached organisms and debris. Directly after rinsing, a portion of the *Ulva* (approximately 20 mg) was used to quantify the culturable bacteria. 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 prior to next-generation sequencing (NGS).

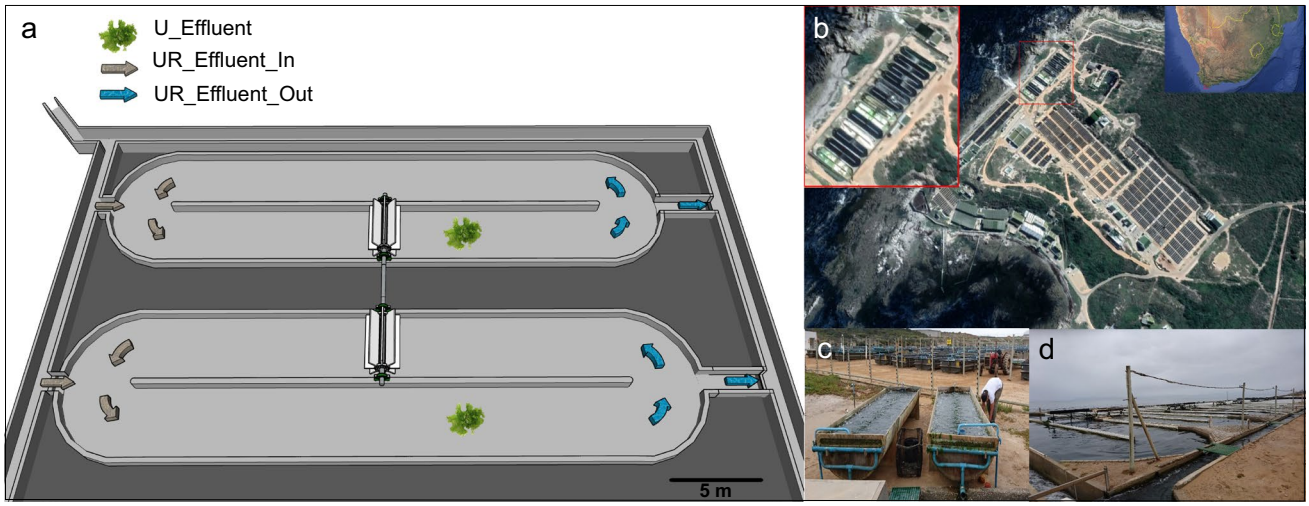


Fig. 1 Schematic of the abalone effluent wastewater *Ulva* raceways (UR) at I&J abalone farm showing the *Ulva* raceways receiving effluent water from the abalone tanks (UR_Effluent_In) and water going out of these tanks (UR_Effluent_Out) (scale bar = 5 m) (a), as well as the location of these *Ulva* raceways (b). Seawater systems consisted of two tanks containing *Ulva* (U_Seawater) that received non-effluent seawater (UR_Seawater_In) and releasing water to the sea (UR_Seawater_Out) (c), as opposed to the *Ulva* raceways bio-remediating abalone effluent water (d)

Inlet and outlet water samples (500 mL) were collected in triplicate from the seawater (non-IMTA) and effluent (IMTA) systems in sterile 500 mL Schott bottles. Samples were stored on ice and transferred to the MRA in Cape Town, where they were processed for enumeration of culturable bacteria and NGS as described below.

Enumeration of bacteria in seawater and on *Ulva*

Three types of microbiological media were used to enumerate live culturable bacteria in seawater and on *Ulva*: Tryptic Soy Agar (TSA; Difco), Thiosulfate Citrate Bile Sucrose Agar (TCBS; Sigma Aldrich), and Ulvan Agar (UA; Jaulneau et al. 2010). TSA is a general-purpose growth medium frequently used for the isolation of a wide variety of marine microorganisms (Lemos et al. 1985; Romanenko et al. 2008; Rodrigues and de Carvalho 2022). TCBS Agar is used for the isolation of potential culturable *Vibrio* spp. (Pfeffer and Oliver 2003), whereas UA is a minimal medium supplemented with Ulvan, the primary carbohydrate of *Ulva* (prepared according to Jaulneau et al. 2010). Though it should be noted that a limited number of bacteria are culturable using standard agar plate techniques and laboratorial conditions (Rodrigues and de Carvalho 2022), this approach allowed for the evaluation of differences in bacterial colony forming units across different system types (IMTA vs non-IMTA) and between samples collected from different system compartments (i.e. inlet vs outlet vs *Ulva*). The culture-based approach also allowed for the isolation, purification and identification of bacteria that appeared in high abundance for comparison with the next-generation sequencing (NGS) approach.

Preparation of media

The TSA and TCBS agar plates were prepared as per the manufacturer's instructions and supplemented with 2.0% and 1.5% NaCl (w/v), respectively. The UA plates consisted of a basic minimal media containing 0.1% (w/v) yeast extract, 0.5% (w/v) ulvan extract (described below), and 15 g L^{-1} of bacteriological agar in 1000 mL sterile autoclaved seawater. Ulvan was extracted from dry *Ulva* by homogenising 50 g of dried *Ulva* into a coarse powder (< 1 cm) in a food processor before diluting the *Ulva* powder with 450 mL Millipore water and autoclaving at 120ºC for 15 min. After allowing partial cooling, the algal suspension was filtered through a sieve (0.25 mm) and centrifuged at 5000×*g* for 5 min. The pellet was discarded and 2.5 volumes (1 125 mL) 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 5000×*g* for 5 min, washed with 96.9% ethanol and vacuum dried in a centrifuge (1 200 rpm) at 30ºC for 4 h. The dry ulvan was ground into a fine powder using a mortar and pestle and aliquots were stored in Eppendorf tubes in a -20ºC freezer until needed. On average, 50 g of dried *Ulva* yielded 4.612 g of ulvan (9.2% dw).

Enumeration of bacteria in seawater and on *Ulva*

For the isolation and enumeration of culturable bacteria from seawater, the 500 mL water samples collected from the seawater (n = 2 per sample type) and effluent water systems (n = 5 per sample type) were aseptically filtered through 0.22 µm-pore-size filter membranes (47 mm diameter, Millipore Corp., USA). 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 10 mL sterile autoclaved seawater (ASW). Tubes were vortexed for 5 min to remove any remaining bacteria attached to the membrane. Thereafter, the membranes were discarded, and 0.1 mL aliquots of the re-suspended cells were serially diluted (10^{-2} – 10^{-5}) and plated in triplicate (100 µL aliquots) on TSA, TCBS and UA plates. The remaining 9.9 mL of seawater containing bacteria from each sample was centrifuged for 10 min at 10 816×g to concentrate the bacterial cells. Following centrifugation, the supernatants were carefully decanted and the tubes containing the pellet were stored at -80°C until required for DNA extractions and NGS.

Bacteria were isolated from fresh vegetative thalli of *Ulva* 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 0.5 g of thallus was vortexed vigorously in 10 mL ASW for 10 min to detach the associated epibacteria. The thallus was then removed, and the remaining supernatant was serially diluted (10^{0} – 10^{-3}) and 100 µL aliquots were plated in triplicate onto the three selective media. Immediately following plating, petri dishes were sealed with Parafilm, and the TSA and TCBS plates were incubated at room temperature for two days, whereas UA plates were incubated at room 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 number of culturable bacteria per mL seawater or g of *Ulva* for each dilution series was calculated and averaged. Thereafter, assay replicates for each experimental replicate (n = 5 for IMTA, and n = 2 for non-IMTA) were averaged and data expressed as the mean ± SE for each media type.

Isolation, identification and storage of culturable bacteria

Following 2 – 7 days of incubation, bacterial colonies of interest were selected from each media type and transferred to fresh plates of the corresponding media type to isolate pure colonies. Bacterial colonies that appeared unique, based on colony form (shape, colour, size and 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 and used for long-term storage. For long-term storage, glycerol socks (20% sterile glycerol in pre-autoclaved Tryptic Soy Broth solution (TSB); 0.625 g NaCl, 0.6 g TSB powder in 20 mL Millipore water) were prepared for each isolate and stored in a -80ºC ultra-freezer.

Genomic DNA was extracted from the chosen bacterial isolates and subjected to 16S rDNA amplification and Sanger sequencing to identify the isolated bacteria (Supplementary Table 1). Genomic DNA was isolated by picking cells from a single colony and suspending them in 300 µL of sterile Millipore water in a 1.5 mL 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 300 µL sterile water. Bacterial pellets were homogenised for 1 min using a hand-held pellet pestle and transferred to new sterile 1.5 mL microcentrifuge tubes containing 0.04±0.05 g Chelex-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 Chelex-100 beads were briefly vortexed and incubated for 20 min at 56ºC on a heating block. Post incubation, samples were briefly vortexed and incubated for a further 30 min at 95ºC to lyse bacterial cells. The lysed samples were placed on ice to cool for 5 min, vortexed briefly and centrifuged at 16 000×*g* for 5 min.

The universal broad-spectrum bacterial primers 16S F-fD1 and 16SR-Rp2 (Supplementary Table 1; Lane 1991) were used to amplify an approximately 1.5 kb fragment of the 16S rRNA gene region of each isolate. PCR reaction mixtures (25 µL; performed in triplicate) consisted of 1 µL genomic DNA (ca. 25 ng), 12.5 µL KapaTaq ReadyMix (Kapa Biosystems; Cat#KK1006), 10.5 µL Millipore water, and 0.5 µL of each primer (400 nM). Amplification was conducted using a Labnet Multigene Thermal Cycler (Labnet International Inc.) and consisted of an initial denaturation at 96ºC for 3 min, followed by 40 cycles of 45 s at 96ºC, 30 s at 57ºC, and 1 min at 72ºC, with a final extension of 72ºC for 5 min. Known amounts of bacterial genomic DNA, previously analysed by PCR and subjected to sequencing, were included as positive controls during the PCR and non-template controls (PCR-grade H_2O) were included as negative controls. The amplified PCR products, as well as the positive and negative controls were visualised on a 0.8% agarose gel electrophoresis to assess reaction specificity and fragment size (ca. 1.5 kb fragment expected). The PCR product obtained from each bacterial colony was purified and sequenced as described above at the Stellenbosch University Central Analytical Facilities

(CAF). The universal 16S forward primer F-fD1 was used for sequencing (Supplementary Table 1). The sequences were edited using CLC Main Workbench and homology searches were carried out using the BLASTN algorithm (Altschul et al. 1990) provided by the National Centre for Biotechnology Information (NCBI).

Statistical analyses for colony forming unit (CFU) data

SigmaPlot 12.0 software was used to perform all CFU data statistical analysis. To determine whether the number of culturable bacteria differed between the inlet (incoming) and outlet (outgoing) water within and between each system (n = 2 per sample type across 2 independent non-IMTA systems; n = 5 per sample type across 5 independent IMTA systems), including the abalone effluent water and seawater system (CFU mL^{-1}), as well as that on *Ulva* from each system (CFU.g^{-1} of *Ulva*), a one-way analysis of variance (ANOVA) was performed (statistically significance at $p < 0.05$), with post-hoc Holm-Sidak tests for comparisons between samples. Data for the different media types (TCBS, TSA and UA) were treated separately. All data were tested for normality (Kolmogorov-Smirnov test) and equal variance. Data were square root transformed prior to statistical analysis. Data values on all figures refer to mean±standard error of the mean (SE).

Bacterial community profiling by 16S rDNA sequencing

DNA extraction, library preparation and sequencing

Microbial genomic DNA was isolated from frozen ground *Ulva* samples (to target both epi- and endophytic bacterial communities) and bacterial pellets, obtained from the seawater samples, 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 5 mg of each frozen ground *Ulva* sample collected from the seawater and effluent water systems, respectively, was used for DNA extraction. A total of 14 frozen bacterial pellets and 7 ground *Ulva* samples were processed. DNA concentration, integrity and fragment size were determined via spectrophotometry and 0.8% agarose gel electrophoresis.

The 16S_341F and 16S_805R primer pair (Klindworth et al. 2013), with added Illumina adapter overhang nucleotide sequences (Supplementary Table 1), were used to amplify the 16S V3-V4 hypervariable 16S gene region of each sample. Each PCR reaction (25 µL; performed in triplicate) consisted of 1 – 5 µL of genomic DNA (ca. 25 ng total), 12.5 µL 2×KAPA HiFi HotStart ReadyMix for hotstart PCR (Kapa Biosystems; Catalog #KM2605), 5.5 – 10.5 µL ddH_2O, and 0.5 µL of each primer (200 nM). 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. The PCR conditions were as follows; an initial denaturation at 95ºC for 5 min, 10 cycles of touchdown PCR (30 s at 95ºC, 30 s at 65ºC, with a 1ºC decrement per cycle, and 30 s at 72 ºC), followed by an additional 25 cycles of PCR (30 s at 95ºC, 30 s at 55ºC, and 30 s at 72ºC), and a final extension step for 10 min at 72ºC. Negative controls, containing all components other than 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 (ca. 460 bp fragment expected).

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 to remove 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, whereafter final library quantification and normalisation were performed. Normalised libraries were pooled prior to paired-end sequencing (2×250 bp) on the Miseq Illumina sequencer, as per manufacturer protocols.

16S raw data processing

Analysis of the 16S V3-V4 rDNA sequences generated using Illumina's MiSeq platform were performed using the open-source Quantitative Insights into Microbial Ecology 2 (QIIME2 version 2020.11) software (Caporaso et al. 2010). The DADA2 pipeline (Callahan et al. 2016) was used to process a total of 6 016 889 demultiplexed sequences across 21 samples (average of 286 519 reads per sample), where forward and reverse reads were trimmed to lengths of 227 and 203 bp, respectively, to remove low quality reads (Q20 score < 20). Subsequently, DADA2 was used to filter, denoise and merge reads, which resulted in reads with a final average read length of 419±11 bp (Supplementary Table 1). An average of 7.63% of the total reads were detected as chimeric and were removed from analyses. After filtering, denoising, merging and removing chimeric reads, a total of 2 138 906 dereplicated sequences were included in subsequent analyses, with an average of 101 853 reads per sample. Taxonomy was assigned using a naive Bayes classifier trained on the SILVA

database (release 132) (Quast et al. 2013; Yilmaz et al. 2014; Glöckner et al. 2017) for each amplified sequence variant (ASV), representing each unique read of the sequenced gene region (Callahan et al. 2017) up to genus-level.

Data filtering and within sample diversity assessments

The ASV dataset was filtered and normalised using MicrobiomeAnalyst (Dhariwal et al. 2017). Data was filtered for low abundance reads (minimum count of 2 with 20% prevalence in samples), whereby ASVs containing mostly zeros were removed to account for potential sequencing errors and low-level contaminations (Dhariwal et al. 2017). This filtered dataset was used to calculate alpha diversity (within sample) statistics (Chao1, Shannon and Simpson index).

Bacterial community alpha diversity was evaluated using the R *phyloseq* (McMurdie and Holmes 2013) and *vegan* (Dixon 2003) packages implemented in MicrobiomeAnalyst. The Chao1 (Chao 1984), Shannon (Shannon 1948) and Simpson (Simpson 1949) non-parametric diversity indexes were evaluated to assess species richness, evenness and uniqueness, respectively. Statistical significance between cohorts was assessed with an analysis of variance (ANOVA; $p < 0.05$) for each index. Lastly, rarefaction curves were constructed in MicrobiomeAnalyst (Dhariwal et al. 2017) by randomly sampling a fixed number of reads to assess whether there was sufficient coverage to capture the bacterial diversity in each sample, which was further assessed by calculating Good's coverage indices across samples.

Normalisation and between sample comparisons

Low variance reads (10% based on standard deviation), unlikely to be associated with study conditions, were removed to reduce the effects of multiple testing on downstream comparative analyses. Filtered data was normalised through relative log expression (RLE) transformationto account for sparsity, under-sampling and uneven sequencing depth in downstream beta diversity analyses.

Beta diversity analyses were conducted in MicrobiomeAnalyst using the R *phyloseq* and *vegan* packages. A NMDS analysis, based on Bray-Curtis dissimilarity indices, was performed using a non-rarefied dataset. These analyses were conducted at the genus level to provide an overview of the microbial community structure in the seawater (non-IMTA) and abalone effluent water (IMTA) systems, as well as the microbiota on the *Ulva* within each system. Statistical significance ($p < 0.05$) was assessed using a permutational multivariate analysis of variance (PERMANOVA; Anderson 2001), which tests for homogeneity across data points. Additionally, to further assess the degree of dissimilarity across samples, a permutational analysis of multivariate dispersions (PERMDISP; Anderson et al. 2006) and an analysis of similarities (ANOSIM; Clarke 1993) was conducted. The online webtool, Jvenn (Bardou et al. 2014), was used to construct a Venn diagram by assessing common bacterial ASVs at genus level in the non-IMTA and IMTA system, respectively, without incorporating relative abundance information. Lastly, 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.

MicrobiomeAnalyst was also used to assess taxonomic abundance across the respective groups, where abundance tables were constructed based on relative abundances (%). The 30 most represented ASVs were displayed and the remaining ASVs were combined and denoted as "Others". Differential abundance across the water and *Ulva* samples was assessed using the DESeq2 (Love et al. 2014). The false discovery rate (FDR) was calculated to adjust *p*-values for multiple comparisons, mitigating the possibility of type I errors (Benjamini and Hochberg 1995).

Functional profiling across water and *Ulva*-associated microbiomes

The functional potential of the bacterial communities from the seawater system, effluent system and *Ulva* in each system was assessed by mapping RLE normalised ASVs to Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) (Kanehisa and Goto 2000) using the QIIME against SILVA database annotation pipeline of the R package *Tax4Fun* (Aßhauer et al. 2015) in the Marker Data Profiling (MDP) module of MicrobiomeAnalyst. Subsequently, the Shotgun Data Profiling (SDP) module was used to analyse the differences in KEGG metabolism, where putative enzymes are mapped onto known metabolic pathways, across water and *Ulva* samples. The statistical software JASP (JASP Team 2017) was used to assess data for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). As data were not normally distributed, a Kruskal-Wallis test with a post-hoc Dunn's test was performed to test for statistically significant ($p < 0.05$) differences in putative functional capabilities between cohorts.

Results

Enumeration of culturable bacteria in seawater and on *Ulva*

Culturable bacterial numbers were significantly ($p < 0.05$; one-way ANOVA) higher in the systems receiving effluent water from the abalone raceways, when compared with the *Ulva* tanks receiving seawater that was fertilised (Fig. 2A). The bacterial abundance associated with *Ulva* cultured in the IMTA systems was also significantly higher than on *Ulva* cultured in the non-IMTA systems on all three selective

media types (Fig. 2B). In both systems, the macroalga *Ulva* appeared to have a strong modulatory effect on bacteria, with culturable numbers on all media types decreasing from the inlets to the outlets of the *Ulva* raceways. This modulatory effect was more pronounced in the IMTA systems, particularly for the total number of potential *Vibrio* (indicated by growth on TCBS), which decreased significantly ($p = 0.032$; one-way ANOVA) from the inlet (150×10^3 cells mL^{-1}) to the outlet (37×10^3 cells mL^{-1}) of the IMTA systems (Fig. 2A). In contrast, there was no significant decline in the total number of general marine bacteria between the inlets and outlets of the IMTA systems ($p = 0.096$), as indicated by growth on TSA (Fig. 2A), though this result could have been influenced by low sample numbers. There was a high abundance of ulvan specific bacteria associated with the *Ulva* collected from both the IMTA and non-IMTA systems, with

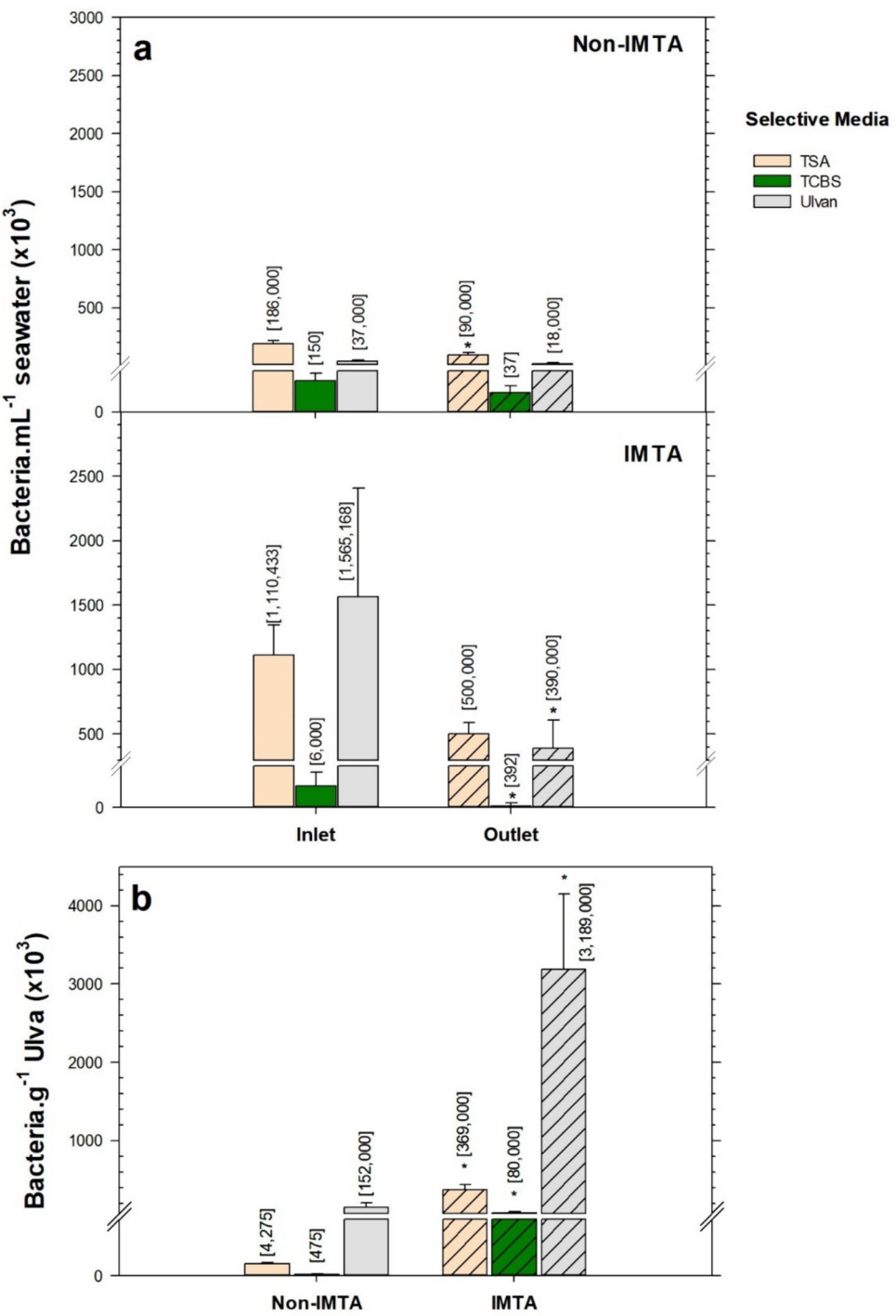


Fig. 2 Number of culturable bacteria per mL seawater (a) collected from the inlets (abalone effluent water entering tanks) and outlets (bioremediated water exiting tanks) of the fertilised (non-IMTA; n = 2 per sample type in triplicate) and effluent (IMTA; n = 5 per sample type in triplicate) *Ulva* raceway systems. (b) Culturable bacteria per gram of *Ulva* collected from the non-IMTA and IMTA systems. Bacteria were cultivated on three selective media: Tryptone Soy Agar (TSA), Thiosulfate Citrate Bile Sucrose agar (TCBS) and Ulvan Agar. All data represents the means (±SEM) of triplicate readings across sample types from the respective systems. An asterisk represents a significant difference ($p < 0.05$; t-test) between the inlet and outlet or non-IMTA verses IMTA *Ulva* on a specific selective media

the abundance of these bacteria shown to be significantly ($p = 0.005$) higher in the IMTA than in the non-IMTA systems (Fig. 2B).

Identification of culturable bacteria

The 11 bacterial isolates evaluated in this study included three isolates from ulvan (U2, U10 and U14), five from TSA (T3, T5, T6, T8, and T10) and three from TCBS (V1, V2, and V3). These isolates were evaluated based either on their unique morphology or their elevated abundance on the three media types. The nucleotide sequences obtained following PCR amplification and sequencing of the forward read using primer (F-fD1) of the 16S rRNA gene region of each of the isolated bacteria of interest resulted in sequences of approximately 460 bp. A BLAST search on GENBANK revealed that most of the sequences showed high similarity to organisms that are frequently isolated and identified from marine environments, including several *Vibrio* and *Pseudoalteromonas* species (Table 1).

Bacterial community profiling by 16S rDNA sequencing

Raw data processing, filtering and normalisation

Microbial diversity in the seawater (non-IMTA) system, abalone effluent water (IMTA) system and on the *Ulva* grown in both systems was assessed using next-generation sequencing of the V3-V4 region of the 16S rDNA. After quality control steps (trimming and filtering), a total of 2 138 906 sequence reads were retained across the 21 samples included in this study, with an average of 101 853±17 703 reads per sample (Supplementary Table 2) and all samples reaching a plateau in the rarefaction analysis (Supplementary Fig. 1). Raw sequence data is publicly available under project accession number

Table 1 The top three sequence similarities as obtained from the BLAST search of the GENBANK database with the ~460 bp 16S rDNA gene sequence of the 11 bacterial isolates. Sample sources are indicated, whereby; UR_Seawater_In = *Ulva* raceway seawater inlet (non-IMTA), UR_Seawater_Out = *Ulva* raceway seawater outlet (non-IMTA), UR_Effluent_In = effluent water inlet (IMTA)

16S rRNA gene sequence similarities			
Sample ID (source)	Closest 3 matches	% Sequence similarity	GENBANK accession number
U2 (UR_Seawater_In)	*Celeribacter* sp. str. 70067	97	MF045112.1
	Celeribacter sp. R-52665	97	KT185135.1
	Tropicibacter napthalenivorans	97	KJ004586.1
U10 (UR_Effluent_In)	Uncultured bac. clone IWNB040	99	FR744587.1
	Marine bac. IVA013	99	KJ814253.1
	Roseobacter sp. pp_D2A 2	99	KC250895.1
U14 (UR_Seawater_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 (UR_Effluent_In)	*Agarivorans* sp. hydD622	99	KM203871.1
	Agarivorans sp. VibC-Oc-061	99	KF577090.1
	Mucus bac. 63	98	AY654801.1
T5 (UR_Effluent_In)	*Pseudoalteromonas* sp. str. 70375	99	MF061293.1
	Pseudoalteromonas sp. str. 70325	99	MF061291.1
	Pseudoalteromonas sp. str. 70320	99	MF061290.1
T6 (UR_Effluent_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 (UR_Effluent_In)	Uncultured bac. AND_GV0309	100	JQ032521.1
	Halamonas ventusa str. NBRC101992	100	AB681651.1
	Halamonas sp. MSSRF DN61	99	KU131286.1
T10 (UR_Effluent_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 (UR_Seawater_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 (UR_Seawater_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 (UR_Seawater_In)	Rhodobacteriaceae sp.	96	AM990859.1
	Celeribacter sp. R-52665	96	KT185135.1
	Celeribacter sp. R-52661	96	KT185131.1

PRJNA1075670 on the NCBI sequence read archive (SRA) database.

Library sizes ranged from 25 765 to 312 621 reads prior to data normalisation (Supplementary Table 2). All rarefaction curves approached the saturation plateau and reached Good's coverage indices ranging from 99.993 – 100%, indicating that there was sufficient sequence reads within each of the 21 samples (Supplementary Fig. 1). Overall, 481 genus-level ASVs were identified after mapping reads to the SILVA reference database. A total of 197 low abundance features were removed based on prevalence prior to conducting alpha diversity analyses and for the construction of relative abundance plots, and a total of 53 low variance features were removed based on standard deviation, resulting in the total removal of 250 features prior to differential abundance assessments using DESeq2. A total of 231 genus-level ASVs were included in downstream analyses.

Within sample diversity

Overall, water samples collected from the seawater tanks had the highest ASV abundance, when compared with *Ulva* samples (Fig. 3). Moreover, bacterial diversity in terms of evenness in the distribution of bacterial genera and the uniqueness of these communities across cohorts did not vary significantly across the samples collected from effluent water, fertilised seawater and *Ulva*, as no significant differences were observed for both the Shannon and the Simpson diversity indexes, with ANOVA F-statistics of 1.13 (p = 0.39) and 1.52 ($p = 0.24$), respectively (Fig. 3). In contrast, the richness estimator Chao1 was significantly higher for the water samples (both IMTA and non-IMTA) than for the *Ulva* samples from both systems (F = 7.11, $p = 0.001$), indicating a high degree of unique ASVs in the water column (Fig. 3).

Bacterial community structure in a non-IMTA vs IMTA system

Clustering analysis, based on Bray-Curtis matrices, showed that the 21 samples are broadly arranged into two clades (Fig. 4A). The first clade is composed of water samples from both the IMTA and non-IMTA systems, whereas the second clade is primarily composed of *Ulva* samples from both systems. It appears as if several subclades exist within the dendrogram. For instance, the inlets and outlets of the effluent water appears to be further subdivided into subclades (Fig. 4A). The *Ulva* samples are also grouped as such and form their own subclades (i.e., *Ulva* growing in fertilised seawater and *Ulva* growing in effluent water). However, the seawater raceway inlet and outlet samples seem to be randomly distributed, which may partly be due to small sample size.

Non-metric multidimensional scaling (NMDS) at genus level shows three clusters, belonging to the effluent system (UR_Effluent_In, UR_Effluent_Out, and U_Effluent), with some degree of overlap for samples collected from inlets and outlets of the *Ulva* raceway (UR) and distinct clustering of the *Ulva* (U) samples (Fig. 4B). It can also be observed that the UR_Seawater_In and UR_Seawater_Out samples cluster closely with the effluent system inlets and outlets. Similar clustering patterns were observed at all other hierarchical levels (data not shown). The NMDS stress of 0.07 implies a good fit, allowing confident inferences. The samples collected from the seawater system did not form clusters with 95% confidence intervals as a result of the small sample size (n = 2 samples per sample type). A shift in the overall structure of the microbiota from the inlets to the outlets of both raceway types, towards a community structure with greater similarity to the *Ulva* samples, can be observed, with some of the outlet samples grouping with the *Ulva* samples to some extent. The bacterial communities associated with *Ulva* from the effluent and seawater systems also cluster together, suggesting that *Ulva* has a distinct microbial profile that is similar across non-IMTA and IMTA environments. These clustering patterns are also supported by the analysis of similarities (ANOSIM) with an R-value of 0.79 ($p < 0.001$) and permutational analysis of multivariate dispersions (PERMDISP) with an F-value of 10.13 ($p <$ 0.001) (Fig. 4B), all of which indicate some degree of dissimilarity in bacterial community profile. As observed in the

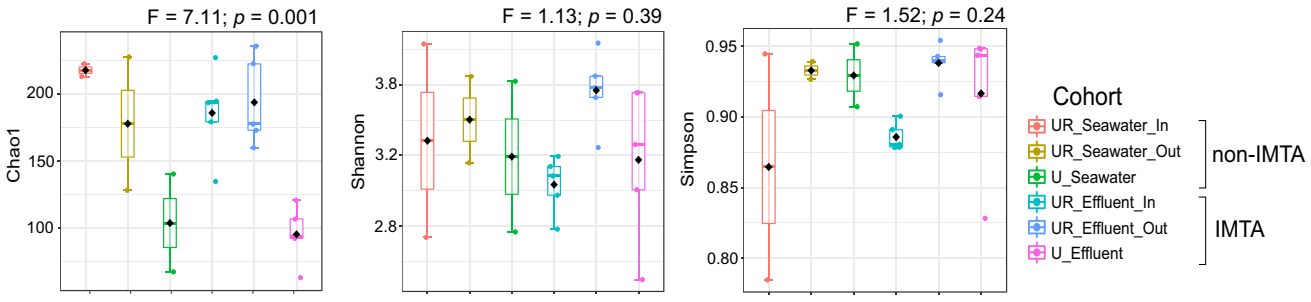


Fig. 3 Average within sample diversity measures (Chao1, Shannon and Simpson) for samples collected from seawater (non-IMTA) and effluent (IMTA) systems at genus level, where the median and standard error for each is indicated. The ANOVA F-values and corresponding p-values are also indicated for each diversity index. UR – *Ulva* raceway; U – *Ulva*

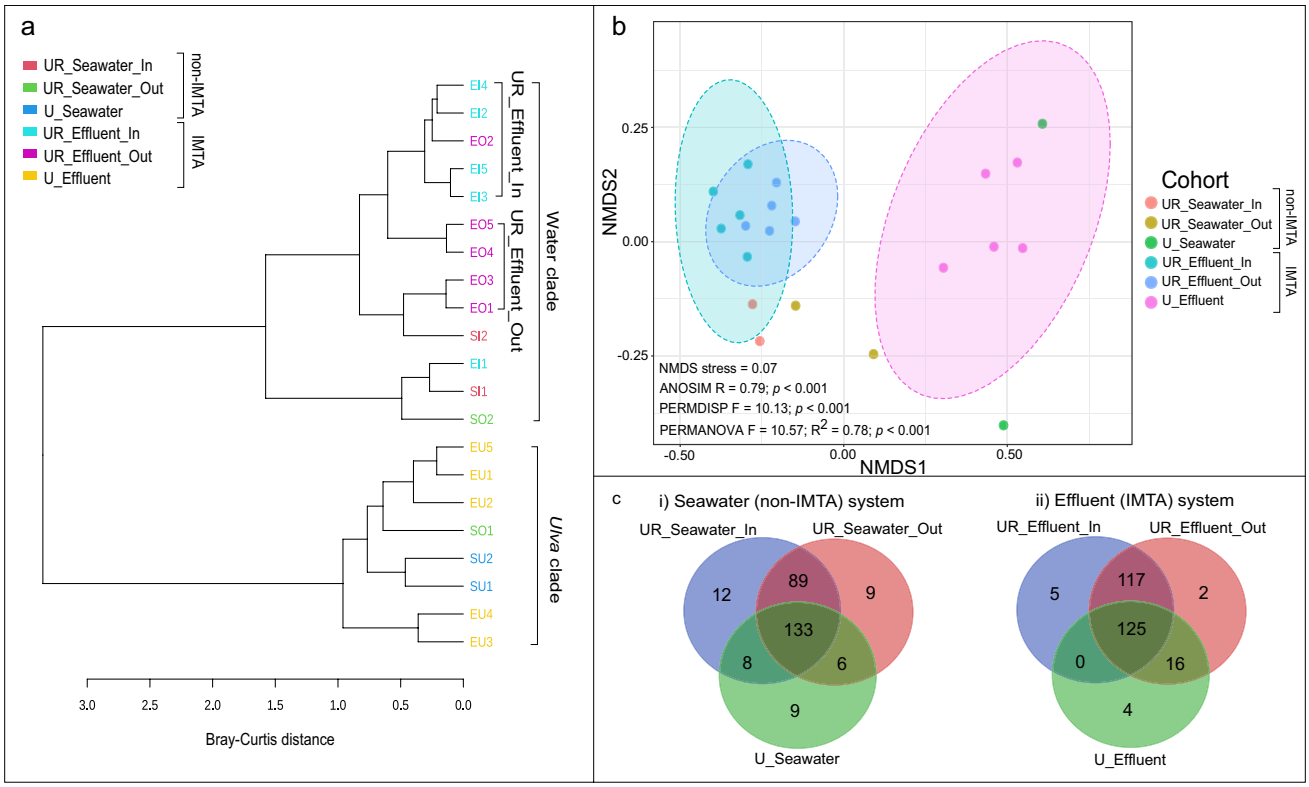


Fig. 4 Between sample (beta) diversity analyses including (a) dendrogram analysis using the Ward linkage method and Bray-Curtis distance measure, where sample sub-clades are also indicated; (b) non-metric multi-dimensional scaling analysis (NMDS) based on Bray-Curtis distance matrices at genus level showing a distinction between water and *Ulva* samples with ellipses indicating clustering at a 95% confidence level; and (c) a Venn diagram of overlapping bacterial communities across water samples collected from inlets and outlets, as well as bacterial communities associated with *Ulva* in the (i) non-IMTA system (n = 6) and (ii) IMTA system (n = 15). UR – *Ulva* raceway; U – *Ulva*

dendrogram analysis, the 21 samples are broadly arranged into two clades, i.e. a water clade, and an *Ulva* clade (Fig. 4). A further separation could be observed for the inlet and outlet water samples collected from the IMTA (effluent) system.

Lastly, Venn diagrams indicated that 133 genera were shared by all samples collected from non-IMTA systems (Fig. 4C). A further 89 genera were shared by water collected from inlets and outlets of the non-IMTA *Ulva* raceway, whereas only 8 and 6 genera were common between the samples collected from *Ulva* and the inlets and outlets, respectively. Similarly, in the effluent *Ulva* raceways, 125 genera are shared by all cohorts, with 117 genera being shared between water samples from inlets and outlets but only 16 genera being shared between outlet and *Ulva* samples (Fig. 4C). No genera were shared between the inlet water and *Ulva* from the IMTA system. The pattern observed in the NMDS plot was also observed here, where the number of genera exclusively associated with the outlet samples (number of genera = 9 and 2, respectively) was lower than those exclusively associated with inlet water samples (number of genera = 12 and 5, respectively) for both the non-IMTA and IMTA systems.

Taxonomic identification and abundance of bacteria associated with *Ulva* and water samples

At phylum level, majority of the bacteria identified in this study belonged to Proteobacteria (60%), which was followed by Bacteroidota (25%) and Fusobacteriota (4%). At class level, the three most abundant classes identified were Gammaproteobacteria (50%), Bacteroidia (25%) and Alphaproteobacteria (10%). At family level, most of the bacteria belonged to the families Pseudoalteromonadaceae (16%), Saprospiraceae (12%), Thiotrichaceae (11%), Flavobacteriaceae (10%), Vibrionaceae (7%) and Rhodomonadaceae (5%).

The three most prevalent ASVs at genus level across samples included *Pseudoalteromonas* (15%), *Leucothrix* (9%) and *Vibrio* (7%) (Fig. 5; Supplementary Fig. 2), all of which vary in abundance across the sample types included in this study. For example, there is a notable reduction in abundance of the genera *Pseudoalteromonas* and *Vibrio* from inlet to outlet samples, across both non-IMTA and IMTA systems. Other high abundance features at genus level consisted of members of the Saprospiraceae family (5%), as

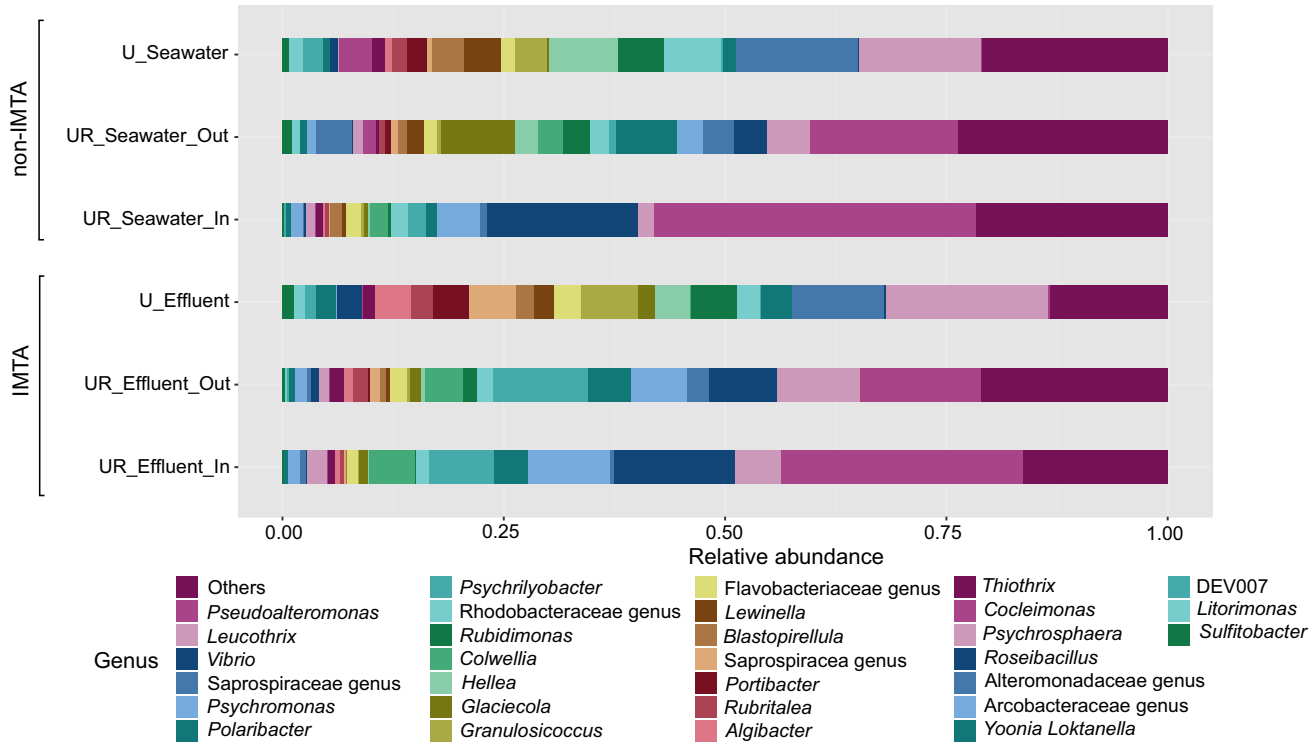


Fig. 5 Relative ASV abundance (percentage abundance) across 21 samples at genus level from *Ulva* (U) samples and water samples collected flowing into and leaving the *Ulva* raceway (UR) in the seawater/non-IMTA and effluent water/IMTA systems, respectively. Less abundant ASVs (counts < 2500) were merged and denoted as "Others"

well as the genera *Psychromonas* (4%), *Polaribacter* (4%) and *Psychrilyobacter* (4%). In total, 125 and 140 significant differentially abundant ASVs (FDR-corrected $p < 0.05$) were observed at genus level. Of these, 99 genus-level ASVs had a higher abundance in water samples (Fig. 6) when compared to the *Ulva* samples, in both the non-IMTA and IMTA systems, with the top 25 differentially abundant genera being displayed. Interestingly, the majority of the bacterial genera isolated and sequenced in the culture-based technique were in low abundance in the Illumina based assessment. For example, the *Celeribacter* (U2), *Roseobacter* (U10), *Modestobacter* (U14), *Agarivorans* (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.

Various marine bacteria, including the genera *Pseudoalteromonas*, *Vibrio*, *Shewanella*, *Psychromonas* and *Tenacibaculum*, were identified as differentially abundant (Fig. 6; Fig. 7). Interestingly, similar levels of abundance of these genera were observed for water samples collected from the non-IMTA and IMTA systems (Fig. 6), with a slightly higher abundance in these genera in the samples collected from the non-IMTA systems in most instances. Some exceptions to this include *Lutibacter*, *Psychrilyobacter* and *Marinifilum* that have a higher abundance in effluent water samples (Fig. 6). Limited differences were observed between non-IMTA and IMTA systems, but in both systems, there is a general decline in abundance of taxa at genus-level when comparing water collected from inlets and outlets. An overall lower abundance of these bacteria in samples collected from *Ulva* was observed. For example, a very low abundance of genera *Shewanella*, *Alteromonas*, *Desulfotalea*, *Halarcobacter*, *Formosa*, *Oleispira*, *Marinicella*, *Lutimonas*, *Wenyingzhuangia* and members of the families Cyclobacteriaceae and Saccharospirillaceae were observed on *Ulva* relative to the water samples (Fig. 6).

In contrast, some bacterial communities had a higher abundance ($p < 0.05$) on *Ulva* when compared to water samples (Fig. 7). This was particularly evident for genera such as *Portibacter*, *Rubidimonas*, *Octadecabacter*, *Hellea*, *Leucothrix*, *Chitinophagales*, *Granulosicoccus*, *Algibacter* and *Lewinella*. Along with this, a higher abundance of these bacteria associated with *Ulva* was observed in outlet samples in both the seawater and effluent systems (Fig. 7), further supporting the modulating effect *Ulva* may have on the bacterial communities in IMTA systems.

Putative functional roles of bacteria in the IMTA system

The functional potential of bacterial communities associated with *Ulva* showed that the microbiome is capable of being involved in various metabolic processes, such as the metabolism of amino acids, carbohydrates, lipids and other secondary metabolites (Fig. 8). An upregulation potential for xenobiotic (chemical or substance that is not naturally produced by the organism) biodegradation and metabolism could be observed for the bacteria associated with *Ulva* samples (Fig. 8). However, these results should be treated with caution given that it is based on 16S data and requires further validation.

Discussion

Enumeration of culturable bacteria in seawater and on *Ulva*

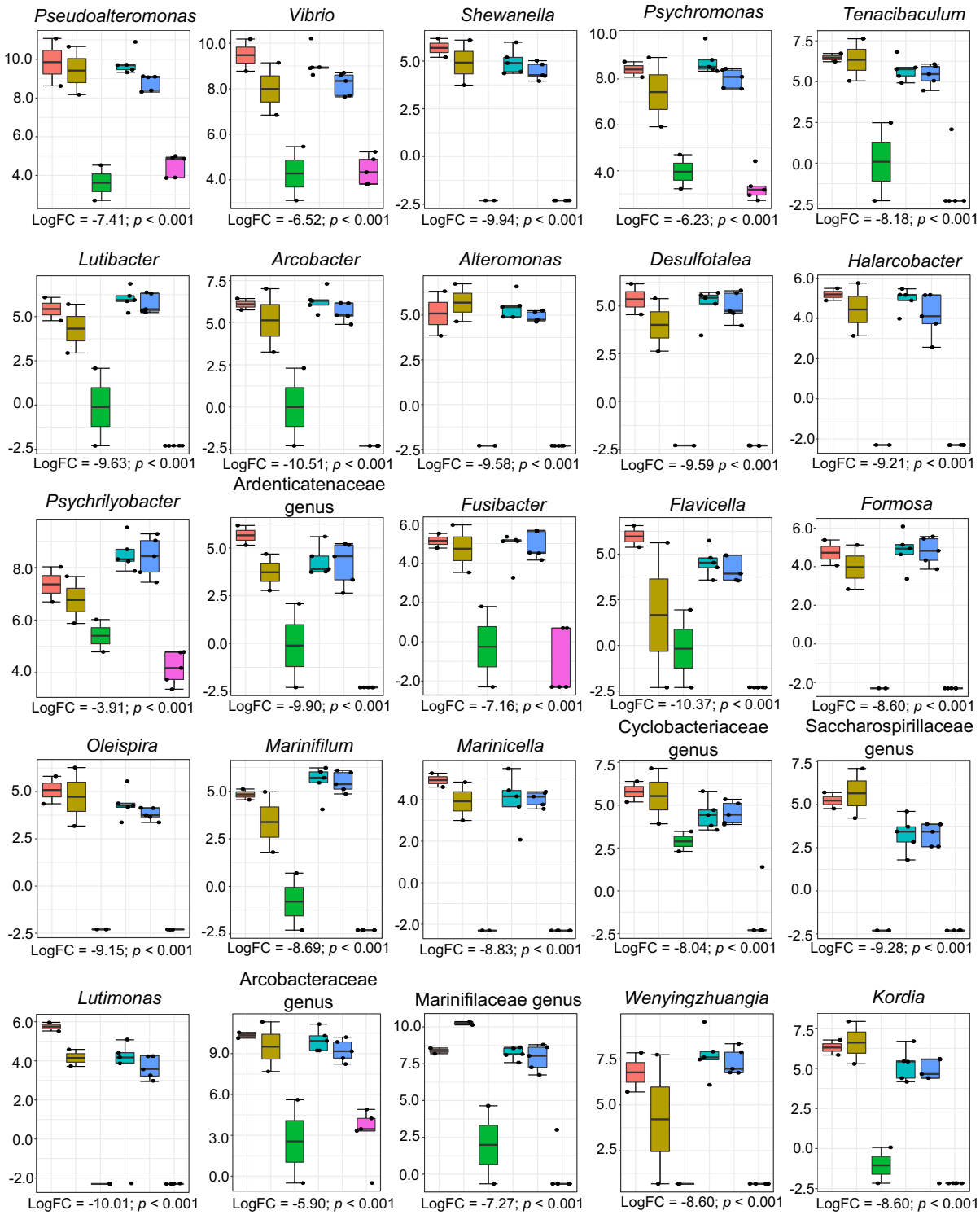
Microbial communities are often host-specific (Taylor et al. 2004; 2005; Reis et al. 2009), and numerous studies have demonstrated host-specific differences between the microorganisms present on macroalgae and in the surrounding seawater (Staufenberger et al. 2008; Lachnit et al. 2009; Califano et al. 2020), between alga of different species and even on a single species from different locations (Bolinches et al. 1988; Tujula et al. 2010; Singh and Reddy 2014; Califano et al. 2020). In the current study, in both fertilised seawater tanks (non-IMTA systems) and abalone effluent raceways (IMTA systems), the macroalga *Ulva* had a strong inhibitory effect on *Vibrio* within the systems, as indicated by the significant decrease in bacteria growing on TCBS agar (a *Vibrio* selective media) between the inlets and the outlets of both systems (Fig. 2). The total number of potential culturable *Vibrio* from *Ulva* samples was higher in the IMTA systems than in the non-IMTA system, and reflect values typically found in seawater and on seaweeds in the natural environment (values range from 10^2 – 10^4 CFU g^{-1} seaweed; Mahmud et al. 2008). Moreover, the inhibitory effect appears to be nutrient dependent, with a substantial decrease in *Vibrio* numbers observed in the effluent water systems. 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. However, this inhibitory effect can be driven by the microbial communities or by *Ulva* itself (Lemos et al. 1985; Egan et al. 2000; Lu et al. 2008; Penesyan et al. 2009).

The high number of bacteria that grew on the minimal media containing ulvan, the main carbohydrate component of *Ulva* (Ray and Lahaye 1995), suggests that there are numerous bacteria capable of growing on *Ulva* within the water column. Several bacteria colonising algal surfaces are known to benefit from the organic components of algae, such as polysaccharides including ulvan, which the bacteria utilise for growth and biofilm formation (Erasmus et al. 1997; Steinberg et al. 2002; Lachnit et al. 2009). The high abundance of ulvan-specific bacteria in the effluent inlet water may be explained by the fact that abalone naturally feed on various wild seaweeds (Simpson and Cook 1998; Naidoo et al. 2006), and abalone in the present study were fed *Ulva* as well as kelp (*Ecklonia maxima*). Therefore, a high abundance of bacteria capable of degrading ulvan is expected in this aquaculture environment. Additionally, the incoming water is pumped from the adjacent coastline where there are kelp beds that could harbour bacterial communities capable of degrading algae.

Identification of culturable bacteria

Most of the marine bacteria identified from the culture plates in this study are frequently isolated from the marine environment, including *Pseudomonas*, *Alteromonas* and *Vibrio* (Rengpipat et al. 2003; Romanenko et al. 2004; Infante-Villamil et al. 2021). A BLAST search of the bacteria isolated on the ulvan substrate, isolates U2 and U10, showed high sequence similarity to *Celeribacter* and *Roseobacter*, respectively (Table 1). 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. 2014) and can cause host-specific adaptations that may be commensal or parasitic (Buchan et al. 2005; Sison-Mangus et al. 2014).

Bacterial isolate T3, isolated on TSA from the effluent system inlet water, showed high sequence similarity to the genus *Agarivorans,* which displays agarolytic activity. Agarolytic bacteria are common inhabitants of marine environments, particularly where there is a high abundance of macroalgae rich in this polysaccharide, such as the globally significant kelp forest ecosystem in South Africa where the farm is located and extracts its seawater from for the abalone raceways (Erasmus et al. 1997; Bolton et al. 2016). Kelp is also used as a feed for the abalone at I&J Cape Abalone Farm. The genus was first isolated from a healthy marine mollusc (*Omphalius pfeifferi*) (Kurahashi and Yokota 2004), and has since been isolated from several marine environments including the marine water column (Park et al. 2014), tidal flats (Kim et al. 2016a; 2016b) and macroalgae (Du et al. 2011). Isolate T8, also obtained from the effluent system inlet water, showed high sequence similarity to the genus *Halomonas* (Arahal et al. 2002). Certain species of *Halomonas* are known to display pathogenic potential in molluscs (Rojas et al. 2009), whereas others have been used as probiotics in aquaculture species (Gao et al. 2019) or to


Pseudoalteromonas
LogFC = -7.41; p < 0.001
Vibrio
LogFC = -6.52; p < 0.001
Shewanella
LogFC = -9.94; p < 0.001
Psychromonas
LogFC = -6.23; p < 0.001
Tenacibaculum
LogFC = -8.18; p < 0.001
Lutibacter
LogFC = -9.63; p < 0.001
Arcobacter
LogFC = -10.51; p < 0.001
Alteromonas
LogFC = -9.58; p < 0.001
Desulfotalea
LogFC = -9.59 p < 0.001
Halarcobacter
LogFC = -9.21; p < 0.001
Psychrilyobacter
LogFC = -3.91; p < 0.001
Ardenticatenaceae genus
LogFC = -9.90; p < 0.001
Fusibacter
LogFC = -7.16; p < 0.001
Flavicella
LogFC = -10.37; p < 0.001
Formosa
LogFC = -8.60; p < 0.001
Oleispira
LogFC = -9.15; p < 0.001
Marinifilum
LogFC = -8.69; p < 0.001
Marinicella
LogFC = -8.83; p < 0.001
Cyclobacteriaceae genus
LogFC = -8.04; p < 0.001
Saccharospirillaceae genus
LogFC = -9.28; p < 0.001
Lutimonas
LogFC = -10.01; p < 0.001
Arcobacteraceae genus
LogFC = -5.90; p < 0.001
Marinifilaceae genus
LogFC = -7.27; p < 0.001
Wenyingzhuangia
LogFC = -8.60; p < 0.001
Kordia
LogFC = -8.60; p < 0.001
Cohort
UR_Seawater_In
UR_Seawater_Out
U_Seawater
non-IMTA
UR_Effluent_In
UR_Effluent_Out
U_Effluent
IMTA

◂**Fig. 6** Differentially abundant ASVs at genus level that had a higher abundance in water samples (UR_Seawater_In; UR_Seawater_Out; UR_Effluent_In; UR_Effluent_Out) when compared with *Ulva* samples (U_Seawater; U_Effluent), where the *p*-value and false discovery rate (FDR) are indicted. Each boxplot corresponds to one of the top 25 differentially abundant ASVs across the cohorts

improve water quality of intensive recirculating aquaculture production systems (Sangnoi et al. 2017; Hastuti et al. 2020).

Isolate T5 shared a high level of sequence similarity with the genus *Pseudoalteromonas*, which belongs to the family Pseudomonadaceae. *Pseudoalteromonas* associated with *Ulva australis* has been shown to inhibit the attachment of fouling organisms and act as a *Vibrio* control agent (Rao et al. 2007; Aranda et al. 2012). This genus was also recently found in relatively high abundance on *U. fenestrata* and *U. linza* in a flow-through land-based laboratory in Sweden (van der Loos et al. 2024). Moreover, several members including *P. auranitia* (Gauthier and Breittmayer 1979), *P. luteoviolacea* (Gauthier and Flatau 1976) and *P. rubra* (Gauthier 1976) release high molecular weight antibiotics. Unlike the *Modestobacter* and *Vibrio* genera, which can be isolated from both terrestrial and marine habitats, the genus *Pseudoalteromonas* is restricted to marine waters and has been isolated from various marine environments, including from sea urchins and macroalgae (Holmström and Kjelleberg 1999; Rao et al. 2007; Brink et al. 2019). Nonetheless, some members of this genus are known to induce disease in macroalgae, including the kelp *Saccharina japonica* (as *Laminaria japonica*, Sawabe et al. 1998) and the red macroalga *Gracilaria gracilis* (Schroeder et al. 2003), but this is mostly when environmental conditions are not optimal and the macroalgae are under stress.

The bacterial isolates T6, T10, V1 and V2 shared high sequence similarity with members of the genus *Vibrio*. 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 (Nicolas et al. 2002), marine bivalves (Paillard et al. 2004), corals (Ben-Haim and Rosenberg 2002), macroalgae (Largo et al. 1995), finfish (Kraxberger-Beatty et al. 1990), prawns (Jiravanichpaisal et al. 1994) and captive bred seahorses (Balcázar et al. 2010). For example, mass mortalities have been previously attributed to *V. harveyi*, which has induced white foot lesions on abalone in Japan (Nishimori et al. 1998) and France (Nicolas et al. 2002). Mass mortality in larvae of the abalone *H. rufescens* has also been observed as a result of *V. alginolyticus* at concentrations above 10^3 cells mL^{-1} (Anguiano-Beltrán et al. 1998). Nonetheless, the isolated *Vibrio* spp. in this study 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; Macey and Coyne 2006; Marshall et al. 2006; Brink et al. 2019). For instance, *V. gallaecicus, V. xuii, V. ichthyoenteri* and *V. parahaemolyticus* have been isolated from marine aquaculture environments, of which only the latter two are often associated with disease (Zorrilla et al. 2003; Gauger et al. 2006; Martins et al. 2013). In the current study the isolated bacteria identified as belonging to the genus *Vibrio* were all isolated from the incoming water samples (abalone effluent), and it was observed that *Ulva* reduces their numbers within the system (Fig. 2). This is not surprising as several species of *Ulva* have been known to display anti-*Vibrio* activity (Lu et al. 2008) or harbour bacteria, as mentioned above, that can produce compounds to restrict their growth (Ismail et al. 2018).

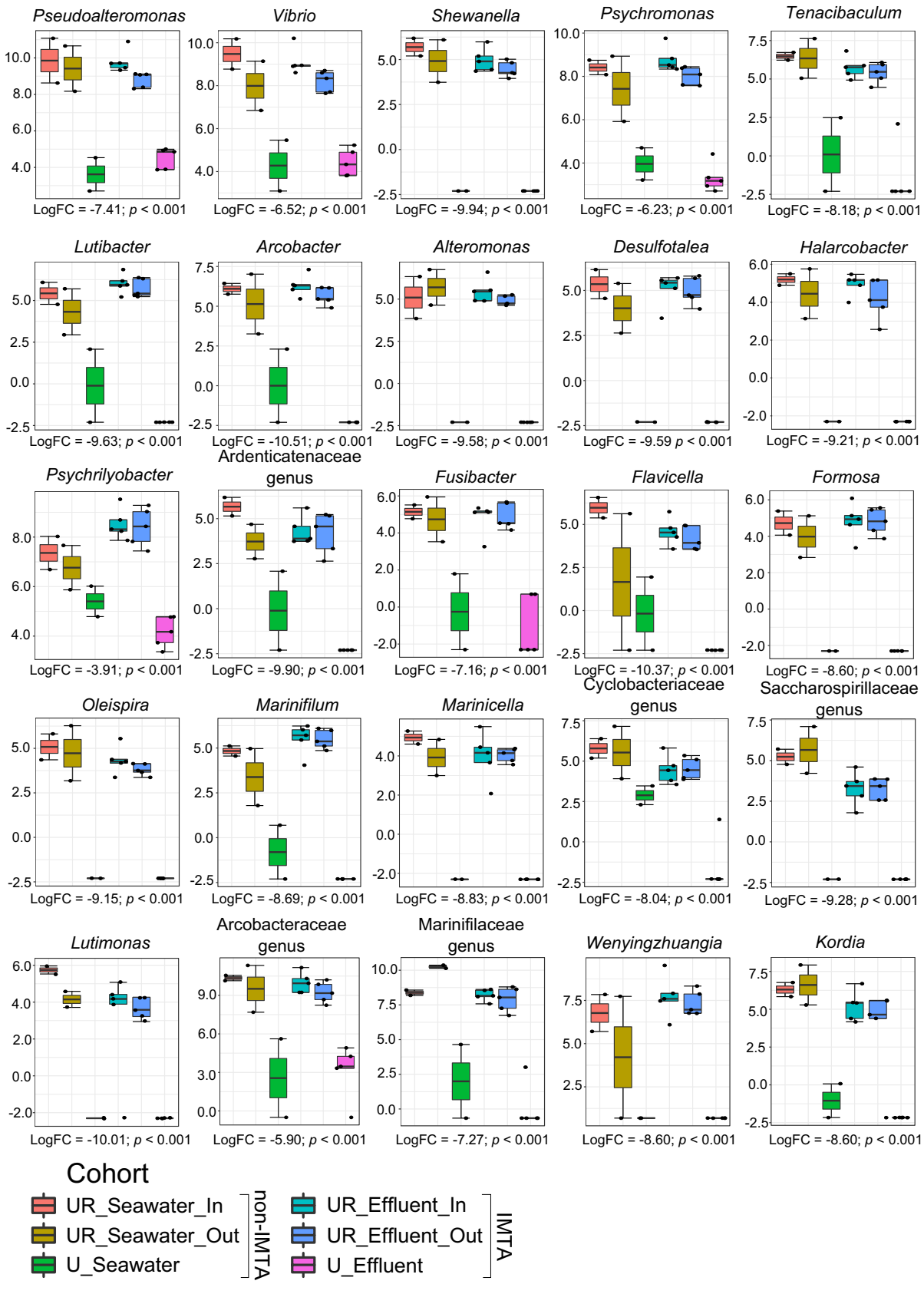
Bacterial community profiling by 16S rDNA sequencing

Bacterial diversity in an IMTA and non-IMTA system

The bacterial diversity remained stable within the samples collected from effluent water, fertilised seawater and *Ulva* as no significant differences ($p > 0.05$) were observed for both the Shannon and the Simpson diversity indexes, with F-statistics of 1.13 and 1.52, respectively (Fig. 3). Interestingly, the richness estimator Chao1 was significantly higher for the water samples across both systems, when compared to *Ulva* samples (ANOVA, $F = 7.11$, $p = 0.001$), indicating a high degree of unique ASVs in the water column. This could be explained by the interchangeable nature of the water with the environment, whereby fresh seawater is constantly pumped in from the ocean and through the abalone raceway systems (in the case of the IMTA), after which it is gravity fed into the *Ulva* raceways. Alternatively, the microbiome modulating effect of *Ulva* and its associated microbiome could be reducing the presence of specific bacteria on the *Ulva* sampled in this study.

Ulva and water bacterial community structure

Several molecular studies have found 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. 2011; Bengtsson et al. 2010; 2012). Cluster analyses comparing the microbial communities of seawater and *U. australis* and *U. rigida*, respectively (Burke et al. 2011; Califano et al. 2020) further support the clear separation observed between the *Ulva* and the seawater observed in the current study. The separate clustering of inlet and outlet water samples from the non-IMTA vs IMTA systems observed in the current study is expected as anthropogenic perturbations, such as the nutrient increases associated with high density aquaculture and recirculating


Pseudoalteromonas
LogFC = -7.41; p < 0.001
Vibrio
LogFC = -6.52; p < 0.001
Shewanella
LogFC = -9.94; p < 0.001
Psychromonas
LogFC = -6.23; p < 0.001
Tenacibaculum
LogFC = -8.18; p < 0.001
Lutibacter
LogFC = -9.63; p < 0.001
Arcobacter
LogFC = -10.51; p < 0.001
Alteromonas
LogFC = -9.58; p < 0.001
Desulfotalea
LogFC = -9.59 p < 0.001
Halarcobacter
LogFC = -9.21; p < 0.001
Psychrilyobacter
LogFC = -3.91; p < 0.001
Ardenticatenaceae genus
LogFC = -9.90; p < 0.001
Fusibacter
LogFC = -7.16; p < 0.001
Flavicella
LogFC = -10.37; p < 0.001
Formosa
LogFC = -8.60; p < 0.001
Oleispira
LogFC = -9.15; p < 0.001
Marinifilum
LogFC = -8.69; p < 0.001
Marinicella
LogFC = -8.83; p < 0.001
Cyclobacteriaceae genus
LogFC = -8.04; p < 0.001
Saccharospirillaceae genus
LogFC = -9.28; p < 0.001
Lutimonas
LogFC = -10.01; p < 0.001
Arcobacteraceae genus
LogFC = -5.90; p < 0.001
Marinifilaceae genus
LogFC = -7.27; p < 0.001
Wenyingzhuangia
LogFC = -8.60; p < 0.001
Kordia
LogFC = -8.60; p < 0.001
Cohort
UR_Seawater_In
UR_Seawater_Out
U_Seawater
non-IMTA
UR_Effluent_In
UR_Effluent_Out
U_Effluent
IMTA

◂**Fig. 7** Differentially abundant ASVs at genus level that had a higher abundance in *Ulva* samples (U_Seawater; U_Effluent) when compared to water samples (UR_Seawater_In; UR_Seawater_Out; UR_Effluent_In; UR_Effluent_Out), where the *p*-value and false discovery rate (FDR) are indicted. Each boxplot corresponds to one of the top 25 differentially abundant ASVs across the cohorts

water within aquaculture systems, are known to influence bacterial communities (Schneider et al. 2007). The three distinct microbial community clusters observed for the inlet, outlet and *Ulva* samples within the effluent water systems further highlight how different systems and species combinations can impact microbial diversity, and in this case indicates that *Ulva* and/or its associated microbiome are capable of altering the microbial community structure within the water column.

The distinct differences in the microbiome observed between the *Ulva* and water samples within a system and between the two systems suggests that *Ulva* can improve biosecurity of aquaculture systems, as it has its own unique microbiota that can aid in modulating the microbiota of the water column, particularly in nutrient rich water (as seen for the abalone effluent in this study). Given the role of microbes in aquatic animal health and the functionality/welfare of ecosystems, understanding the complexities underlying the differences in microbial community structure between *Ulva* and the surrounding seawater and between IMTA and non-IMTA systems is essential.

Water column bacteria in an IMTA vs non-IMTA system

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 (Schneider et al. 2006; Wietz et al. 2009; Schreier et al. 2010).

In the current study the bacterial communities associated with the seawater samples from both systems (Fig. 5) showed similarities to seawater samples collected from two different rock pools containing *Ulva* at Bare Island, La Perouse (Burke et al. 2011). Bacteria belonging to Gammaproteobacteria, Flavobacteriaceae, Alphaproteobacteria and Rhodobacteriaceae were identified in the current study, with communities belonging to the genera *Pseudoalteromonas*, *Vibrio*, *Shewanella*, along with 99 other genera, exhibited higher abundance in water samples, when compared with *Ulva* (Fig. 6). Water samples from both the non-IMTA and IMTA systems in this study had a high abundance of ASVs belonging to the heterotrophic bacterial genus *Pseudoalteromonas*, which was not reported by Burke et al. (2011) but has been found in association with wild-collected *Ulva* from

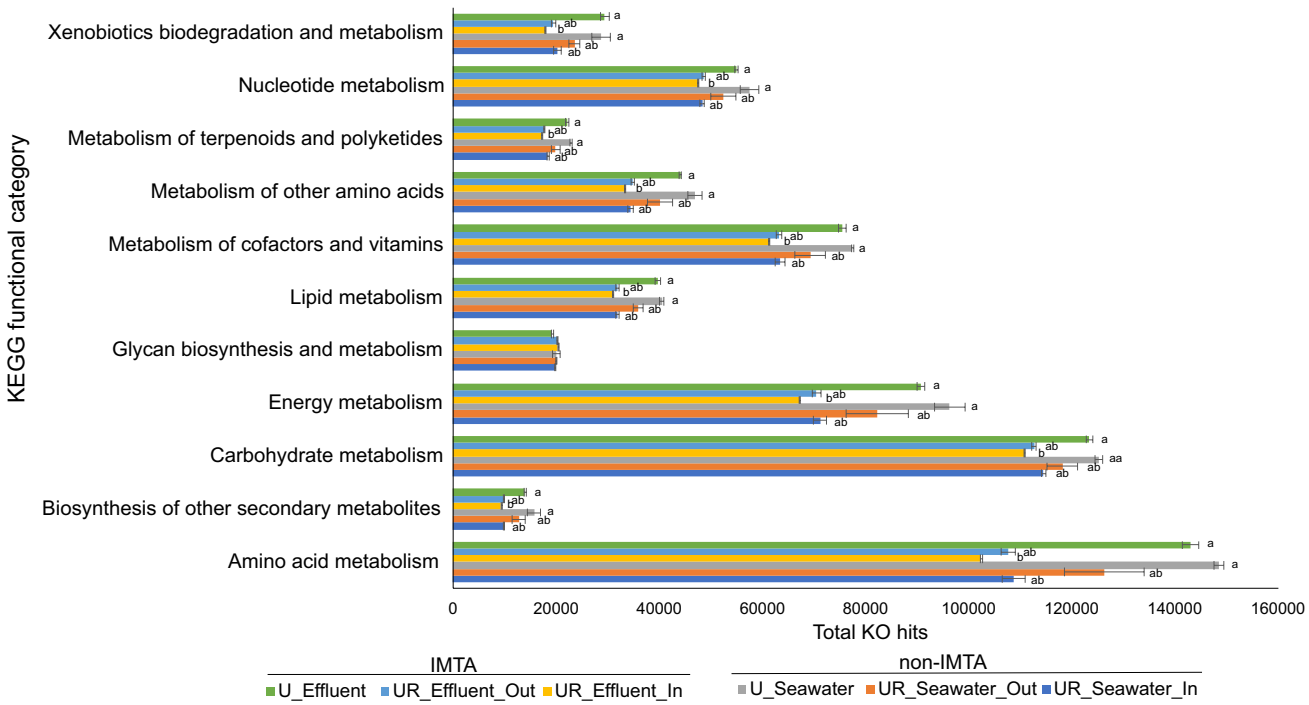


Fig. 8 The Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) metabolic pathways based on total KO hits averaged across inlets (UR_Seawater_In; UR_Effluent_In), outlets (UR_Seawater_Out; UR_Effluent_Out) and associated with *Ulva* (U_Seawater; U_Effluent) in the IMTA and non-IMTA systems, respectively, where the standard error for each mean is indicated. Letters indicate statistically significant differences ($p < 0.05$; Kruskal-Wallis test with Dunn's post-hoc test)

Portugal (Grueneberg et al. 2016). This genus has previously been associated with healthy recirculating aquaculture systems (Schreier et al. 2010). Previous studies have suggested that *Pseudoalteromonas* acts as an inducer for larval settlement for various marine invertebrates (Hadfield 2011). Therefore, bacterial communities associated with the water may not only influence the *Ulva* growing in the raceways but could also positively impact abalone larval settlement.

The seawater entering an abalone farm could be a vector for both opportunistic bacterial pathogens, as well as naturally occurring bacteria that may be neutral or probiotic. Overall, the bacteria associated with the non-IMTA seawater samples belonged predominantly to genera common in the marine environment, such as *Pseudoalteromonas*, *Vibrio*, *Sulfitobacter, Tenacibaculum* and *Psychrobium* (Fig. 6). This was also observed for the inlet effluent water samples of the IMTA systems that contained the bacteria *Psychromonas*, *Psychrilyobacter*, *Colwellia* and *Psychrosphaera*. The presence of the genus *Vibrio* in both the IMTA and non-IMTA systems could be a cause for concern, as some members of this genus are known potential pathogens (Rodrick 1991).

Vibrio are ubiquitous in the marine environment and the potential risk they may pose for disease in the integrated systems appears to be mitigated by the modulatory effect of *Ulva* on the microbiome, as they have a statistically significantly lower (FDR-corrected $p < 0.05$) abundance in samples collected from *Ulva* (Fig. 6) and the abundance of *Vibrio* spp. decreases in effluent water following bioremediation with *Ulva*. Previous studies have found that certain probiotic bacteria, such as *Lactobacillus* and *Phaeobacter*, have the ability to reduce the abundance of *Vibrio* in brine shrimp (*Artemia*) and turbot (*Psette maxima* and *Scophthalmus maximus*) aquaculture systems (Prol-Garcia and Pintado 2013; Ofelio et al. 2021; Pintado et al. 2023), where *Phaeobacter* has been used to colonise *U. ohnoi* in IMTA systems to improve biofiltration capabilities. However, neither of these taxa were found in the dataset generated in the current study. Given the reduction in *Vibrio* following bioremediation with *Ulva*, this study provides additional evidence for the microbial gardening capabilities of *Ulva* as described in an IMTA-based algal production system with seabream (Califano et al. 2020), without the need to supplement with probiotic bacteria.

This idea is further supported by the differential abundance analysis (Fig. 6), where it could be observed that the majority of bacteria that had a significantly ($p < 0.05$) higher abundance in the water samples, had a very low abundance on *Ulva* samples. In contrast, the bacteria that had a higher abundance on *Ulva* samples (Fig. 7) were still present in the water column, suggesting that bacteria in the water column do not influence those of *Ulva* as much as *Ulva* modulates the microbiota of the water column. It has also been suggested that moderate levels of fertilization, either chemical or via the addition of agricultural waste, can act as a feed for microbial communities, promoting a balanced ecosystem in nutrient deprived recirculating aquaculture systems, and therefore, preventing shifts from neutral bacterial consortia to potentially pathogenic communities (Bentzon-Tilia et al. 2016). Of the differentially abundant bacteria in the IMTA and non-IMTA systems, many genera have previously been detected in marine environments and do not appear to be pathogenic. For example, *Lutimonas*, which was in higher abundance in the water samples of both systems (Fig. 6), are members of the family Flavobacteriaceae and have been isolated from the abalone *Haliotis tuberculata*, polychaetes and tidal flat sediments. They are believed to exclusively inhabit marine invertebrates and seawater and have not been associated with algae (Yang et al. 2007; Kim et al. 2011; 2016a; 2016b; Gobet et al. 2018). The genus *Psychrilyobacter*, which also had a higher abundance in seawater, are common marine bacteria that have been isolated from seawater, sediment and crustacea (Romanenko et al. 2004; 2009) and have been studied for their bioremediatory potential (Abd-Elnaby et al. 2016) and roles in abalone digestive tracts (Gobet et al. 2018).

At the studied site, the abalone effluent water originates from the nearby shallow coastal environment, passes through the abalone rearing tanks and then flows into the *Ulva* raceways. This means that the bacterial communities present in the inlet water of the abalone effluent *Ulva* raceways are derived from the incoming seawater but are also influenced by the abalone and their associated microbiota (Schreier et al. 2010), as well as by the feed used in the system. Moreover, the presence of other organisms in abalone rearing tanks, such as polychaetes (Ruck and Cook 1998) may further shift the microbial community structure. The primary nutrients entering the abalone effluent raceways are in the form of particulate and dissolved organic matter that originates from digested feed and faeces/dissolved excreta from the abalone, as well as any uneaten feed that may remain in the wastewater. Bacteria responsible for the degradation of organic matter are known as heterotrophs and in RAS, heterotrophs are the dominant water column bacteria and aid in maintaining water quality (Itoi et al. 2006; Attramadal et al. 2012). Heterotrophic bacterial communities in aquaculture systems have been found to be dominated by Alphaproteobacteria and Gammaproteobacteria (Sugita et al. 2005; Wietz et al. 2009), which were also detected in the current study as dominant phyla. Opportunistic bacteria, which could include heterotrophs, are capable of proliferating rapidly (r-strategists) by using available resources, and are gradually outcompeted by the slower growing specialists, such as the nitrifying bacteria (k-strategists) (Salvesen et al. 1999; Defoirdt 2016).

Common nitrifying bacteria in aquaculture systems include species of the genera *Nitrosomonas, Nitrosococcus,*

Nitrobacter, Nitrosolobus, Nitrosovibrio and *Nitrococcus* (Belser 1979; Teske et al. 1994). The low abundance of nitrifying bacteria in the abalone wastewater raceways in the current study is noteworthy as nitrifying bacteria have been frequently associated with effluent water (Harms et al. 2003; Lydmark et al. 2007; Paungfoo et al. 2007). The particulate organic matter released by the abalone in the effluent water could be enhancing the growth of heterotrophic bacteria which, in turn, outcompete 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 become problematic if nitrification rates are restricted when oxygen levels are low (Michaud et al. 2006). However, on the studied farm, the bubbler systems in the seawater non-IMTA system, the paddlewheel systems used in the abalone effluent IMTA raceways, and the aeration in the abalone tanks, aid in oxygenating the water column, in addition to the oxygen produced by *Ulva* from photosynthesis during the day. Macroalgae also release superoxide ions and hydrogen peroxides as a defence mechanism against bacteria, and the production of these reactive oxygen species (ROS) could reduce the number of associated bacteria on the surface of the algae (Weinberger 2007; Egan et al. 2013; Zhao et al. 2021). Alternatively, the low abundance of nitrifying bacteria may also be attributed to the systems design (i.e., land-based pump-ashore systems with high flow/water replacement rates, typically around 0.25 to 0.5 tanks volumes per hour) and the biofiltration capacity of *Ulva*, which has previously been reported to outperform traditional bacterial biofilms (Bolton et al. 2016). *Ulva* has in fact been shown to consistently remove more that 70% of available nitrogen, mostly ammonia (the main excretory product of abalone), when grown in integrated system, possibly outcompeting bacteria for this nutrient source.

Benefits of the bacterial communities associated with *Ulva* in an IMTA and non-IMTA system

The differences in abundance of several genera on the *Ulva* in both the IMTA and non-IMTA system in the current study concur with studies showing that the microbiome of *Ulva* is distinct to that of the surrounding water (Pintado Valverde et al. 2018; Califano et al. 2020). This is evident in the current study considering that only 25 ASVs were in higher abundance in *Ulva* samples (Fig. 7), as opposed to the 99 identified as having a higher abundance in water samples (Fig. 6). In general, cultivating *Ulva* changes the diversity and abundance of ASVs in laboratory and aquaculture settings when compared to newly obtained specimens (Ghaderiardakani et al. 2022), consequently harbouring beneficial bacteria (Califano et al. 2020).

Both *Ulva* libraries (IMTA and non-IMTA) were characterised by ASVs from common marine environmental bacterial genera, such as *Leucothrix*, *Polaribacter*, *Hellea*, *Glaciecola*, *Granulosicoccus*, *Rubidimonas*, as well as members of the families Saprospiraceae and Rhodobacteraceae. Of these, *Leucothrix* and *Granulosicoccus* have been found multiple times worldwide in other land-based *Ulva* systems in Belgium, Portugal and Israel (Califano et al. 2020; Nguyen et al. 2023; van der Loos et al. 2024). *Granulosicoccus* is a gammaproteobacterium that comprises two species that have been documented exclusively in marine environments, where 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), as well as with other *Ulva* species (van der Loos et al. 2024). The presence of *Rubidimonas* is interesting given that previous studies have found this genus in high abundance in association with *Ulva* towards the end of an experiment (after 32 weeks) in a land-based laboratory facility, but almost absent at the start of the experiment (van der Loos et al. 2024). This suggests the *Ulva* sampled in the current study displayed a mature microbial profile. In the current study, a higher abundance of the genera *Portibacter*, *Sulfitobacter*, *Rubidimonas*, *Octadecabacter*, *Hellea* and *Granulosicoccus* were observed in *Ulva* samples (Fig. 7), across both non-IMTA and IMTA systems. Interestingly, *Portibacter* are members of the Saprospiraceae family and have also been isolated from RAS biofilters (Yoon et al. 2012; Li et al. 2016; Nguyen et al. 2023), and *Sulfitobacter* has been found in high abundance in association with *Ulva* samples collected from the previously mentioned land-based laboratory facility, where it was present throughout the 32 week experiment (van der Loos et al. 2024) and has been identified in association with *Ulva* in other aquaculture environments (Ghaderiardakani et al. 2019; van der Loos et al. 2021), where they are likely promoting *Ulva* growth (Amin et al. 2015; Califano et al. 2020).

Genera belonging to the family Arenciellaceae and class Flavobacteriales, as well as the genera *Reinchenbachiella* and *Maribacter,* exhibited an increased abundance in the effluent water systems from the inlet to outlet samples and were prevalent in high abundance on the *Ulva* (Fig. 7). Notably, *Maribacter* strains have previously been identified as important inducers for *Ulva* morphogenesis (Weiss et al. 2017), releasing high amounts of the morphogen thallusin in algal culture (Ulrich et al. 2022), particularly when present in combination with *Roseovarius* strains (Spoerner et al. 2012). This genus could also play a role in *Ulva* morphogenesis in the studied aquaculture environment, as *Roseovarius* was also identified, albeit in relatively low abundance (denoted as "Others" in Fig. 5). In the current study, the addition of inorganic phosphorus and nitrogen to the non-IMTA system and the dissolved organic matter that the IMTA system receives likely positively impacts bacteria associated with *Ulva*, as fertilisation is known to promote beneficial interactions (Lara-Anguiano et al. 2013; Cooper

and Smith 2015). Moreover, 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.

A comparison across the dominant bacterial taxa present on the *Ulva* from the respective systems showed that the same bacterial taxa have a similar abundance in both systems, further highlighting that using effluent water as a means to fertilise *Ulva* does not introduce, nor increase the abundance of, harmful bacteria. In the current study, the bacteria present on the effluent-grown *Ulva* also shared similarities with those identified in RAS biofilter studies, including the genera *Planctomycetes, Roseobacter, Pseudoalteromonas, Desulfovibrio* and *Vibrio* (Schreier et al. 2010). Bacteria associated with *Ulva*, when utilised as a feed on an abalone farm, may also positively impact the microbiome of abalone. Ten Doeschate and Coyne (2008) noted that *H. midae* fed kelp cakes supplemented with *Pseudoalteromonas* sp. strain C4, isolated previously by Erasmus et al. (1997), grew faster than abalone fed a diet of kelp without this probiotic. The colonisation of the intestinal tract with probiotic bacteria has numerous positive effects on host function and regulation (van Baarlen et al. 2013; Bhatnagar and Lamba 2015). Moreover, Macey and Coyne (2005) indicated that abalone fed probiotic feed supplemented with *Vibrio midae* SY9 increased protease activity in the intestine of the abalone and improved growth, general health and disease resistance of cultured abalone. Effluent grown *Ulva* could be beneficial in aquaculture systems, as the *Ulva* may be colonised by and inoculated with probiotic bacteria from the faeces, which could in turn, re-inoculate the gut of the abalone in a microbial loop, limiting the need for continual probiotic supplementation. Probiotic bacteria within the water column have several benefits, including nutrient and niche competition (Callaway et al. 2008), aid in enzymatic digestive processes and improved growth rates (Erasmus et al. 1997; Macey and Coyne 2005), enhance immune responses and have antipathogenic properties (Nogami and Maeda 1992; Nakayama et al. 2009).

Putative functional capabilities of bacterial communities in water and *Ulva* samples

Microbiota are known to strongly influence 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 in abalone feed and faeces are commonly decomposed by heterotrophic bacteria to ammonia, after which nitrifying bacteria convert the ammonia to nitrate (Bender et al. 2004; Itoi et al. 2006). The latter also applies to the dissolved ammonia directly excreted by then abalone. The co-existence of bacterial genera, with different functional roles, is often desirable, as can be observed in the symbiotic relationship between heterotrophic bacteria and nitrifiers, allowing for simultaneous carbon and ammonia oxidation (Kindaichi et al. 2004; Dong and Sun 2007). Similar to a study conducted by Schreier et al. (2010), bacteria with the potential for nitrification, denitrification and nitrate producers were also identified in the current study, including *Pseudomonas*, and various members belonging to the classes Bacteroidetes, Proteobacteria and Firmicutes. The differential presence of the bacterial genus *Desulfotalea* in the current study, which are sulfate-reducing bacteria, also indicates that there is potential for organic substrates to be oxidised to produce acetate and CO_2 (Widdel and Bak 1992; Inagaki et al. 2002).

In the current study the inlet seawater is more susceptible to change as a result of the variation introduced by the water being pumped on land from the adjacent ocean, as this seawater interacts with various other biotic factors, such as the nearby kelp forest for example. Putative functional capability profiles showed that the bacteria associated with *Ulva* across both systems had greater metabolic potential across majority of the KEGG metabolic pathways identified in this study (Fig. 8). Though limited inferences can be made given that this study was based on 16S data alone, an upregulated potential capability for the metabolism of lipids, carbohydrates, energy, terpenes, amino acids, cofactors and vitamins, as well as the biosynthesis of secondary metabolites was observed for *Ulva*-associated taxa (Fig. 8), which could influence the health of the *Ulva* by improving nutrient availability. Additionally, these bacteria showed potential capabilities for xenobiotic degradation and metabolism, whereby bacteria make use of substances that would otherwise be toxic to them by breaking them down and using them as carbon, phosphorus, sulphur or nitrogen sources (Datta et al. 2020). This potential of the bacterial communities to degrade potentially toxic compounds and their metabolites could aid in the biofiltration and bioremediation action of the *Ulva* in these IMTA systems.

It is possible that the use of *Ulva* as a biofilter maintains a microbial balance in the water column of aquaculture systems through microbial antibiotic biosynthesis, however, a shotgun metagenomics approach would need to be implemented to assess this further and confirm the functional roles of the microbial communities in these systems. The bacteria inhabiting the *Ulva* could protect the *Ulva* holobiont, as well as the abalone by inhibiting bacterial species that could be pathogenic, such as *Vibrio*, without the need to administer antibiotics. This is beneficial considering that administering antibiotics in high dosages could negatively affect beneficial microflora and has the potential to result in the development of antibiotic resistance and the potential

spread of these resistance genes to human and animal pathogens (Handlinger et al. 2006).

Implications for farm management

A common concern with biofilters in integrated aquaculture systems, such as the *Ulva* used in the current study, is their potential to harbour bacterial pathogens that may either infect the co-produced organisms within the system, or consumers after processing. In the current study, evidence was provided to support that the presence of *Ulva* in the raceways does not act as a sink for harmful bacteria and instead is capable of positively impacting the microbiome through antimicrobial action and therefore, would not act as a biosecurity risk when used as a means of bioremediation in IMTA systems or when used as an abalone feed. For example, members of the genus *Vibrio*, displayed a reduction in abundance from the inlets to the outlets of an *Ulva* raceway, following bioremediation by *Ulva.* It was further demonstrated that the modulatory effect of *Ulva* and/or its associated microbiota is increased with increasing nutrient levels, as the aforementioned effect was more pronounced in the IMTA systems.

Seawater is thought to have a lower proportion of opportunistic microorganisms when sufficient time is provided for it to mature (k-selection) and reach a stable microbial community (Blancheton et al. 2013). It is likely that IMTA systems are capable of promoting similar microbial maturation benefits, whereby bacterial communities may establish a stable carrying capacity within the system and be composed of dominant organisms that remain balanced under the given environmental conditions (Attramadal et al. 2012).

Similarly, *Ulva* that has remained in a system for sufficient time to enable microbial maturation may also serve as a stable source of host-specific microbes that positively interact with the water column, the *Ulva* growing in a system, as well as the abalone co-produced within a system when provided as a feed. *Ulva* also improves farm productivity through its interaction with beneficial microorganisms (Wichard 2015; 2023; Alsufyani et al. 2020) and the continual vegetative growth of *Ulva* in effluent water raceways will facilitate the microbial maturation process. If this is the case, microbially matured *Ulva* should exhibit less of a biosecurity concern because as a stable carrying capacity on the host (*Ulva*) is reached, there will be less niche availability for colonisation and growth of opportunistic pathogens. Therefore, there is potential for microbial manipulation in IMTA systems by incorporating *Ulva*, but an improved understanding of these mechanisms in response to different environments is required (Ghaderiardakani et al. 2020; van der Loos et al. 2024). Beneficial microorganisms have the potential to improve future farming practices (Li et al. 2023) and can support growth even under environmental stress (Hmani et al. 2023).

The ability of *Ulva,* as well as its associated microbiota, to act as a natural disinfectant and antifouling agent (Egan, et al. 2000; Ravikumar, et al. 2016) further reduces the risk for proliferation of opportunistic bacteria and the spread of disease should effluent grown *Ulva* be used as a feed. Nonetheless, the extent of *Ulva'*s ability to act as a bioremediation unit under varying physiochemical conditions, including seasonal assessments and microbiome characterisation across increasing recirculation rates in abalone-*Ulva* IMTA systems, requires further investigation, particularly given that the bacteria present on biological filters, such as *Ulva*, rely on interactions with the environment as a function of nutrient input.

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Code availability All code used in this manuscript is described in the manuscript. Any additional information is available from the corresponding author upon request.

Declarations

Competing interests The authors have no competing interests to declare.

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