The Role of Seaweed Antimicrobials in Selection for Antibiotic Resistance

Volume 1

Submitted by Tiffany Morcom to the University of Exeter

as a thesis for the degree of

Masters by Research in Medical Studies

In February 2018

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Antibiotic resistance is quickly becoming one of the biggest modern-day threats to human health. It has not only been observed in the clinic but in natural environments as well. Selection for antimicrobial resistant bacteria in the marine environment has been shown to be driven factors such as low concentrations of antibiotics entering the environment through discharge from wastewater treatment plants and run off from agricultural sites. However, antimicrobial resistance is likely to not be solely due to anthropogenic pollution, as it is an ancient mechanism and has been found in environments with minimal human exposure. Here we investigated whether natural antimicrobial producers, i.e. seaweeds, select for antibiotic resistant bacteria.

We used both culture-based and molecular techniques to characterise the bacterial communities associated with different seaweed species, focusing on the human pathogens *Vibrio*, *E. coli* and *S. aureus*. *Vibrio* was harboured by all the seaweeds tested but *E. coli* and *S. aureus* were not. For the first time, we tested if *Vibrio* isolated from seaweed are locally adapted to their host macroalgae using a novel seaweed media assay. Our results showed *Vibrio* did not display local adaption. We tested the resistance profiles of bacteria isolated from seaweeds and found *Vibrio* showed cross-resistance to antibiotics and natural antimicrobial, in the form of methanolic seaweed extracts. We can conclude seaweeds harbour antibiotic resistant bacteria, but specific species of seaweeds do not select for specific antibiotic resistance.

We quantified the prevalence of a biomarker for antibiotic resistance, the *intI1* gene, and found seaweed select for antibiotic resistant bacteria independent of anthropogenic pollution, suggesting seaweed-associated bacterial resistance is an intrinsic mechanism. Using metagenomics, we characterised possible antimicrobial resistance genes associated with different seaweed species from which we were able identify eflamycin, aminocoumarin and fluoroquinolone resistance genes on all the seaweeds tested. Two of the antibiotic classes are produced by Streptomyces, which is present on seaweeds. Suggesting resistance on seaweeds is selected for by bacterial community or the genes characterised show cross-resistance to seaweed antimicrobials.

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Acknowledgments

First and foremost, I would like I would like to thank my supervisor's Dr Mick Vos and Dr Will Gaze for all their help, guidance and support over the last year and a half and for giving me the opportunity to undertake my MSC by Res under their supervision. I would like to show my appreciation for my pastural tutor Dr Andrew Williams who has always made time for and supporting me with any personal issues.

I am hugely appreciative of all the member of the Coastal Pathogen laboratory group who have helped me with my questions and made the atmosphere here so friendly. Specifically, I would like to mention Issy, Aimee and Anne, founding member of the Poo Crew who have supported me throughout this process, made me laugh and even took me to my first conference. I am very grateful for the work Paul and Karen from the Exeter Sequencing Service contributed and appreciate that they let me help with what I could allowing me to learn techniques I wouldn't have been able to otherwise. I would also like to thank all the other lab users, staff and students at the ESI, ECHH and Exeter Medical School.

Last but not least I would like to express how grateful I am to all my family and friends who have given their support over the last year and half. I would especially like to thank my mum, dad, brother, Perran and Diesel who have put up with all my stress and mood swings but put the smile back on my face and kept me going.

Chapter 1: General Introduction

1.1 Brief History of Antibiotics

The 'antibiotic era' is thought to have started at the beginning of the 20th century with Paul Ehrlich's search for the 'magic bullet' for which he argued that "chemical compounds could be synthesized to exert their full action exclusively on the parasite harboured within the organisms" (1). In 1904, this idea led to Ehrlich developing a large-scale screening programme to find a drug to treat syphilis, which is caused by the bacteria *Treponema pallidium*. It took five years of testing derivatives of arsenic to discover a compound, arsphenamine, which could be used to treat syphilis (2). It first showed promise in infected rabbits and treated patients with venereal disease (3). The drug was a derivative of the highly toxic drug atoxyl and was marketed by Hoechst under the name salvarsan, however it was insoluble in water making it hard to administer and had toxic and painful side effects (2). In 1912 salvarsan was replaced with the less toxic more soluble drug neosalvarsan, which is today classed as a prodrug and used for chemotherapy (4).

Ehrlich's systematic screening programme has since helped in the discovery of thousands of drugs, including the class of drugs we know to today as the sulphonamides. In 1932 the first sulpha drug, Prontosil, was synthesised by a group of Bayer researchers testing dye derivatives for antimicrobial properties (5). However, the active compound sulphanilamide had been used in the dye industry for many years therefore could not be patented. Many companies started producing their own derivatives of the drug leading to a 'sulpha craze'. Unfortunately, this craze lead to the elixir sulphanilamide disaster in 1937 from which 100 people died, sparking the Federal food, drug and cosmetic act in 1938 (6). Despite the disaster, sulphonamides were widely used throughout World War II but due to high instances of resistance the use of sulpha drugs was reduced by 97% in the 1990's. The prevalence of sulphonamide resistant *Escherichia coli* in the clinic is still 40%-45% (7).

In the late 1940's penicillin took over as the most frequently prescribed drug and it is still one of the most well-known antimicrobial agents. It was initially isolated from *Penicillium notatum* mould by Alexander Fleming in 1928 (8). It was not until 1945 that penicillin could be mass produced as it took over 12 years from discovery for a purification method to give a sufficient yield to undergo clinical trials (9). Initially, penicillin could only treat a narrow range of infections therefore new derivatives of the drug were needed which sparked the

synthesis of ampicillin in 1961, which had better bioavailability and a broader spectrum (10). Penicillin and it derivatives are part of the β-lactam group of antibiotics, other examples of β-lactam antibiotics include carbapenems, cephalosporins and monobactams (11).

The approaches used to discover and develop Salvarson, Prontosil and penicillin led to the discovery of over 20 novel classes of antibiotics between 1950 and 1970 (2). Since that time no new classes have been found and only variations on existing compounds have been approved (12). The main reasons no new antibiotic classes have been discovered is because there is a limited number of metabolic pathways which can be targeted (13) and the huge costs involved in getting a drug to market (14). A lack of new antibiotics is becoming a massive problem due to the emerging issue of resistance to the drugs currently used to treat bacterial infections (15-17).

1.2 The Antimicrobial Resistance Epidemic

In 2014 the UK government commissioned a review on antimicrobial resistance conducted by Sir Jim O'Neill which estimated that antimicrobial resistance could be the cause of 10 million deaths per year by 2050, overtaking cancer as the leading cause of death, and potentially costing the world $100 trillion (15). In the same year the World Health Organisation (WHO) released a statement saying, "*A post antibiotic era- in which common infections and minor injuries can kill, far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century"* (WHO, 2014). Even though we are currently still able to effectively treat most bacterial infections the fears of a post antibiotic era are quickly becoming a reality with the first case of *Acinebacter baumannii* displaying resistance to 'last resort' drug colistin (16). In 2016, a strain of *Klebsiella pneumonia* was isolated from an American woman who had recently returned from India. The strain was shown to be resistant to 26 antibiotics and the woman sadly died (17). The Infectious Diseases Society of America has predicted that at least ten more classes of antibiotics, which are effective against multi-drug resistant bacteria, are needed within the next 10 years to keep the issue of resistance under control (18). However, this will only be a short-term fix and new methods are needed to overcome the problem of antibiotic resistance in the long-term. Unfortunately current avenues, such as the genomic approach, to discovering new classes of antibiotics are yet to provide any results (19). A possible option is to explore ecological niches, such as the marine environment, to try and uncover novel antimicrobials (20, 21).

1.3 Mechanisms of Resistance

Bacteria can acquire resistance to antibiotics in two ways: through point mutations and through horizontal acquisition of resistance genes. Horizontal gene transfer occurs through three main mechanisms: conjugation (via plasmids and conjugative transposons), transduction (bacteriophages) and transformation (uptake and incorporation of extracellular DNA) (22). Acquisition of different resistance genes or altering genetic material can result in different resistance mechanisms. Some resistance genes encode enzymes which degrade antibiotics before they can have an effect, e.g. *Pseudomonas aeruginosa* produces AmpC β-lactamase which inhibits penicillin and cephalosporins which induce the enzyme (23). Another mechanism of resistance is altering the target or binding site, for example the *penA* gene encodes an altered penicillin-binding-protein (PBP)-2 in penicillin resistant *Neisseria gonorrhoeae*, causing a 10 fold decrease in acetylation of penicillin (24). Upregulation of efflux pumps is a common mechanism seen in multi-drug resistant bacteria e.g. multidrug transport (Mdt) A efflux pump, which is expressed in *Lactococcus lactis* and *E. coli*, that provides resistance to chloramphenicol, tetracycline and streptomycin (25). Downregulation, exchange or mutation of porins prevents antibiotics reaching intracellular targets preventing them for exerting an effect, for example β-lactam resistant *Klebsiella pneumoniae* has been to shown exchange the larger porin OmpK35 for the smaller porin OmpK36 preventing β-lactam antibiotics entering the cell (26).

Another inherent mechanism of antimicrobial resistance is the formation of a biofilm (27-29). Bacterial biofilms are formed from a hydrated matrix of polysaccharides and proteins which encase bacteria in a slimy layer (30). Bacteria which can form biofilms are often associated with infections caused by medical instruments such as ocular implants and prosthetic heart valves (29) and are also linked with diseases such as cystic fibrosis (31) and periodontitis (32). *Pseudomonas aeruginosa*, which is responsible for pneumonia, is one of the best known bacteria to form a biofilm (33). Antimicrobial resistance due to biofilms is different from the other resistance mechanisms mentioned above as it is not thought to be acquired through mobile genetic elements or genetic mutation (27, 30). This theory comes from the fact that bacteria with no known genetic basis for resistance have reduced susceptibility to antibiotics when growing in biofilms (30, 34), and when the biofilm is dispersed the bacteria rapidly becomes susceptible again (35). One study showed bacteria surrounded by a biofilm could survive after treatment with antibiotics hundreds and even thousands of times the

minimum inhibitory concentration (MIC) (36).There are three main theories about why biofilm formation causes antimicrobial resistance in bacteria (27): the first is slow or incomplete penetration of the biofilm by the antibiotic; the second theory suggests biofilms form their own microenvironment which antagonises the antibiotic; the third theory is that micro-organisms within the biofilm form a unique, highly protective, spore-like state. Infections due to biofilm forming bacteria can persist for months, years or even a lifetime if they can't effectively be treated with antibiotics or the cause of infection can't be removed by surgery (37).

1.4 Selective Pressures for Antimicrobial Resistance

Different anthropogenic selective pressures have rapidly increased the rate of antibiotic and antimicrobial resistance over the last 50 years. The most recognised selective pressure of antibiotic resistance is over-prescription of antibiotics in the clinic, with a strong correlation between antibiotic consumption and infection with resistant bacteria (38). In 2015, the National Institute for Health and Care Excellence (NICE) released guidelines on prescribing antibiotics to help preserve their future effectiveness. These guidelines included discussing alternative treatments with patients, not prescribing antibiotics to patients with infections which will likely get better on their own and if antibiotics are needed the shortest effective dose should be prescribed (39). However, general practitioners (GPs) are reporting pressure from patients to prescribe antibiotics. 45% of GPs said they prescribed antibiotics for viral infection knowing they would not be effective, while 44% of GPs said they prescribed antibiotics to get a patient to leave their consulting room (40). However, it has been shown that when GPs are able to spend longer with a patient they are less likely to prescribe an antibiotic (41). This evidence suggests that to put a stop to the over-prescribing of antibiotics the public should be better educated about when antibiotics are needed and the risks of taking antibiotics unnecessarily e.g. for a viral infection.

Antibiotics are also extensively used prophylactically in agriculture and aquaculture to promote growth of livestock and improve feed efficacy in agriculture (42, 43). Humans and animal have similar microbiomes meaning bacteria and bacterial resistance genes can readily pass between the two, for instance through human consumption of meat or fish (42). Antibiotics have been shown to be poorly absorbed by animal's guts with 30%-90% of the parent compound being excreted (44) and as most of the drugs are water soluble 90% of

the active drug can be excreted in urine and 75% can be excreted through faeces (45). Land animal faeces and closed aquaculture systems wastewater are used as supplements in fertilizer which is spread over crops. This can cause the transfer of resistance genes and antibiotic residues to crops, soil and the aquatic environment through farm-land run off (46), and may subsequently impact open aquaculture system located in shallow coastal waters and sheltered bays (47). To try and reduce the effect agriculture has on driving antimicrobial resistance the Veterinary Feed Directive (VFD) was approved by the FDA in 2015 (48). The VFD is used to instruct veterinarians, pharmaceutical companies and producers on how to administer drugs in animal feed and water (48). It is also now illegal to use antimicrobial agents which relate to human antibiotics for anything other than to treat and prevent the spread of infection. In the US, producers also need a veterinary signed form to purchase, store and administer those drugs (48). Regulations on antimicrobial use in aquaculture vary dependant on location. In Europe the Veterinary Medicine Products Derivative (VMPD) banned the use of antibiotics to prevent disease and very few antimicrobial agents are licensed for the use in aquaculture to treat infection when it occurs (49). However, 90% of aquaculture production takes place in developing countries where there is little regulation on the use of antimicrobial agents. One study investigating shrimp farmers on the Thai coast found that 74% of farmers used antibiotics daily for pond management with over 13 different antibiotics being used (50). This indicates that stricter regulations and dissemination of the effects antibiotic use has on resistance are needed in developing countries to try and combat the spread of resistance genes from aquaculture.

Another selective pressure for antimicrobial resistance is pollution from wastewater treatment plants (51). Resistance genes and antibiotic residues can enter aquatic environments through wastewater, especially from hospital waste (52). Wastewater is disinfected using a range of techniques including chlorination and photogeneration, which has been shown to remove antibiotics such as β-lactams, fluoroquinolones and tetracycline (53-55). Despite this, low concentrations of antibiotics and resistant bacteria have been found in wastewater samples released into the environment. Even at low concentrations, antibiotics can inhibit sensitive bacteria allowing resistant bacteria to persist in the aquatic environment (56).

1.5 Natural Antimicrobials

In the past, antibiotics have been discovered through intensive screening of terrestrial organisms such as microbes, plants and animals. The bioactive compounds were then altered or were the basis for synthetic derivatives of new antibiotics in existing classes (57). However, over the last 20 years discovery of terrestrial-borne natural products with unique scaffolds has decreased dramatically, resulting in a lack new antibiotic classes (57, 58). This coupled with the rapid emergence of resistance to current antimicrobial agents has meant modern medicine has turned to the marine environment to search for new bioactive compounds and products which can work in synergy with existing drugs (57, 59). Marine organisms, which contribute to approximately half the world's biodiversity, have been classified as the largest remaining untapped reservoir of novel compounds which have the potential to be developed into pharmacological agents (60). The unique bioactive compounds produced by marine organisms are a consequence of their harsh living environments, in which they must compete for space, adapt to tide variations, face high salinity and protect against predation (61). Previous studies have shown marine sessile organisms, such as sponges, coral and algae, produce peptides with diverse pharmacological effects including: antineoplastic agents, antimicrobial agents, HIV-inhibitors, as well as the diagnosis and treatment of certain central nervous system disorders (62).

1.6 Seaweed as a Model

For this project we are interested in the antimicrobial effect of macroalgae (seaweeds), which can be classified by three groups: Rhodophyta (red seaweeds), Phaeophyceae (brown seaweeds) and Chlorophyta (green seaweeds). Seaweeds are constantly exposed to a wide range of micro-organisms that are present in the ocean, some of which are potentially harmful. It has therefore been hypothesised that their metabolites and secondary metabolites have strong antimicrobial effects to prevent disease, fouling and the settlement of unwanted epiphytes (63, 64). Bioactive compounds within structural elements of seaweeds include: alginates in brown seaweeds (64, 65), carrageenans in red seaweeds (64, 65), ulvans in green seaweeds (64, 66), and carotenoids which are present in all types of seaweeds (64, 67). Seaweed secondary metabolites which exert an antimicrobial effect include: lectins (68), alkaloids (69) and terpenes (70). Studies have shown that factors such

as season, geographical location and age of the seaweed can affect its antimicrobial activity (64).

1.7 Seaweed-Associated Bacterial Communities

Despite seaweeds exerting antimicrobial effects they also secrete nutrients which attract the settlement of certain microorganisms and stimulate the formation of biofilms (71). Studies have shown that between 10^2-10^7 cells per cm^2 can settle on seaweed, depending on macroalgae species, section of thallus and season (72-74). One of the first studies to investigate the composition of seaweed associated bacterial communities used scanning electron microscopy to examine the brown macroalgae *Aschophyllum nodosum* (75). The study found notable differences in bacteria populations of the hold-fast, internodal regions of the stipe and apical tips, with the most diverse population being found on the internodal regions of the stipe (75). Staufenberger et al., (2008) used denaturing gradient gel electrophoresis (DGGE) and 16srRNA gene clone libraries to discover that the bacterial communities on *Laminaria saccharina* were more diverse at the older blades compared to younger sections of the plant (76). It was hypothesised that this was due to more damaged cells, which are susceptible to bacterial decomposition being present in older sections of the macroalgae. This hypothesis is backed by previous research which showed a higher bacterial density on diseased or bleached macroalgae compared to healthy plants (77, 78).

Multiple studies have found differences in the type and abundance of bacteria which colonise macroalgae in different seasons, with a higher abundance and diversity of bacteria being observed in summer compared to winter (79-81). One study looked at the effect temperature had on the abundance and diversity of bacteria settled on *Fucus vesiculosus*, and found an increase in temperature increases the diversity of the bacterial community but not the abundance of bacteria present (82). This suggests temperature could be behind the change in bacterial communities from summer to winter. The same study also found seaweed bacterial communities are species specific (79). Another study found that the type of bacteria which settle on seaweed is unique to its specific ecological niche, independent of seaweed species (83). However, other research has found that seaweed of the same species in different geographical location has more similar epiphytic

bacterial communities than seaweeds of differing species in the same ecological niche (83, 84).

Many studies have used different culture-based and molecular techniques to investigate the composition of seaweed associated bacterial communities. Techniques such as 16s rRNA gene sequencing and before that DGGE have revealed that the most common classes of seaweed epiphytic bacteria include *Alphaproteobacteria, Gammaproteobacteria Bacteroidetes, Planctomycetes* and *Actinobacteria* (85). Despite seaweed associated bacterial communities vary depending on factors such as seaweed species, geography and temporal changes these classes have been isolated from different seaweed species from around the world (85). Although these different classes of bacteria have been isolated on green, red and brown seaweed, one review has found some bacterial classes are more common on some seaweed groups than others. *Alphaproteobacteria* mainly isolated from green seaweeds, and *Actinobacteria and Firmicutes* are more commonly associated with red and brown seaweeds (86).

One review found that only 33 different bacterial genera were detected across red, green and brown seaweeds, these included *Bacillus*, *Pseudomonas* and *Vibrio* (86), however this number could be an underestimation due to the majority of studies using culture-based techniques. The same review also found that the same species of bacteria are rarely isolated from different species of seaweed, even when the macroalgae are from the same genus (86). For example, the *Flavobacteria* bacterium *Zobellia russellii* has only been found to be present on *Acrosiphonia sonderi*, *Octadecabacter antarcticus* has only been isolated from *F. vesiculosus* (79) and *Acinetobacter lwoffii* has exclusively been identified on *Ulva australis* (86, 87). However there has been a handful of bacterial species which has been reported on multiple macroalgae, including seaweed from different genera (86), these include *E. coli* (88-91), *Vibrio tasmaniensis* (92, 93) and *Bacillus licheniformis* (92, 94). The variety of culture-based and molecular techniques used to identify seaweed associated bacteria has led to the discovery of over 50 new bacterial species (95). *Cullulophaga baltica* and *Cellulophaga fucicola* were initially discovered through 16s rDNA sequencing of bacteria isolated from *Fucus serratus* (96). *Cullulophaga baltica* has only shown to be present on the surface of *F. serratus* however *Cellulophaga fucicola* has been isolated from *Ulva australis* in addition to *F. serratus* (97). *Pseudoalteromonas ulvae* is another example of bacteria which was first discovered after being isolated from the surface of *Ulva lactuca*, *(98).*

Most of the studies characterising the epiphytic bacterial communities of seaweed have focused on the classification of bacteria which settle on the surface of macroalgae. However, one study has indicated that it may be the function of the bacteria which determines the seaweed microbial community rather than taxonomical group (99). Burke et al., 2011, used metagenomic sequencing to analyse the bacterial communities of six *Ulva australis* plants collected from the same location. They found that the functional composition of the bacterial communities were a lot more similar compared to the species of bacteria present on each sample, indicating a core set of functional genes (99). For example, proteins associated with flagellum-mediated motility were highly expressed in all the bacterial communities tested, those proteins will allow the bacteria to move towards the host and will also help with the formation of bacterial biofilms (99, 100). Other proteins, which are associated with the metabolism of water-soluble polysaccharides (e.g. rhamnose and xylose) produced specifically by *Ulva australis,* were also abundant across the bacterial communities of all six samples, presumably aiding adaptation to the host (99, 101).

1.8 Local Adaptation

Many studies have focused on the composition of microbial communities associated with different seaweeds and have found bacteria which settle on seaweeds are host specific (85, 86), which could indicate the bacterial population is locally adapted to its seaweed host. Local adaptation is defined as a population which is fitter in its home habitat than populations from foreign habitats (102, 103). To our knowledge there has been no previous research into whether bacteria which settle on seaweeds are locally adapted, however a handful of studies have demonstrated local adaption of invertebrates on seaweeds (104-107). Most local adaption studies focus on terrestrial and aquatic environments but not many have looked at the marine environment. This is in part due to the pre-conceived idea that organisms in the marine environment evolve to have phenotypic-plasticity because of their hostile, ever-changing living environments (107, 108). Marine organisms are also dispersed over broad spaces dependent on sea currents and their ability to settle, which would indicate a preference to evolve more generalized (107, 109). In contrast, more recent research has shown that the evolution of marine organisms is a lot more restrictive and can depend on local currents, behaviour of the organism and its ability to find a suitable habitat (107, 109-111).

1.9 Cross-Resistance

We are interested in whether bacteria that are resistant to seaweed extracts show cross-resistance to clinically used antibiotics. Cross-resistance is defined as the occurrence of resistance to one antimicrobial agent accompanied by resistance to another distinct antimicrobial agent (112). Cross-resistance occurs when different antimicrobial agents share the same route of access into the cell or initiate a common pathway to cell death (112). This interaction can be seen between different classes of antibiotics as well as antibiotics and other substances, such as heavy metals, biocides, disinfectants or solvents (112-115). An example of a cross-resistance mechanism is the multi-drug resistant pump present in some *Listeria monocytogenes* strains which has the ability export both antibiotics and heavy metals (116).

Previous studies have shown seaweed extracts can inhibit human pathogens, for instance *Sargassum vulgare* ethanol extracts have shown to be effective against *Klebsiella pneumoniae* (117) and *S. aureus* growth is inhibited by *Sargassum fusiforme* diethyl ether (117) and *Chaetomorpha aerea* methanol extracts (118). These results are promising as they demonstrate seaweeds could be a potential avenue for the development of new antibiotics. However, it is important to identify whether bacteria that are resistant to conventional antibiotics are cross-resistant to natural antimicrobials.

1.10 Natural Antimicrobial Resistance

Despite human activity having huge impact on the acceleration of antimicrobial resistance, it is not the only relevant driver of its evolution. Phylogenetic reconstruction has revealed antimicrobial resistance genes have been present in the environment well before the introduction of antibiotics (119). For example, protein structure-based phylogeny has been able to identify that serine β-lactamases originated over 2 billion ago (120), and metagenomic clones derived from 10,000-year-old ‘cold-seep’ sediment suggests that the evolution of the enzyme is ancient (121). Resistance genes have been found in pristine environments with no human contact or pollution from antibiotics such as ice (122), deep terrestrial subsurface (123), and permafrost (124), indicating the genes have not originated through anthropogenic activity. As genes that encode for different resistance mechanisms originated and evolved before the use of antimicrobial agents they could have had a different original purpose. Genes found in natural antimicrobial producers are likely to of evolved to

prevent the host being harmed by its effects, for example actinomycetes, producers of antibiotics such streptomycin, express aminoglycoside-modifying enzymes (125). Also, efflux-pumps have been shown to remove metabolites and are involved in cell signalling (126) and β-lactamases are involved in the biosynthesis of the cell wall (127).

1.11 Aims and Objectives

The aim of this project is to use both culture-dependent and culture-independent techniques to identify:

- Whether seaweeds can select for human pathogens and bacteria which are resistant to both natural antimicrobials, in the form of seaweed extracts, and clinically used antibiotics.
- Relative abundance of antibiotic resistance in seaweed-associated communities and characterise what resistant genes are present in the seaweed microbiome and whether they vary depending on seaweed species.

Chapter 2: Culture-based Characterisation of Seaweed-Associated Bacteria and Their Resistance Profiles

2.1 Introduction

2.1.1 Local Adaptation and Cross-resistance

In the previous chapter we discussed the hypothesis of seaweed-associated bacterial communities being locally adapted to their host (see section 1.9). To our knowledge there has been no previous research into whether bacteria which settle on seaweeds are locally adapted. During this chapter we will, for the first time, measure local adaption of seaweed-associated bacteria by testing *Vibrio, E, coli* and *S. aureus* isolated from specific seaweeds grow better in seaweed-based broth produced from their host species compared to media made from other seaweeds. We shall also use a disc diffusion assay to test whether bacterial strains are less susceptible to methanolic extracts derived from the seaweed species they were sampled from compared to extracts of different seaweed species.

In section 1.10 we discussed bacteria being cross-resistant to both clinically used antibiotics and natural antimicrobials (see section 1.10). A previous study conducted in our laboratory used disc diffusion assays to investigate cross-resistance and collateral sensitivity between methanol seaweed extracts and conventional antibiotics. A positive correlation between resistance to seaweed extracts and clinically used antibiotics was demonstrated by hospital isolated *S. aureus*, indicating cross-resistance (128). 48 seaweeds extracts were tested against 28 *S. aureus*. 37.5% of the seaweeds extracts were able to inhibit all the *S. aureus* strain, those extracts included: *Asparagopsis armata, Cystoseira baccata, Himanthalia elongate, F. vesiculosus, F. serratus, Furcellaria lumbricalis* and *Sargassum muticum* (128). In this chapter we shall use *Vibrio, E. coli* and *S. aureus* isolates obtained from different seaweed species and employ the same techniques to investigate cross-resistance towards clinically used antibiotics and methanol seaweed extracts.

2.1.2 Community Resistance Assay

We shall look at resistance to specific antibiotics in general bacteria isolated from seaweed as *Vibrio*, *E. coli, S. aureus* will not be the only bacteria present on the surface of our seaweeds. This shall be done using a community resistance assay. This will involve cultivating bacteria isolated from different seaweeds species on marine agar. The bacterial communities will be plated on plain marine agar and agar amended with antibiotics. This will allow to see if seaweeds select for antibiotic resistant bacteria. By investigating resistance to antibiotics in seaweed bacterial communities, including cross-resistance to conventional antibiotics and natural antimicrobials, we can determine if seaweeds are hotspots for

antibiotic resistant bacteria. This is important as the seaweeds we are testing are abundant across the UK coastline therefore humans may be exposed to them, and the antibiotic resistant bacteria settled on their surface, posing a potential risk to their health. If we can show seaweed selects for antimicrobial resistant bacteria, we can use them to determine which marine waters of safe for recreational use i.e waters with a high abundance of seaweeds may not be safe for humans.

2.1.3 *Vibrio species*

Vibrio spp are gram negative, curved rod-shaped bacteria which can be both pathogenic and non-pathogenic and are mainly found in marine environments (129). *Vibrio* species are commonly divided into two groups: *Vibrio cholerae*, responsible for cholera infections, and non-cholera *Vibrio* (130). The most common non-cholera *Vibrio* species include *V. parahaemolyticus, V. alginolyticus* and *V. vulnificus* (130). These species are considered foodborne pathogens, with *V. parahaemolyticus* being the leading cause of seafood-associated bacterial gastroenteritis in the United States and making up approximately half of the foodborne outbreaks in some Asian countries (131). However *Vibrio* infections can also be caused by exposing open wounds to warm seawater (132). Symptoms of *Vibrio* gastroenteritis include stomach cramps, nausea, vomiting, diarrhoea and haematochezia. Patients presenting with a *Vibrio* infected wound often have an immunocompromising condition making it essential for quick diagnosis and treatment. The infection can often lead to sepsis and severe cellulitis around the abrasion. Patients should be treated with both doxycycline and ceftazidime, as well as direct treatment of the wound, such as drainage of abscesses or even amputation. However, even with efficient diagnosis and treatment 30-40% of *Vibrio* infections are fatal and if infections are not treated within 72hrs the fatality rate can rise to 100% (133).

The Centre for Disease Control and Prevention (CDC) have been voluntarily assessing culture confirmed *Vibrio* infections from the Gulf coast region of America since 1998, expanding to nation-wide in 2007. *Vibrio* infections have increased by 115% between 1998 and 2010, whereas other common infection such as Shiga toxin–producing *Escherichia coli*, *Campylobacter* and *Salmonella* have decreased over the same timeframe (134). In 2011 there were an estimated 8000 cases of *Vibrio* infections of which 4500 were cause by *V. parahaemolyticus* and around 100 were due to *V. vulnificus*, however this is thought to be

an underestimate due to under reporting (135). Although *V*. *parahaemolyticus* is the most commonly reported non-cholera infection *V. vulnificus* contributes to 94% of non-cholera *Vibrio* infection related deaths, this is thought to be due to *Vibrio* gastroenteritis going undiagnosed as thiosulfate-citrate-bile salts-sucrose (TCBS) medium is not routinely used to test stool samples (136).

There is relatively little information on *Vibrio* infections in Europe as there are no regulations in place for *Vibrio* controls in seafood traded within the EU, therefore there is no legal obligation to test for the bacteria. This is partly due to the EU expert scientific committee on veterinary measures related to public health (SCVMPH) deeming current internationally recognised methods for monitoring *Vibrio* infections not fit for purpose. SCVMPH instead suggested *V. parahaemolyticus* and *V. vulnificus* should be included in microbiological sentinel surveillance systems for infectious gastroenteritis and should be included in the European Network for Epidemiologic Surveillance and Control of Communicable Diseases (9118/98/CE) (137). Another reason there are no regulations is because incidences of *Vibrio* infection are low in Europe and the cases seen are mainly related to traveling to high risk areas such as the US and Asia (138). However, in recent years reports of *Vibrio* infections in countries surrounding the Baltic sea have increased (139-141). One study has suggested this may be due to rising sea temperatures linked to global warming (142). The Baltic sea is believed to be one of the largest, fastest warming, low salinity marine ecosystems on the earth, giving *Vibrio* the perfect condition to thrive (142). This coupled with the fact that more than 30 million people live within 50 km of the Baltic sea significantly increases the clinical risk of *Vibrio* infection (137).

V. vulnificus has shown sensitivity to a range of antibiotics including tetracycline, aminoglycosides, chloramphenicol, fluoroquinolones and third generation cephalosporins (143-145). However, *Vibrio* strains CMCP6 and YJ016 contain active drug transporters, enzymatic-modification systems and permeases giving them the perfect tools to develop resistance against antimicrobial agents (146). Unfortunately, extensive use of antibiotics in aquaculture to prevent disease has seen a recent rise in multi-drug resistance *V. parahaemolyticus* and *V. vulnificus*. In the US, studies found 57%-90% of the *Vibrio* strains expressed resistance to ampicillin (146, 147) and studies in Italy also found ampicillin-resistance in 100% of the *Vibrio* strains isolated (148). Ampicillin-resistant *V. parahaemolyticus* dates as far back as 1978 (149). 45% of the US *Vibrio* isolates were also

found to be resistant to three or more antibiotics including doxycycline and tetracycline, which are currently used to treat *Vibrio* infections (146), however the Italian isolates were susceptible to chloramphenicol and doxycycline in all its isolates (148). The worldwide emergence of antibiotic-resistant *Vibrio* proves there should be a reassessment of how infections are currently treated in clinics to ensure effective treatment can be maintained.

2.1.4 *Escherichia coli*

E. coli is a gram negative, facultative anaerobic, rod shaped bacteria commonly found in the lower gastrointestinal tract. The majority of *E. coli* strains are harmless and form part of normal, healthy gut flora. However, a few virulent strains can cause infections such as urinary tract infections (UTI's), gastroenteritis and even Crohn's disease. Faecal-oral transmission is the most frequent route of infection. UTI's cause by *E. coli* are one of the most common types of infections and are often treated with antibiotics. Antibiotics used to treat UTI's include trimethoprim, nitrofurantoin and broad-range antibiotics such amoxicillin and ampicillin (150). The high number of antibiotic prescriptions for UTI's has contributed to the rapid emergence of antimicrobial resistance seen in the clinic (151, 152). For example, the 'English Surveillance Programme for Antimicrbial Utilisation and Resistance' (ESPAUR) found that a third of UTI samples analysed by the NHS in 2016 displayed resistance trimethoprim (153). This indicates more needs to be done to reduce the number of antibiotic prescriptions for UTI's. One way in which this could be done is by using urine dipstick tests to confirm whether antibiotics are the best method of treatment (154).

E. coli are used as bacterial indictors for contamination in aquatic environments and are monitored to ensure bathing waters do not pose a threat to human health (155). Increased levels of *E. coli* in marine waters have been shown to directly correlate with a higher prevalence of gastroenteritis seen in bathers exposed to polluted waters (156-158). Studies have shown the presence of *E. coli* in aquatic environments could be due to multiple factors. One study found that within 15 minutes bathers can shed up to $6x10^5$ and colony forming units (CFU) of *E. coli* and (159). Multiple studies have been able to isolate *E. coli* from treated wastewater meaning they could potentially be released into the environment and pose a threat to human health (160, 161). The bacteria were also found in storm water (162) and streams which drain into the coast (163), giving other possible sources of contamination in coastal waters.

It was initially thought that *E. coli* could not survive in seawater as after exposure they could no longer form culturable colonies. It is now known *E. coli* enter a viable but nonculturable (VBNC) state however there is controversy about whether they pose a threat (164). Some studies have found that the cells are dead or cannot be resuscitated (165, 166). However, other evidence suggests that not only can the *E. coli* survive but the pathogenic strains can still be infective (167), meaning they could pose a threat to human health. Salinity does not appear to have a significant effect on the survival of *E.coli* in seawater (164), however many other factors can influence survival rates including pH, nutrient availability, temperature and light radiation. *E. coli* prefers lower, more acidic pH levels (5-0.9) meaning the neutral pH (7.5-8.5) of seawater has a detrimental effect on survival rates (168). Light radiation also has a negative effect as it leads to oxidative stress, this is especially seen in shallow waters (169, 170). The optimal temperature for *E. coli* growth in the laboratory is 37°C, however it has been shown that in the marine environment survival rate of the bacteria is higher at lower temperature (168, 171).

2.1.5 *Staphylococcus aureus*

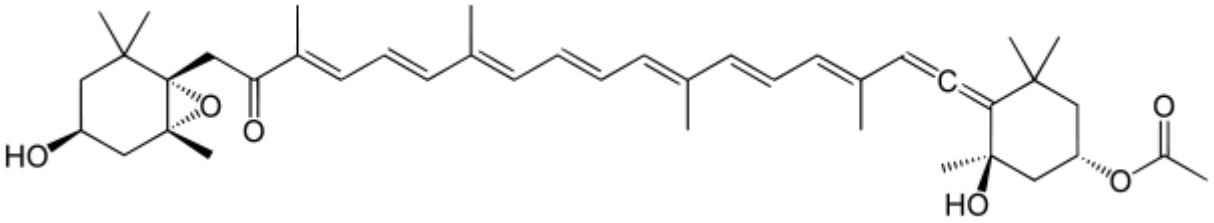
S. aureus is a facultative anaerobic, gram positive, cocci shaped bacterium which is most commonly found in the nose, respiratory tract and on the skin. *S. aureus* commonly colonises the upper digestive tract and skin without issue. However pathogenic strains can result in infections ranging from common skin conditions, such as eczema and impetigo, to rarer more severe infections, such as endocarditis and pneumonia. These diseases can spread through close skin contact, sharing of linen, coughing, sneezing and unwashed hands. Like *Vibrio*, *S. aureus* is also considered a food-borne disease which is normally found in dairy products and processed meats which have not been handled and stored correctly. The bacteria produces toxins known as enterotoxins, which when consumed cause rapid-onset of symptoms such nausea and sickness with or without diarrhoea, these are normally self-limiting and rarely result in hospitalization (172). Methicillin-resistant *S. aureus* (MRSA) is a type of *S. aureus* which has acquired resistance to β-lactam antibiotics, such as methicillin and penicillin, through HGT and natural selection. When MRSA is exposed to methicillin the normal penicillin-binding proteins (PBP) are inactivated and taken over by PBP-2a, which is coded for by the *MecA* gene (173). PBP-2a has a low affinity for methicillin rendering the antibiotic useless and allowing the bacteria to multiply (173).

Antibiotics currently used to treat MRSA include vancomycin and the drug combination trimethoprim and sulfamethoxazole (174).

High concentrations of *S. aureus* in coastal waters have shown to cause an increase in bathers contracting a skin, eye or ear infection (156, 157, 175). Bathers themselves have been shown to shed up to $6x10^6$ CUF's of *S. aureus* within 15 minutes of entering water. Like *E. coli*, *S. aureus* are also thought to enter the marine environment through contamination from waste water treatment plants (176), run off after storms (162) and streams leading to the ocean. Research investigating survival of *S. aureus* in marine environments found the bacteria favoured seawater over fresh water due to the higher salinity (177). It also showed *S. aureus* could survive up to 11 days at 13°C compared to only 7 days at 20°C, indicating it prefers colder climates (177, 178).

2.1.6 Fucoxanthin

As well as looking at cross-resistance to seaweed extracts and clinically used antibiotics in bacteria isolated from seaweeds we shall use a disc diffusion assay to investigate whether clinical *S.aureus* strains are cross-resistant to antibiotics and Fucoxanthin. One study found



that Fucoxanthin is used by the brown seaweed *F. vesiculosus* as a chemical defence against microfouling (179). The same study also used anti-settlement assays to demonstrate Fucoxanthins ability to inhibit the settlement of bacteria isolated from *F. vesiculosus*, indicating its potential as a surface-associated antimicrobial agent (179). Fucoxanthin is a yellow accessory pigment commonly found in the chloroplasts of brown algae and it is one of the most abundant carotenoids, contributing to over 10%, in the marine environment (180). Due to its chemical structure (Fig 1.0), Fucoxanthin has ability to scavenge reactive oxygen species which contributes to its disease preventing effects (181). Peng et al (2011) summarised the numerous studies which have investigated its promising effects as an antioxidant, anti-inflammatory, anticancer, anti-obesity, antidiabetic, antiangiogenic and antimalaria agent (182).

Figure 1 Chemical structure of Fucoxanthin (183)

2.1.7 Aims and objectives

The aims of this chapter are to:

- Identify whether *Vibrio*, *E. coli* and *S. aureus* are present on the surface of different seaweeds using selective agars.
- Test if *Vibrio* isolated from seaweeds are locally adapted to their host species using a novel seaweed media assay.
- Test if *Vibrio* obtained from different seaweed species show cross-resistance to natural antimicrobial agents and clinically used antibiotics.

2.2 Materials and Methods

2.2.1 Seaweed Collection

Samples of different seaweed species were collected at multiple intervals between October 2016 and May 2017 at Castle Beach, Falmouth (51.6719° N, 4.6954° W) and The Greenbank Hotel, Falmouth (50.1612° N, 5.0744° W). Each sample was placed into a sterile plastic bag and transported to the laboratory. The seaweeds were thoroughly inspected, and any necrotic areas or epiphytes were removed before being gently washed with ddH_2O. Table 1 displays the different seaweed species, the location they were sampled and how they were used over the course of this project.

Table 1. Species of seaweed used for each experiment and the location they were sampled.

Seaweed Species	**Experimental Use**
Asparagopsis armata (Harpoon Weed) (Rhodaphyta) (C)	• Bacterial isolation • Disc diffusion assay: *Vibrio* isolation & extract • Prevalence of *IntI1** • Metagenomic sequencing*
Calliblepharis jubata (False Eyelash Weed) (Rhodaphyta) (C)	• Disc diffusion assay: extract • Seaweed media assay: *Vibrio* isolation & media

Corallina officinalis (Common Coral Weed) (Rhodaphyta) (C)	• Bacterial isolation • Disc diffusion assay: Extract • Seaweed media assay: *Vibrio* isolation & media • Prevalence of *IntI1** • Metagenomic sequencing*
Cystoseira baccata (Bushy Berry Wrack) (Phaeophyceae) (C)	• Bacterial isolation • Disc diffusion assay: extract
Cystoseira tamariscifolia (Rainbow Wrack) (Phaeophyceae) (C)	• Bacterial isolation
Fucus serratus (Serrated Wrack) (Phaeophyceae) (C & GB)	• Bacterial isolation (C) • Disc diffusion assay: *Vibrio* isolation & extract (C) • Seaweed media assay: *Vibrio* isolation & media (C) • Prevalence of *IntI1** (C & GB)
Fucus vesiculosus (Bladder Wrack) (Phaeophyceae) (C & GB)	• Bacterial isolation (C) • Extract for disc diffusion assay (C) • Prevalence of *IntI1** (C & GB)
Furcellaria lumbricalis (Clawed Fork Weed) (Rhodaphyta) (C)	• Bacterial isolation • Disc diffusion assay: *Vibrio* isolation & extract • Seaweed media assay: *Vibrio* isolation & media • Prevalence of *IntI1** • Metagenomic sequencing*
Himanthalia elongate (Thong Weed) (Phaeophyceae) (C)	• Bacterial isolation • Disc diffusion assay: *Vibrio* isolation & extract
Polyides rotundus (Discoid Fork Weed) (Rhodaphyta) (C)	• Bacterial isolation • Disc diffusion assay: *Vibrio* isolation & extract
Sargassum muticum (Wireweed) (Phaeophyceae) (C)	• Bacterial isolation • Disc diffusion assay: extract
Ulva lactuca (Sea Lettuce) (Chlorophyta) (C)	• Bacterial isolation • Disc diffusion assay: *Vibrio* isolation & extract • Prevalence of *IntI1** • Metagenomic sequencing*
Rhodaphyta= red seaweeds, Phaeophyceae= brown seaweeds and Chlorophyta= green seaweeds. C= Castle beach, GB=Greenbank Hotel * experiments featured in chapter 2	

2.2.2 Bacterial Isolation and Culture Conditions

Bacteria were isolated from the seaweed surface using sterile cotton swabs. 15 cm^2 sections were swabbed for 10 seconds in three places: the tips, thallus and base of each seaweed sample, using a single swab. Bacteria were transferred onto selective agar by swabbing the agar surface for 30 seconds. Five different selective agars were used: Marine agar (MA2216; Difco) General marine bacteria, TCBS agar (Oxoid) for *Vibrio*, Chromocult®

(enhanced selectivity) agar for *E. coli* (EMD Millipore), mannitol salt agar (10 g/L proteose peptone (Oxoid), 1 g/L meat extract (Fluka analytical), 75 g/L sodium chloride (Fisher Scientific), 10 g/L D-mannitol (Sigma Aldrich), 0.025 g/L phenol red (Sigma Aldrich) and 15 g/L agar powder (Alfa Aesar) adjusted to pH 7.4±0.2) and readymade sheep blood agar (Oxoid) both for *S. aureus* .

2.2.3 *Vibrio* Freezer stocks

After bacterial isolation from TCBS agar individual yellow and green *Vibrio* colonies were picked. *V. cholerae* and *V. alginolyticus* present as yellow colonies (184). *V. parahaemolyticus* and *V. vulnificus* form green colonies (184). Isolates were incubated at 28 °C at 180 rpm overnight in 5 ml of marine broth (MA2216; Difco). 1 ml of each overnight culture was centrifuged for 2 minutes at 2500 rpm and the supernatant was removed. The pellet was resuspended in 1 ml of 20 % glycerol solution, made from 250 µl of 80 % glycerol and 750 µl of marine broth (MA2216; Difco) and the glycerol stock was stored at -80 °C.

2.2.4 *S. aureus* Isolates

Eleven antibiotic resistant and susceptible *S. aureus* strains were obtained from Dr Ruth Massey (University of Bath, UK). A further seven pathogenic *S. aureus* strains were obtained from the Royal Cornwall Hospital (Truro, UK). Resistance properties of each strain can be found in table S1.

2.2.5 Seaweed Extracts

Seaweed samples were lyophilized using a freeze drier (Scanvac, Labogene) and ground to powder using a household spice grinder (Cookwork Coffee and Herb Grinder). 5 g of powder was mixed with 50 ml of 60 % methanol which was incubated for 2 hours at 40 °C at 100 rpm. The solution was centrifuged for 15 minutes at 2500 rpm after which the supernatant was decanted, evaporated overnight and resuspended in 5 ml of 60 % methanol (Fisher Scientific). Fucoxanthin (carotenoid antioxidant) (Sigma Aldrich) was dissolved in acetonitrile and diluted using ddH_2O to 1600 µg/ml and 800 µg/ml.

2.2.6 Antibiotics

Table 2 shows the stock concentrations, working concentrations of the different antibiotics and the solvents used. Antibiotics dissolved in ddH_2O were sterilised using a 0.22 µm filter (Millex).

Table 2. Antibiotics used for each experiment, their solvents and concentrations

Antibiotic	**Supplier**	**Stock Solution Conc. (mg/ml)**	**Solvent**	**Working Solution Conc.**		
				***Vibrio* disc diffusion assay (mg/L)** (146)	***S. aureus* disc diffusion assay (mg/L)**	**Community resistance assay (mg/L)**
Ampicillin sodium salt	Sigma Aldrich	10	ddH2O	4, 8, 16	**-**	2
Azithromycin	Sigma Aldrich	10	Ethanol	2, 4, 8	-	-
Chloramphenicol	Sigma Aldrich	10	Ethanol	8, 16	5, 10, 20	2
Ciprofloxacin	Fluka analytical	1	0.1N HCl	-	**-**	2
Erythromycin	Sigma Aldrich	10	Ethanol	-	2.5, 5, 10	-
Gentamicin sulfate	Amresco	10	ddH2O	2, 8, 16	**-**	16
Kanamycin Sulfate	Fisher Scientific	10	ddH2O	**-**	25, 50, 100	-
Tetracycline hydrochloride	Fisher Scientific	10	ddH2O	**-**	2.5, 5, 10	24

2.2.7 Kirby-Bauer Disc Diffusion Assay

Vibrio isolates grown for 18 hours at 28 °C in marine broth (MA2216; Difco) and *S. aureus* strains grown for 18 hours at 37 °C in LB broth were diluted to the McFarland standard (0.5-0.8 at 625 nm) (185) measured using a spectrophotometer (Jenway 7315, Bibby Scientific). 1.5 ml of diluted *Vibrio* was added to 28.5 ml of marine agar (MA2216; Difco) and mixed thoroughly before pouring into square plates (Gosselin). 400 µl of *S. aureus* dilution was added to 30 ml Mueller-Hinton agar (Oxoid) and mixed thoroughly before pouring into a square plate (Gosselin). Whatman antibiotic assay discs (Whatman international) were soaked for 5 hours in seaweed extract (table 1) or antibiotics stock (table 2) and dried in a laminar flow hood for 15 minutes before being placed on the surface of the agar using sterile tweezers (186). Negative controls (60 % methanol, ethanol, ddH_2O, acetonitrile and 0.1 N

HCl) were prepared in the same way (186). *Vibrio* plates were incubated overnight at 28 °C and *S. aureus* plates were incubated overnight at 37 °C. The diameter of each halo (zones of no bacterial growth around disc) were measured in millimetres using a ruler.

2.2.8 Seaweed Medium Assay

50 g (wet weight) of seaweed (table 1) was blended using a household hand blender (Braun, 250 watt). The blended seaweed was mixed with seawater and vortexed for 5 minutes. The mixture was strained using a 63 µm sieve (Endecotts). The filtrate was sterilised using a 0.22 µm filter (Millex) attached to a 10ml syringe. 200 µl of *Vibrio* isolate, cultured in marine broth (MA2216; Difco) overnight at 28 °C at 180 rpm, was added to 2 ml of seaweed medium and incubated for 18 hours at 28 °C at 100 rpm. Any turbidity was noted before adding 400 µl of LB broth (Fisher Scientific) and incubating for another 18 hours under the same conditions. After checking for turbidity, a further 600 µl of LB broth (Fisher Scientific) and incubating for a further 18 hours under the same conditions. Any culture which showed turbidity was plated on LB agar (Fisher Scientific) and incubated overnight at 28 °C to check for growth.

2.2.9 Community Resistance Assay

15 cm^2 from the tips of each seaweed, indicated in table 1, were cut and placed in 5 ml of marine broth (MA2216; Difco) containing 10 glass beads and vortexed for 30 seconds, after which the seaweed was removed. The remaining liquid was diluted 1:10 with marine broth (MA2216; Difco). 100 µl per plate of the diluted solution was plated onto marine agar plates (MA2216; Difco), amended with antibiotic (table 2) and a control plate containing no antibiotic. All plates were incubated for 18 hours at 28 °C before counting. Counts from amended plates were divided by the count from non-amended plate to calculate the proportion of resistant bacteria.

2.2.10 Statistical Analysis

All statistical tests were performed in R studio (version 1.0.153) (187). A binomial generalised linear model was used to identify the influencing factor on halo size. The linear

relationship between the number of seaweed extracts and conventional antibiotics different *Vibrio* strains were resistant to was classified using Pearson's correlation coefficient. One-way ANOVA, post hoc Tukey HSD tests and unpaired t-tests were used to identify significant differences in the average colony counts, disc diffusion assays, seaweed media assay and community resistance assay. Significance was tested at a 95 % confidence level. ggplot2 (188) was used to enhance basic R graphics.

2.3 Results

2.3.1 Presence of *E. coli*, *S. aureus* and *Vibrio* on seaweeds

The initial stage of our investigation was to isolate potential human pathogens from different species of seaweeds. We attempted to isolated *Vibrio*, *E coli* and *S. aureus* from different seaweed species during September 2016, November 2016 and March 2017. We were unable isolate any bacteria during November 2016, we could not isolate any *S. aureus* and we were only able to isolate very low number of *E. coli* from seaweeds samples in September 2016. As *E. coli* and *S. aureus* abundances seemed to be very low, we focused the rest of our experiments on *Vibrio*. Table 3 shows the average number of yellow and green *Vibrio* colonies isolated from 5 samples of different seaweed species during September 2016 and March 2017. Overall the average number of yellow colonies was higher than the average number of green colonies and there was more of both colonies colours in March 2017 compared to March 2017. *A. armata* and *C. baccata* had by the far highest number of colonies in March 2017 and *Cystoseira tamariscifolia* had the highest number of counts in September 2016. One-way ANOVA (Y: $p<2.00x10^{-16}$, G: $p= 1.13x10^{-13}$, n=5, ANOVA) and a post hoc Tukey tests (*p* summaries in Table S2, n=5, TukeyHSD) were used to identify significant differences in the number of *Vibrio* colonies isolated from each seaweed species in September 2016. The same tests were also used to compare the number of *Vibrio* colonies isolated in March 2017 (Y: $p<2.00x10^{-16}$, G: $p<2.00x10^{-16}$, n=5, ANOVA, *p* summaries in Table S2, n=5, TukeyHSD). The average number of yellow *Vibrio* colonies isolated from *Cystoseira tamariscifolia* and *S. muticum* in September 2016 were significantly higher compared to the other seaweeds (*p* values: see Table S2, n=5 TukeyHSD). *Cystoseira tamariscifolia* also had a significantly higher number of yellow colonies than *S. muticum* (*p*=0.00, n=5, TukeyHSD). *F. serratus* and *C. baccata* had a significantly greater green *Vibrio* colony counts than the other seaweed species sampled in

September 2016 (*p* values: see Table S2, n=5 TukeyHSD). *F. serratus* and *C. baccata* had statistically similar green colony counts (*p*=0.38, n=5, TukeyHSD). whereas *Cystoseira tamariscifolia* showed to be significantly higher (*p* values: see Table S2, n=5 TukeyHSD). The average yellow and green colony counts from *A. armata* and *C. officinalis* in March 2017 were significantly higher than the other seaweeds (*p* values: see Table S2, n=5 TukeyHSD). Both coloured colony counts from *S. muticum* were significantly different from the other seaweeds in March 2016 (*p* values: see Table S2, n=5 TukeyHSD). The exception to this was the green counts from *C. jubata* (*p*=0.67, n=5, TukeyHSD) *and P. rotundus* (*p*=0.09, n=5, TukeyHSD). The *C. jubata* colony counts were also statistically indistinguishable to the average number of colonies isolated from *P. rotundus* and *H. elongate.* The average number of green colonies from *P. rotundus* was statistically higher than the *H. elongate* green colony count. Statistical differences in the number of colonies isolated from each species of seaweed in September 2016 and March 2017 were tested using unpaired t-tests. The *Vibrio* colony counts from *F. serratus, S. muticum and U. lactuca* were significantly higher in March 2017 compared to September 2016 (*p* values summarised in table S3, n=5, unpaired t-test). The number of green colonies isolated from *F. serratus* were also significantly higher in March 2017 than September 2016 (*p*=0.01, n=5, unpaired t-test).

Table 3: Average number of *Vibrio* colonies isolated on TCBS agar from different seaweed species (five samples of each species) collected during September 2016 and March 2017.

	Average Colony Count			
	September 2016		**March 2017**	
	Yellow	**Green**	**Yellow**	**Green**

Asparagopsis armata	-	-	780.0 ± 25.0	146.0 ± 8.6
Calliblepharis jubata	-	-	65.4 ± 4.2	25.0 ± 2.3
Corallina officinalis	-	-	790.0 ± 10.2	154.0 ± 3.8
Cystoseira baccata	27.0 ± 1.0	4.0 ± 0.6	-	-
Cystoseira tamariscifolia	536 ± 14.0	11.0 ± 0.9	-	-
Fucus serratus	14.6 ± 0.4	5.6 ± 0.2	15.6 ± 0.8	7.6 ± 0.5
Fucus vesiculosus	6.2 ± 0.2	0.8 ± 0.3	14.8 ± 0.5	5.8 ± 0.3
Furcellaria lumbricalis	-	-	32.0 ± 2.2	13.0 ± 0.4
Himanthalia elongata	2.6 ± 0.2	1.0 ± 0.3	4.0 ± 1.0	1.0 ± 0.4
Polyides rotundus	-	-	35.6 ± 1.4	19.0 ± 0.8
Sargassum muticum	57.2 ± 1.5	1.6 ± 0.2	102.8 ± 1.5	34.4 ± 1.2
Ulva lactuca	8.0 ± 0.3	0.6 ± 0.4	14.2 ± 0.6	4.6 ± 0.5

Average ± SEM. – represents seaweed which were not sampled at that time. One-way ANOVA and post hoc Tukey HSD tests were used to identify statistical differences between counts in the same month (*p* values summarised in Table S2, *n*=5). Unpaired t-tests were used to compare the average number of *Vibrio* colonies isolated from a species in different months (*p* values summarised in Table S3, *n*=5).

2.3.2 Local Adaptation

We used seaweed media assays to investigate local adaption of *Vibrio* isolated from different seaweed species. Table 4 shows the growth of individual yellow or green *Vibrio* strains, isolated from *C. jubata, C. officinalis, C. baccata, F. serratus and F. lumbricalis,* inoculated in seaweed media prepared from the same species. Each strain was grown over four days. On day one 200 µl of LB broth was added to the seaweed media, on day two a further 400 µl of LB broth was added, then on day three 600 µl of LB broth was added. On day four we plated the mixture on LB agar. This four-day approach was done due to the lack of growth observed in the first couple of days. We were unable to demonstrate local adaptation due to difficulty in growing *Vibrio* strains in the seaweed media. None of the isolate grew in the seaweed media prepared from *F. lumricalis* or *C. officinalis*. There was growth of yellow and green *Vibrio* observed in the *C. jubata* and *C. Baccata* media. However, the *Vibrio* isolated from *F. serratus* and the yellow strain isolated from *F. lumbricalis* did not grow in the *C. jubata* media and *Vibrio* from *C. officinalis* did not grow in the *C. baccata* media. The green Vibrio isolated from *C. baccata* was the only to grow in the *F. serratus* media.

Table 4: Growth of a yellow and a green *Vibrio* strain, isolated from different seaweed species, in various 2 ml seaweed medias over four days

			Seaweed Media									
			Callibleph aris jubata		*Corallina officinalis*		*Cystoseira baccata*		*Fucus serratus*		*Furcellaria lumbricalis*	
			Y	G	Y	G	Y	G	Y	G	Y	G
***Vibrio* Origin**	*Calliblepharis jubata*	Day 1	+	-	-	-	-	-	-	-	-	-
		Day 2	+	+	-	-	+	+	-	-	-	-
		Day 3	+	+	-	-	+	+	-	-	-	-
		Day 4	+	+	-	-	+	+	-	-	-	-
	Corallina officinalis	Day 1	+	-	-	-	-	-	-	-	-	-
		Day 2	+	+	-	-	-	-	-	-	-	-
		Day 3	+	+	-	-	-	-	-	-	-	-
		Day 4	+	+	-	-	-	-	-	-	-	-
	Cystoseira baccata	Day 1	-	-	-	-	-	-	-	-	-	-
		Day 2	-	+	-	-	+	+	-	-	-	-
		Day 3	+	+	-	-	+	+	-	+	-	-
		Day 4	+	+	-	-	+	+	-	+	-	-
	Fucus serratus	Day 1	-	-	-	-	-	-	-	-	-	-
		Day 2	-	-	-	-	+	+	-	-	-	-
		Day 3	-	-	-	-	+	+	-	-	-	-
		Day 4	-	-	-	-	+	+	-	-	-	-
	Furcellaria lumbricalis	Day 1	-	-	-	-	-	-	-	-	-	-
		Day 2	-	+	-	-	+	+	-	-	-	-
		Day 3	-	+	-	-	+	+	-	-	-	-
		Day 4	-	+	-	-	+	+	-	-	-	-
Day 1=+200µl LB broth, day 2=+400µl LB broth, day 3=+600µl LB broth and day 4=plated on LB agar) + indicates growth. – indicates no growth. Y=yellow colonies, G=green colonies.												

Another way we looked at local adaption was by investigating whether *Vibrio* strains isolated from seaweed were less sensitive to methanol extracts of their host seaweed compared to extracts from other seaweeds. Figure 2 displays the effects of A: *A. armata,* B*: F. lumbricalis,* C: *P. rotundus* and D: *U. lactuca* extracts on green and yellow *Vibrio* isolated from: *A. armata*, *F. lumbricalis*, *P. rotundus* and *U. lactuca*. Again, we were unable to show local adaptation of bacteria isolated from seaweed. Bacteria isolated from *U. lactuca* displayed the opposite effect to cross-resistance, as isolates from other seaweed were resistant to the *U. lactuca* extract whereas both green and yellow *Vibrio* isolated from the seaweed were susceptible to the extract (Figure 2D). All the *Vibrio* isolates tested were highly susceptible to *A. armata*, with average halo sizes > 10 mm (Figure 2A). *Vibrio* colonies isolated from *P. rotundus* were the only isolates resistant, except for yellow *F. lumbricalis* isolates*,* to the *P. rotundus* extract which could indicate local adaptation. However, the isolates were also resistant to *F. lumbricalis* and *U. lactuca* extracts. One-way ANOVA found there were no

significant differences between the average halo sizes caused by the inhibitory of specific seaweed extracts on green and yellow *Vibrio* isolated from different seaweeds (*p* values summarised in Table S2, ANOVA).

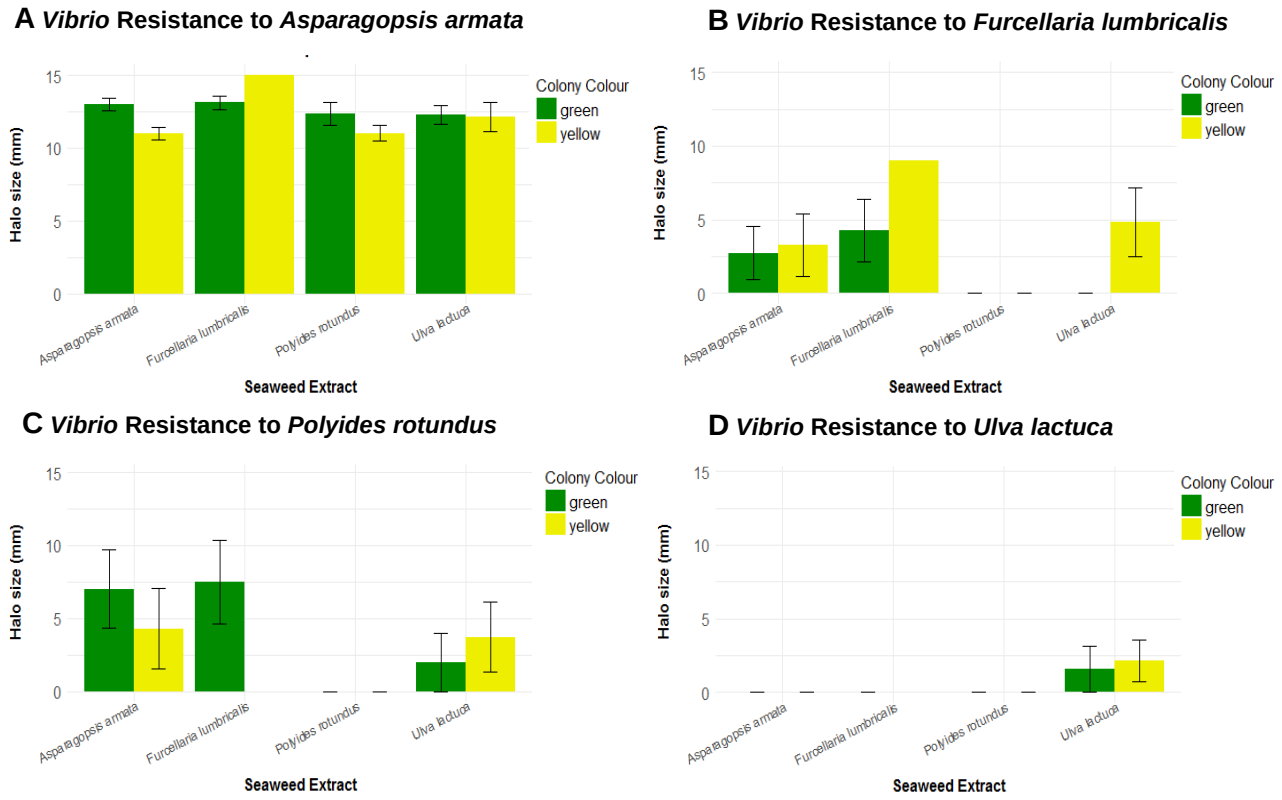


Figure 2

Bar chart showing the average halo sizes cause by **A** *Asparagopsis armata;* **B** *Furcellaria lumbricalis*; **C** *Himanthalia elongate* and **D** *Ulva lactuca* inhibiting the growth of yellow and green *Vibrio* strains (n=number of isolates) isolated from *Asparagopsis armata* (G: n=8, Y: n=7), *Furcellaria lumbricalis* (G: n=8, Y: n=1), *Polyides rotundus* (G: n=8, Y: n=7) and *Ulva lactuca* (G: n=7, Y: n=7). One-way ANOVA was used to identify significant differences in halo size of green and yellow vibrio colonies isolated from the different seaweed species in response to each seaweed extract (**A**: G: *p*=0.69, Y: *p*=0.20, **B**: G: *p*=0.11, Y: *p*=0.19 **C**: G: *p*=0.06, Y: *p*=0.47 **D**: G: *p*=0.34, Y: *p*=0.22, ANOVA).

2.3.2 Cross-resistance Between Clinically used Antibiotics and Seaweed Extracts

We investigated cross-resistance of *Vibrio* isolated from different seaweeds (Table 1) to conventional antibiotics (Table 2) and seaweed extracts (Table 1) using a disc diffusion assay. Figure 3 displays the correlation between the number of clinically used antibiotics (four classes at different concentration, total=eleven) and seaweed extracts (total=11) each *Vibrio* isolate (green= 38, yellow=27) showed resistance to in the disc diffusion assay. Pearson's correlation coefficient showed a significant positive correlation between resistance to seaweed extracts and clinically used antibiotics (r=0.24, p=0.05, n=65, Pearson's), indicating cross-resistance. All isolates were resistant to at least two seaweed extracts and six out of the eleven different concentrations of antibiotics used. Only one of the 65 *Vibrio* strains was resistant to all four antibiotics at the highest concentrations we used. None of the isolates were resistant to all the seaweed extracts. Most of strains were resistant to more antibiotic concentrations than seaweed extracts. On average, the *Vibrio* strains tested were resistant to 8.57 ± 0.82 (SD) of the total number antibiotics concentrations used (Table 2) and 5.37 ± 2.19 (SD) seaweed extracts (Table 1). The standard deviations demonstrate that there is more variation in the number of seaweed extracts each *Vibrio* isolate was resistant to compared to the number of conventional antibiotics.

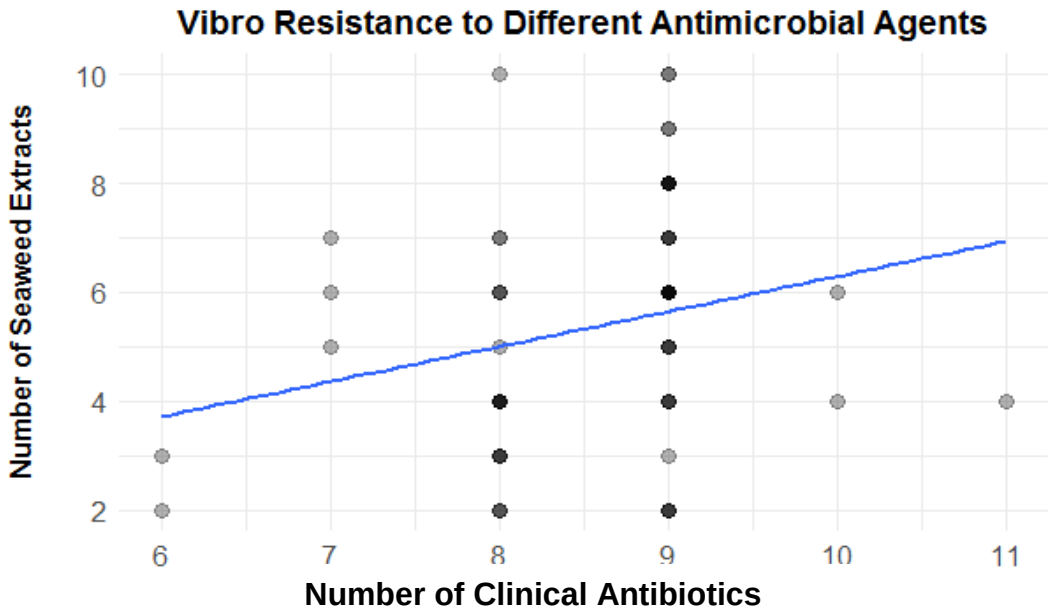


Figure 3

Scatter graph displaying the total number of seaweed extracts (Table 1) and clinically used antibiotics (Table 2) *Vibrio* isolates used for the disc diffusion were resistant to (n=65). Pearson's coefficient used to determine correlation (r=0.24, p=0.05, n=65). The shade of each dot represents the number of isolates with that resistance profile, darker dots=more isolates (min=1, max=9).

We used a binomial generalised linear model (GLM) to identify whether colony colour, antimicrobial agent or a combination of both factors had a significant effect on halo size. Colony colour (p=0.13, n=44, binomial GLM) and a combination of colony colour and antimicrobial agent (p=0.44, n=44, binomial GLM) did not have a significant effect on halo size, whereas antimicrobial agent did have a significant effect ($p<2x10^{-16}$, n=44, binomial GLM). Therefore, we investigated the effects of different antimicrobial agents further. Figure 2.1 displays the average halo sizes produced by green (Fig 2.1A) and yellow *Vibrio* (Fig 2.1B) colonies in response to clinically used antibiotics and seaweed extracts. Overall, both colony types were resistant to more antibiotics than seaweed extracts. High levels of resistance (average halo size<2.11±1.04 mm (SEM)) were observed towards: azithromycin 2 mg/L, azithromycin 4 mg/L, ampicillin 4 mg/L, ampicillin 8 mg/L, ampicillin 16 mg/L, gentamicin 2 mg/L, gentamicin 8 mg/L, gentamicin 16 mg/L, *C. officinalis* and *U. lactuca*. Both colonies types were highly susceptible (average halo size>9.79±1.19 mm (SEM)) to: Chloramphenicol 8 mg/L, Chloramphenicol 16 mg/L, *A. armata, C. baccata*, *F. serratus*, *F. vesiculosus* and *Himanthalia elongata*. Susceptibility to each antimicrobial agent was roughly the same for both yellow and green colonies, except for azithromycin 8 mg/L and *F. lumbricalis.* In figure 2.1A the average halo sized produced by azithromycin 8 mg/L (4.59±1.39 mm (SEM)) and *F. lumbricalis* (3.26±0.98 mm (SEM)) was larger compared to the halo size produced the same agents in figure 2.1B, 2.29±0.80 mm (SEM) and 1.47±0.60 mm (SEM) respectively. One-way ANOVA (A: $p<2x10^{-16}$, n=38, B: $p<2x10^{-16}$, n=27, ANOVA) and post hoc Tukey HSD tests were performed for both colony types to identify significant differences in halo size caused by each antimicrobial agent. Lower case letters were used to highlight antimicrobial agents which were not significantly different from each other. Results from the post hoc Tukey HSD test are summarised in Table S2

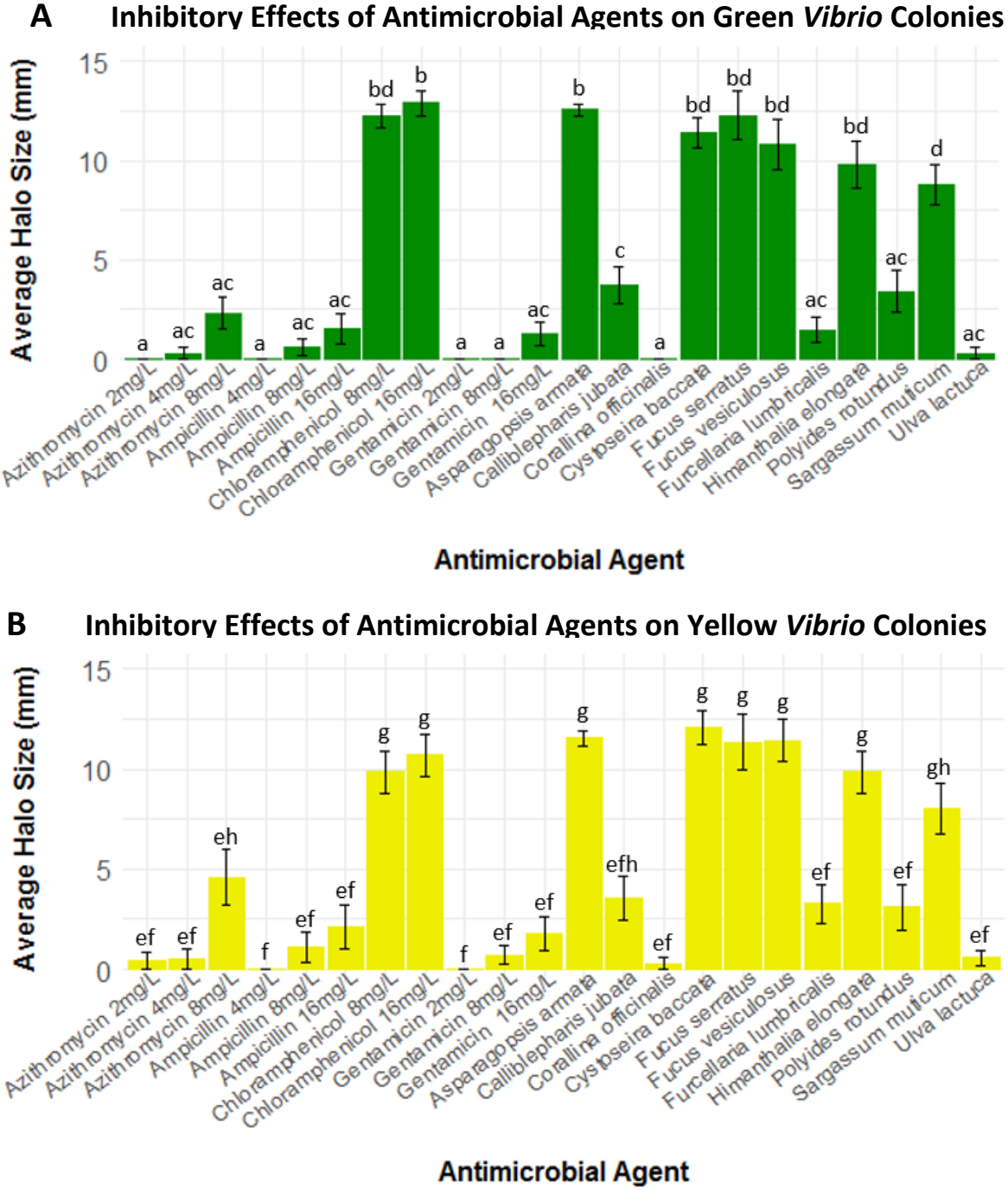


Figure 4

Bar charts displaying the average halo size (mm) produced by green (A) and yellow (B) *Vibrio* colonies in response to different antimicrobial agents. One-way ANOVA (A: $p<2x10^{-16}$, $n=38$, B: $p<2x10^{-16}$, $n=27$, ANOVA) and post hoc Tukey HSD test (p values summarised in table S2) used to detect significant differences. a, b, c and d represent antimicrobial agents which do not have significantly different effects on green vibrio colonies. e, f, g and h represent antimicrobial agents which do not have significantly different effects on yellow vibrio colonies.

2.3.3 *S. aureus* Resistance to Fucoxanthin

We used a disc diffusion assay to look at the resistance profiles of 18 clinical *S. aureus* strains to different conventional antibiotics of varying concentrations (Table 2) and two concentrations (800 mg/L and 1600 mg/L) of Fucoxanthin, a yellow accessory pigment found in brown algae. Figure 5 displays the results of that disc diffusion. All the *S. aureus* strains were resistant to Fucoxanthin and the majority were not susceptible to the lower concentrations of chloramphenicol. Around half of the strains were not susceptible to all three erythromycin concentrations and 2.5 mg/L of tetracycline. None of the isolates were resistant to kanamycin at the concentrations used (Table 2). A One-way ANOVA ($p<2x10^{-16}$, $n=3$) and post hoc Tukey HSD test (p values summarised in Table S2) were used to examine significant differences in the number of *S. aureus* isolates each antimicrobial agent could inhibit. The average number of *S. aureus* strains sensitive to tetracycline 2.5 mg/L and tetracycline 5 mg/L were significantly different from all the other antimicrobial agents and each other (p values summarised in Table S2). Susceptibility to tetracycline 10 mg/L and kanamycin was statistically indistinguishable, as was susceptibility to chloramphenicol 20 mg/L and erythromycin (p values summarised in Table S2). Chloramphenicol 5mg/L and 10mg/L had statistically indistinguishable numbers of sensitive *S. aureus* strains as Fucoxanthin (p values summarised in Table S2).

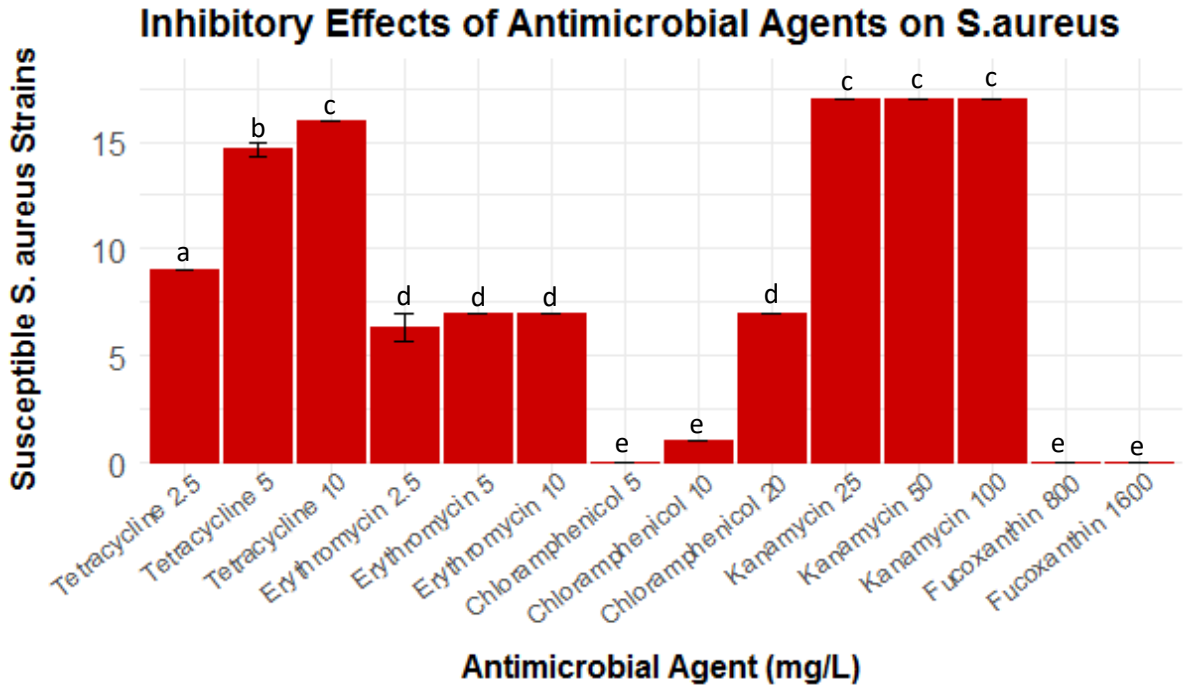


Figure 5

Bar chart displaying the average number of *S. aureus* strains (out of 18) susceptible to different clinically used antibiotics and fucoxanthin. A one-way ANOVA ($p<2x10^{-16}$, $n=3$, ANOVA) and post hoc Tukey HSD test (p values summarised in table S2, $n=3$) were used to test for significant differences between susceptibility to each antimicrobial agent. a, b, c, d and e signify the agents which were statistically similar.

2.3.4 Clinically used Antibiotic Resistance in Seaweed-Associated Bacteria

To extend our findings on AMR on seaweed-associated bacteria we also wanted to investigate antibiotic resistance of general bacterial communities isolated from different species of seaweed. This was done by isolating bacteria from seaweed on marine agar with and without antibiotics (Table 2). Figure 6 displays the proportion of bacteria resistant to ampicillin (2 mg/L), chloramphenicol (2 mg/L), ciprofloxacin (2 mg/L), gentamicin (16 mg/L) and tetracycline (24 mg/L) isolated from *C. officinalis* (A), *F. lumbricalis* (B) and *U. lactuca* (C). We also attempted to isolate antibiotic resistant bacteria from *A. armata* but found no resistance to any of the conventional antibiotics we used. One-way ANOVA and a post hoc TukeyHSD tests were used find significant differences between the proportion of bacteria resistant to different antibiotics isolated from specific seaweeds (p= Table S2, $n=6$, ANOVA and TukeyHSD). We used the same statistical tests to compare significant differences

between the proportion of bacteria resistant to a specific antibiotic isolated from different species of seaweeds (*p*= Table S2, *n*=6, ANOVA and TukeyHSD). There were very low proportions of ciprofloxacin and chloramphenicol resistant bacteria which were statistically similar for all three seaweeds (ciprofloxacin: *p*=0.30, chloramphenicol: *p*=0.09, n=6, ANOVA). The proportion of bacteria resistant to ampicillin and gentamicin was statically indistinguishable on all three seaweeds (*p*= Table S2, n=2, TukeyHSD). However, ampicillin resistance in *U. lactuca* associated bacteria was statistically higher than that in bacteria isolated from *C. officinalis* and *F. lumbricalis*. x and y. Surprisingly, the proportion of ampicillin resistant bacteria isolated from *U. lactuca* and the proportion of tetracycline resistant bacteria isolated from all three seaweeds exceeded one. This means the presence of tetracycline, and ampicillin for bacteria isolated from *U. lactuca*, increased the number of colonies which grew compared to the plates with no antibiotic.

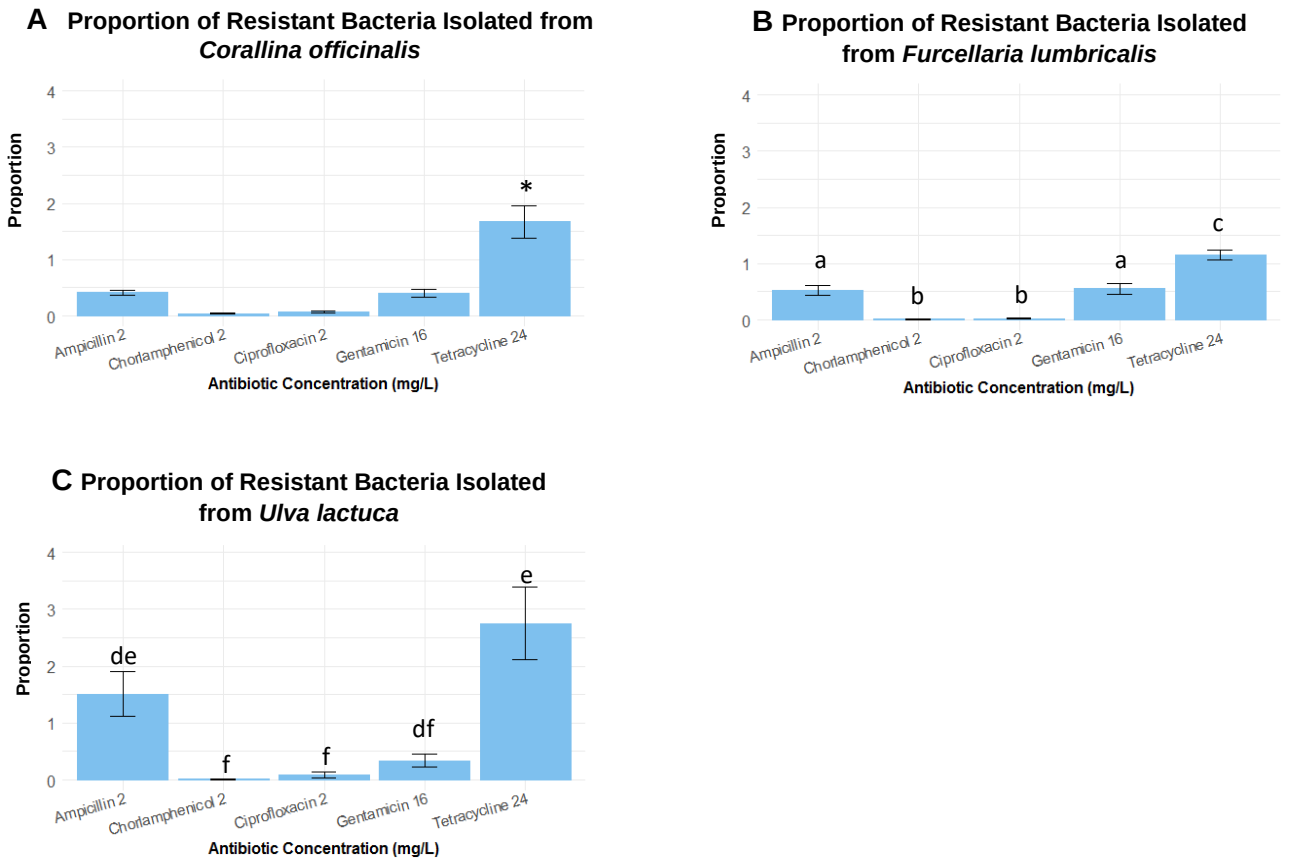


Figure 6

The proportion of antibiotic resistant bacteria isolated from three different seaweed species: **A** *Corallina officinalis*, **B** *Furcellaria lumbricalis* and **C** *Ulva lactuca*. One-way ANOVA (**A**: p=2.48x10^{-8}, **C**: p=1.83x10^{-11}, **D**: p=1.33x10^{-5}, n=6, ANOVA) and post hoc Tukey HSD tests were used to identify significant difference in the proportion of bacteria resistant to different antibiotics isolated from a seaweed species (p= table S2, n=6, Tukey HSD). **B:** * signifies statistically different result. **C:** a, b and c indicate proportions which are statistically indistinguishable. **D:** d, e and f indicate proportions which are statistically indistinguishable. One-way ANOVA (amp: p= 0.01, chlor: p= 0.09, cip: p= 0.30, gent: p= 0.30 and tet: p= 0.04, n=6, ANOVA) and post hoc-Tuckey tests were also used to compare the proportion of bacteria resistant a single antibiotic isolated from multiple seaweeds (p= table S2, n=6, Tukey HSD).

2.4 Discussion

2.4.1 Isolation of Human Pathogens from The Surface of Seaweeds

Our first aim was to try and isolate potential human pathogens, specifically *Vibrio*, *E. coli* and *S. aureus,* from the surface of seaweeds using culture-based techniques. We sampled different seaweeds species at different times of the year (September, November and March). We were able to isolate *Vibrio* from all the seaweeds sampled, but we were unable to isolate *S. aureus* and could only grow very low number of *E. coli*. Previous studies in our laboratory have looked at the effects of seaweed extracts on clinical *S. aureus* strains (128), for this investigation we wanted to see if *S. aureus* found in the marine environment is cross-resistant to clinically used antibiotics and natural antimicrobials, however we were unable to cultivate any of the bacteria. To our knowledge, *S. aureus* has never previously been identified in seaweed associated bacterial communities which is surprising due to its high salt tolerance (177). There were also very few *E. coli* colonies isolated from the different seaweed species. *E. coli* has been identified in the bacterial communities of multiple seaweed species (86, 88-91, 189) and so the inability to isolate this species is also somewhat surprising. However, we sampled the seaweeds from Castle Beach which is thought to be a relatively 'clean' beach, i.e. it is not in close proximality to any wastewater treatment plants. Therefore, we would expect very low levels of *S. aureus* and *E. coli* to be present in the water surrounding the seaweed meaning the bacteria is not available to settle on the macroalgae. Other studies also have shown difficulty isolating *E. coli* from marine environments using standard laboratory media (164, 190-192), which could explain the relatively low *E. coli* colony count.

We were able to isolate varying numbers of *Vibrio* colonies from different seaweeds in both September 2016 and March 2017. Many studies have previously isolated *Vibrio* spp. from multiple seaweed species, including *Corallina* sp., *Ulva* sp., *Fucus* spp. and *Sargassum* sp. (193-196). The *Vibrio* colony count from each seaweed species was higher in March 2017 compared September 2016, this could be due to the change in season, which previously has been shown to alter seaweed bacterial communities (79-81). The highest number of colonies were isolated from *A. armata C. officinalis, C. baccata* and *S. muticum*. This could be due to the shape of those seaweed species. *A. armata* and *C. officinalis* have lots of small, fine branches and *C. baccata* and *S. muticum* have small air bladders on their

branches. This means when we swabbed the 15cm^2 section of those seaweeds a higher surface area was covered, equating to more bacteria, compared to seaweed with large flat blades. We attempted to identify the specific *Vibrio* spp. we isolated using Sanger sequencing of the 16s rRNA gene. Unfortunately, this method failed therefore we continued to use colony colour to distinguish between the different *Vibrios* used in our experiments.

Our approach could have been improved by sampling all the seaweed species examined at all three timepoints. However, not all the seaweed species were available for sampling at each timepoint, due to seasonal changes in macroalgae. If we had more time we could have sampled the same seaweed species during the same months but a year later. This would have allowed us to see if the abundance of bacteria on the surface of different seaweeds follows a yearly pattern, like the trend observed in Lachnit et al., (2011) for *F. serratus* (99). We will complement the culture-based approach with culture-independent approaches (metagenomic sequencing) in the following chapter.

2.4.2 Local adaptation of Seaweed-associated Bacterial Communities

This was, to our knowledge, the first-time local adaption of seaweed associated bacterial communities has been tested therefore there were no previously established methods. We used both a seaweed media assay and a disc diffusion assay, but we were unable to show local adaption of bacteria settled on the surface of different seaweed species. Seaweed extracts have different physiological and chemical properties to fresh seaweeds, this is also true for the seaweed media. Therefore, the results of both seaweed media assay and disc diffusion assay may not truly reflect whether bacteria are locally adapted to seaweeds in the natural environment. The most accurate way to study local adaptation would be to see if bacteria isolated from one seaweed species could settle on other seaweed species in the marine environment, however this would be practically challenging. To recreate the marine environment microcosms containing seawater and seaweed could be used. An alternative method to study local adaption could be to use a settlement assay similar to the one used in Saha et al., 2011 (179). This could be done using bioactive compounds which allow bacteria to settle on specific seaweeds, such as Fucoidan (85, 197-199). Fucoidan is a polysaccharide commonly found in brown seaweeds which attract the settlement of fucoidanase bacteria such as *Verrucomicrobia* (85, 197-199).

2.4.3 Antimicrobial resistance in Seaweed-associated Bacteria

We used disc diffusion assays and a community resistance assay to test for antimicrobial resistance in seaweed associated bacterial communities. We found a positive correlation between resistance to conventional antibiotics and resistance to natural antimicrobials in *Vibrio* spp. isolated from seaweeds, which is consistent with cross resistance. Our results reflect those of Colclough et al., (128), and are suggestive that seaweeds can select for bacteria that are resistant to their secondary metabolites and that these same bacteria are also more resistant to clinically used antibiotics. These results have possible implications for human health. Potential cross-resistant pathogens settled on the surface of seaweeds may spread resistance genes to other bacteria colonising the macroalgae or to bacteria present in the surrounding water through horizontal gene transfer. Humans may be exposed to those resistant pathogens through bathing or consumption of seafood, if an infection develops from those pathogens it will make treatment difficult thus resulting in a major health risk (131, 167).

We could also use our results to identify seaweed species which have the potential to be developed into new antimicrobial agents. The occurrence of resistance to some of the seaweed extracts tested suggests those species may not be ideal candidates for potential antimicrobial agents. However, both yellow and green *Vibrio* showed high levels of susceptibility to some of the brown seaweed extracts tested, such as *C. baccata* and the *Fucus* spp. A previous study also showed brown seaweeds in general have higher antimicrobial activity compared to red and green macroalgae (200). Fucoxanthin is a carotenoid commonly found in brown seaweeds which has previously shown to inhibit the settlement of bacteria on *F. serratus* (179). Here, we tested the antimicrobial properties of fucoxanthin against clinical *S. aureus* strains resistant to various antibiotics. We found all strains were resistant to fucoxanthin at the concentration used, regardless of the resistance profile of the *S. aureus* strain. Although the pigment has anti-settlement properties, it is probably not behind the antimicrobial activity of brown seaweeds observed in the disc diffusion assays.

Both the disc diffusion assay testing different seaweeds and antibiotic classes against *Vibrio* and the assay testing fucoxanthin against *S. aureus* had the limitation of only testing one genus or species of bacteria. It would be beneficial to investigate the effects of brown algae

extracts and fucoxanthin against multiple bacterial species from both clinical and environmental sources, to obtain a more in-depth insight into the antimicrobial activity of brown seaweeds. Another option could be to test the antimicrobial properties of other specific secondary metabolites produced by brown macroalgae. Some studies have identified antimicrobial activities of laminarans (201) and phlorotannins (202), which are secondary metabolites specific to brown seaweeds.

Bacteria resistant to antibiotics were observed in the microbial communities isolated from different seaweed species tested in the community resistance assay. Over all, we found that bacteria isolated from different seaweed species are generally more susceptible to the same classes of antibiotics. The results reflected the resistance and susceptibility profiles of the *Vibrio* spp. tested in the disc diffusion assay. This suggests that the bacteria which grew on the marine agar were predominately *Vibrio*. To confirm this, we could have re-plated the bacteria on TCBS agar. The microbial communities isolated from all four seaweeds had very low numbers of bacteria resistant to chloramphenicol and ciprofloxacin. This suggests that chloramphenicol and ciprofloxacin resistant bacteria are either unable to colonise the seaweeds sampled or they are generally rare in the marine environment. To examine these ideas we could have used the community resistance assay to test for resistant bacteria in the water surrounding the seaweed samples or used a microbial inhibition assay to test for chloramphenicol and ciprofloxacin residues at the sampling site (203). The presence of tetracycline showed an overall increase in counts in three of the four seaweed culturable microbiomes. Tetracycline has previously been shown to cause a hormetic, unexpected growth stimulating, response in *E. coli* at low concentrations (204), which could explain our results. It would be interesting to plate the same bacteria on agar containing lower or higher concentrations of tetracycline to see if there is still an overall increase in bacterial growth.

Bacteria isolated from *A. armata* were susceptible to all the antibiotics tested as well as the *A. armata* extract. These results are surprising as we would expect the bacteria isolated from *A. armata* to be resistant to its host extract. If we assume cross-resistance, which we have demonstrated, we would expect the bacteria to be resistant to other antimicrobial agents due to the highly potent antimicrobial effects of the *A. armata* extract, which has shown to be down to it halogenated metabolites, such as bromoform and dibromoacetic acid (205). However, turning fresh seaweed into a methanolic extract significantly alters the physiological and chemical properties of the plant meaning when macroalgae is in extract

form it loses some of the metabolites. Therefore, the results displayed by bacteria isolated from *A. armata* may be a true representation of the bacteria in the natural environment.

CHAPTER 3: Characterisation of Seaweed-Associated Bacterial Communities and their Antibiotic Resistance Genes

3.1 Introduction

In the previous chapter we used culture-based techniques to identify specific pathogens present on the surface of seaweeds. In this chapter we use molecular techniques to characterise the bacterial communities of different seaweed species and identify specific genes present in those communities. Only a small percentage of bacteria are culturable (164, 190-192) and therefore we may not get an accurate representation of bacterial communities using purely culture-based methods. Molecular techniques have been shown to be better suited than culture-dependent techniques to the identification of a wide range of bacteria (206). It is difficult and time consuming to characterise the diverse bacterial phenotypes present in bacterial communities using culture-based techniques, therefore we shall use metagenomics during this project to characterise resistance genotypes. One gene we are particularly interested in is the class 1 integron-integrase gene (*intI1*), which we will quantify using qPCR.

3.1.1 The Class 1 Integron-Integrase Gene

The *intI1* gene is one of three features which makes up the genetic platform known as an integron, the other two are the recombination site (*attI*) and promoter (P_C). Integrons can acquire, stockpile and express exogenous genes which reside on gene cassettes, allowing bacteria which contain this genetic mechanism to adapt and evolve rapidly (207, 208). Class 1 integrons are often carried on transposons and plasmids allowing rapid dissemination of the genes they carry throughout both gram negative and gram-positive bacteria (209, 210). The *intI1* gene has been recovered from both environmental and clinical bacterial isolates, with DNA sequences from clinical settings being almost identical, suggesting that all class 1 integrons share a common and relatively recent ancestor (209). Many of the cassettes carried by class 1 integrons code for antibiotic resistance determinants (211), therefore the *intI1* gene is often used as a biomarker for antibiotic resistance. As anthropogenic factors have shown to be the driving force behind the increase in antimicrobial resistance (see section 2.4), it has been proposed the *intI1* gene could also be a biomarker for human pollution in the environment (212). Studies have already found increased levels of the gene

in sewage sludge, pig slurry and run off from wastewater treatment plants (212-216), which have all been shown to increase levels of ARG's in the environment.

To our knowledge there have been no previous studies investigating the presence of the *intI1* gene in seaweed surface associated bacteria, and there have been very few studies on the *intI1* gene in the marine environment. One of the first studies to do so surveyed 3000 gram-negative strains from multiple estuaries isolated over a two-month period. 3.6% of the isolates contained *intI1*, of which only 19 contained gene cassettes conferred antibiotic resistance (217). Another study used PCR and found 11% of *E. coli* isolates isolated from multiple estuaries in France contained the *intI1* gene. The class 1 integrons predominately contained the *dfr* cassette gene, which confers resistance to trimethoprim (218). Wang et al., (2008) also used PCR to detect the *intI1* gene in *Enterobacteriaceae* isolated from Jiaozhou Bay, China and found 68.8% of the isolated tested positive for the gene (219). The *intI1* gene was also found to be present in gram-negative ampicillin resistant bacteria isolated in Ria de Aveiro, the gene was found in 29.6% of the *Enterobacteriaceae* isolates and in 21% of the *Aeromonas* isolates (220). We will use PCR and qPCR to quantify the presence of the *intI1* gene in bacterial DNA isolated from the surface of different seaweed species. This should allow us to quantify the prevalence of antimicrobial resistant bacteria in seaweed associated bacterial communities.

3.1.2 Antibiotic Resistance Genes in the Marine Environment

There has been very little research into ARGs in the marine environment. Most of the studies which have been conducted have focused on resistance in aquaculture systems in Asian countries. One study used PCR to identify resistance genes in bacteria isolated from marine aquaculture sites in both Japan and Korea. 22.5% of the isolates carried *tet*(M), a gene which confers resistance to tetracycline antibiotics, with most of the isolates being *Vibrio* sp., *Lactococcus garvieae* or *Photobacterium damsel (221)*. *Tet*(S), another tetracycline resistance gene, was also detected in *Vibrio* sp. sampled in Korea (221). Nonaka et al., (2007) also used PCR and 16s rDNA sequencing to analyse bacteria sampled from aquaculture sites in Japan and found the presence of *tet*(M) in *Vibrio* sp. (222). The bacteria were collected from a site which had used oxytetracycline therapy 92 days before sampling. Oxytetracycline is a tetracycline antibiotic which is used as a prophylactic measure in aquaculture (223), which may explain why there is an abundance of tetracycline resistance

genes present in those environments. Oxytetracycline-resistant bacteria were also detected in Chinese mariculture environments. The bacterial species included *Vibrio*, *Pseudoaltermonas* and *Altermonas* and contained *tet*(A), *tet*(B) and *tet*(M) resistance genes (224). The same study also detected *cat II* and *floR* genes which could be the determinates of the chloramphenicol-resistant *Pseudoaltermonas* isolated from the mariculture environments (224). Another study also isolated chloramphenicol-resistant bacterium from Chinese coastal waters. *Cat I* and *catt III* genes were detected in human pathogens such as *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis and Shewanella algae* (225).

3.1.3 Metagenomic Sequencing

Metagenomic sequencing is defined as the analysis of genetic material contained within an environmental sample. Previous metagenomic studies on soil samples have revealed culture-based techniques and PCR have overlooked huge reservoirs of ARGs in the natural environment (226-229), for this reason we opted to use metagenomic sequencing to characterise seaweed associated bacterial communities and identify any ARGs present. To identify ARGs within our samples we compared our sequence data to The Comprehensive Antibiotic Resistance Database (CARD) a reference sequence database which compiles data from the last five decades and provides information on antibiotics and their targets, antibiotic resistance genes, associated proteins and associated literature (230). All sequences are imported from GenBank (231) and are associated with peer reviewed PubMed publications, which contributes to the more than 1600 (as of 2013) known antibiotic resistance genes available in the database (230). CARD is preferred to other similar databases as it is under constant curation and has developed a new ontology to standardise the language used to classify and describe ARGs (230). This is important when compiling data from multiple sources as it allows for accurate, comparable results being returned from the database. The features stated above are why we selected CARD, opposed to other databases, as our reference sequence database for our metagenomic data. We shall use Krona (232) to help present our metagenomic data. Krona is an online tool which can be used to visualise complex hierarchical data. Here we shall use it to explore the proportions of different taxonomic groups assigned to the genes present in each of our samples.

Previous research on ARGs in the marine environment have predominately used PCR and 16s rDNA sequencing to identify resistance genes and host species identity (221-225).

However, those techniques only allow the identification of known resistance genes as they use targeted primers. Metagenomic sequencing relies on DNA extraction directly from environmental samples and sequencing of a subsample of all bacteria and genes within that subsample, therefore it can access bacteria which may not be readily culturable on standard agar media. This is especially important as the vast majority of marine bacteria cannot be cultured using standard laboratory practice (190-192). In contrast to the above culture-based studies focusing specifically on fish pathogens, a more recent study used functional metagenomics to analyse the resistance genes present in a range of marine environments located around the USA (233). Only 28% of the genes classified as (potential) resistance genes detected were previously known from clinical studies, for example *bcr* (tetracycline resistance), *sul1*(sulphonamide resistance) and *tem1* (beta-lactamase resistance) (233). The other 72% of genes detected were unassigned, of those genes 44% were categorised as tetracycline resistant and 27% were thought to be ampicillin resistant. This meant that over half the ampicillin resistant genes were classified as unknown (233). One study used metagenomics to identify antibiotic resistance genes in bacterial communities associated with *Asparagopsis taxiformis* and *A. armata* (234) and found the presence of genes conferring resistance to fluoroquinolones on *Asparagopsis taxiformis* and methicillin resistance on *A. armata* (234). In this study, we use metagenomics to characterise the bacterial communities present on *A. armata, C. officinalis, F. lumbricalis* and *U. lactuca* and identify the presence and abundance of ARG's in those communities.

3.1.4 Aims and Objectives

The aims of this chapter are to:

- Detect and quantify the prevalence of *intI1* in seaweed associated bacterial communities using PCR and qPCR
- Identify the presence of our focal human pathogens on different seaweed species and characterise any ARG's associated seaweed bacterial communities using metagenomics.

3.2 Methodology

3.2.1 DNA Extraction

15 cm^2 sections of the tip, thallus and base of each seaweed (Table 1) was swabbed for 10 seconds using two sterile cotton swabs to obtain bacteria. Bacterial DNA was extracted using a QiaAMP DNA mini kit (Qiagen) following the manufacturers protocol for buccal swab with the following modifications (235): after the addition of protease K, the incubation time was increased to 60 minutes at 56 °C, and incubation with AL buffer was changed to 10 minutes at 70 °C followed by 5 minutes at 95 °C (236).

Quality and Quantity of DNA was measured using a NanoDrop 2000/2000c Spectrophometer (Thermo Scientific). All DNA samples had a concentration above 1µg/ml, 260/280 values between 1.6-2.0 and 260/230 values between 1.9-2.3.

3.2.2 Polymerase Chain Reaction (PCR)

PCR reaction mix was prepared on ice and consisted of: 7.5 µl of OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (BioLabs), 0.75 µl of forward primer (able 5), 0.75 µl of reverse primer (table 5), 3.8µl ddH_2O and 0.1 5µl of bovine serum albumin (Fluka analytical). 2 µl of bacterial DNA was mixed with 13 µl of PCR reaction mixture. A Veriti® 96-Well Thermal Cycler was used to run the following programme: 95 °C for 2 minutes (one cycle), 95 °C for 20 seconds, 55 °C for 30 seconds then 72 °C for 30 seconds (35 cycles) and then 72 °C for 5 minutes (one cycle). 6 µl of GeneRuler 1 kb Plus DNA Ladder (Thermofisher scientific) was loaded into first well of 1 % agarose gel (1 g agarose (Fisher Scientific), 100 ml of TAE buffer (Fisher Scientific) and 5 µl ethidium bromide (Fisher Scientific)). 5 µl of PCR mix was loaded into subsequent wells. Gel was run for 90 minutes at 80 volts. PCR products were imaged using a Gbox (Syngene) and captured using G box Chemi XLI (V1.280) (Syngene).

Table 5: 10 µM of primers (Integrated DNA Technology) used to target 16s gene and intI1 gene for PCR and qPCR.

Target	**Primer name**	**Sequence (5'-3')**	**Size (BP)**	**Reference**
16s	1369F	CGGTGAATACGTTCYCGG	124	(237)
16s	1492R	GGWTACCTTGTTACGACT	124	(237)
intI1	intI1-LC1	GCCTTGATGTTACCCGAG AG	196	(238)
intI1	intI1-LC5	GATCGGTCGAATGCGTGT	196	(238)

3.2.3 Quantitative PCR (qPCR)

Each 20 µl qPCR reaction mixture contained: 10 µl Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Agilent), 1 µl of forward primer (table 5), 1 µl of reverse primer (table 5), 2.4 µl ddH_2O, 0.6µl reference dye (Agilent) and 5µl of bacteria DNA (diluted 5-fold from original concentration). Reactions for each sample were performed in duplicate. A StepOnePlus Real-Time PCR System (Applied Biosystems) was used to run the following programme: holding stage: 95 °C for 20 seconds (one cycle), cycling stage: 95 °C for 10 seconds then 60 °C for 30 seconds (50 cycles) and melt curve: 95 °C for 15 seconds, 60 °C for 60 seconds then 95 °C for 15 seconds (one cycle). 16s rRNA gene copy number was used a proxy for bacterial cell count and used to calculate prevalence of the *intI1* gene. Copy number of each gene was determined by comparing to a standard curve, which was made from a serial dilution of each target gene (16s: $1x10^8$-$1x10^4$, intI1: $5x10^6$-$5x10^3$).

3.2.4 Metagenomic Sequencing

Library preparation, metagenomic sequencing and the bioinformatics was performed by Dr Karen Moore and Mr Paul O'Neil at the Exeter Sequencing Service & Computational Core Facility the University of Exeter. A Nextera XT DNA library preparation kit (Illumina) was used to prepare bacterial DNA isolated from three samples of four different seaweed species (Table 1).

Tagmentation of Input DNA

10 µl of tagment DNA buffer and 5µl of amplicon tagment mix were added to 5 µl of input DNA (1 ng total). Mixture was places in thermocycler for 5 minutes at 55 °C and then held until it reached 10 °C. 5 µl of neutralize tagment buffer was added to each sample and incubated at room temperature for 5 minutes.

PCR Amplification

15 µl of Nextera PCR master mix, 5 µl of a N7 index primer (Table 6) and 5µl of a S5 index primer (Table 6) were added to each sample.

Table 6. N7 and S5 index primer added to each DNA sample

Sample	N7 Index Primer	N7 Sequence	S5 Index Primer	S5 Sequence
1	N701	TAAGGCGA	S517	GCGTAAGA
2	N702	CGTACTAG	S502	CTCTCTAT
3	N703	AGGCAGAA	S503	TATCCTCT

4	N705	GGACTCCT	S504	AGAGTAGA
5	N709	GCTACGCT	S505	GTAAGGAG
6	N710	CGAGGCTG	S506	ACTGCATA
7	N711	AAGAGGCA	S507	AAGGAGTA
8	N712	GTAGAGGA	S508	CTAAGCCT
9	N716	ACTCGCTA	S513	TCGACTAG
10	N718	GGAGCTAC	S515	TTCTAGCT
11	N719	GCGTAGTA	S516	CCTAGAGT
12	N720	CGGAGCCT	S518	CTATTAAG

The following PCR programme was run: 72 °C for 3 minutes (one cycle), 95 °C for 30 seconds (one cycle), 95 °C for 10 seconds, 55 °C for 30 seconds then 72 °C for 30 seconds (12 cycles) and then 72 °C for 30 seconds (one cycle).

PCR Clean-up

90 µl of AMPure XP beads were added to each sample and incubated for 5 minutes at room temperature. Samples were placed on magnet for 2 minutes, after which the supernatant was removed and discarded. Beads were washed by adding 200 µl of 80 % ethanol and leaving for 30 seconds, after which the ethanol was removed. This stage was repeated twice. Beads were then left at room temperature for 10 minutes. They were then resuspended in 10 µl resuspension buffer before being incubated for 15 minutes at room temperature. The resuspended beads were again placed on the magnet for a further 2 minutes. 5 µl of the supernatant of each sample were removed and used for library pooling.

Library Pooling and Sequencing Loading

5 µl of supernatant of each sample were all added to a 1.5 ml Eppendorf. 576 µl of hybridization buffer was added to 24 µl of the pooled sample. The mixture was incubated for 2 minutes at 96 °C before being inverted twice and placed in an ice water bath for 5 minutes. The pooled sample was loaded into a MiSeq reagent cartridge. The library was then sequenced using a MiSeq (Illumina) machine according to the MiSeq system user guide (239). The use of index primers in the previous step allows multiple samples to be sequenced at the same time. The MiSeq (Illumina) machine uses the unique index primer sequence attached to each sample to distinguish which read is associated with which sample,

Bioinformatics

The raw Illumina reads were filtered then adaptor and quality trimmed (<Q20) using fastq-mcf (ea-utils) using the following parameters: -q 20 -l 35 -k 0 -p 15 –max-ns 0 -m 1 -t 0.01. The trimmed reads were aligned and compared to CARD (230) using double index alignment of next-generation sequencing data (DIAMOND) (University of Tübingen) (240) Similar reads were clustered and assembled into contiguous fragments of DNA sequence (contigs) using SPAdes 3.11.0 (St. Petersburg State University) (241) using the k-mer lengths: 21, 33, 55, 77, 99, 121. Contigs <500bp were removed. Basic local alignment search tool (protein) (BLASTX) (242) was used to compare the contigs to the CARD databases. CARD contains multiple detection models: 'homolog' which compares sequence data to reference antimicrobial resistance protein data and 'variant' which compares sequence data to reference data conferring mutations and give information on host bacteria as mutations are often host-specific (243). Results from the CARD detection models were combined and any replicates or hits with an E value ≤$1x10^{-5}$ (244) or an amino acid identity ≥90% were removed (245). Krona (232) was used to assign and visualise the taxonomic identities of different genes present in each sample, based on the results from CARD.

3.2.5 Statistical Analysis

All statistical tests were performed in R studio (version 1.0.153) (187). One-way ANOVA and post hoc Tukey HSD tests were used to detect significant differences in the prevalence of the *intI1* gene associated with different seaweed species from different locations and the average number of hits per kilobase for both the read and contig metagenomic data. Significance was tested at a 95 % confidence level, $P<0.05$ was considered a significant result. ggplot2 (188) was used to enhance basic R graphics. Krona was used to visualise the hierarchies within the metagenomic data (246).

3.3 Results

3.3.1 Abundance of *IntI1* in Bacterial DNA from Seaweeds

We used PCR and qPCR to detect and quantify the abundance of the *intI1* gene used as biomarker for antibiotic resistance in bacterial DNA swabbed from different seaweed species which had been washed to ensure only the seaweed microbiome was isolated. This gave an indication of whether there were any genes conferring to antibiotic resistance in our

samples before continuing with more expensive and time-consuming methods such as metagenomics. Figure S1 is a visualisation of PCR reaction used to detect the number of 16S rRNA and *intI1* genes in bacterial communities recovered from: A: *U. lactuca* B: *F. serratus* C*: F. vesiculosus,* D: *H. elongate* and E: *C. baccata*. The 16S rRNA controls only worked for samples A, B and C. *U. lactuca* was the only sample to have a faint band at 200bp, confirming the presence of the *intI1* gene for bacteria isolated from *U. lactuca* (A2 in Figure S1). The *intI1* gene was undetectable in bacterial DNA isolated from the other seaweed species.

PCR did not allow us to quantify the abundance of the *intI1* gene in bacterial DNA obtained from seaweeds therefore we used qPCR in addition to PCR. qPCR has a lower detection limit compared to PCR meaning we were able to quantify low levels of the gene. The *intI1* gene was amplified from all the seaweeds sampled. Figure 7 displays the prevalence of *intI1* in bacterial DNA isolated from different seaweed species. Bacterial DNA obtained from *A. armata, C. officinalis* and *U. lactuca* had a lower prevalence of the gene compared to the control, which was seawater sampled from the same rockpool as the seaweeds, indicating their bacterial communities contain a low abundance of resistance genes. The prevalence of the *intI1* gene was highest in bacterial DNA isolated from *F. serratus*, followed by *F. serratus* and then *F. lumbricalis*. Therefore, we would expect those seaweeds to have the highest abundance of ARGs in their bacterial communities. A post hoc Tukey HSD test found the prevalence of the gene obtained from *F. serratus* was significantly higher than *A. armata* and *U. lactuca* (*p*= see Table S2, TukeyHSD).

In addition to comparing the prevalence of the *intI1* gene in bacterial DNA isolated from different seaweed, we investigated the prevalence of the gene obtained from seaweeds sampled from different sites. As *F. serratus* and *F. serratus* showed the highest prevalence of the gene we decided to compare these results to the prevalence of the gene detected in bacterial communities of seaweeds of the same species sampled from another location (Figure 8). We chose The Greenbank hotel as it is more likely be impacted by pollution than castle beach. We also tested for the gene in seawater sampled from The Greenbank hotel harbour. The prevalence of the gene obtained from all four seaweeds were relatively similar, despite being sampled from different locations (Figure 8). The prevalence of the *intI1* gene was significantly higher in the water sample from The Greenbank Hotel (*p*= Table S2,

TukeyHSD). This implies that the water at The Greenbank Hotel contains more ARGs compared to the water at Castle Beach.

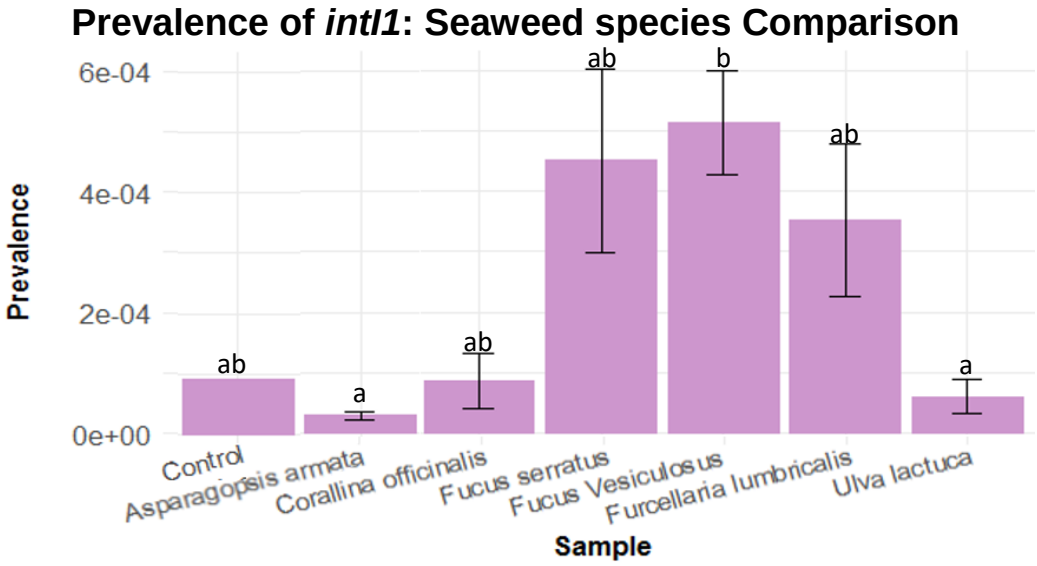


Figure 7

Prevalence of the *intI1* gene obtained from bacterial DNA isolated from: *Asparagopsis aramata, Corallina officinalis, Fucus serratus, Fucus vesiculosus, Furcellaria lumbricalis* and *Ulva lactuca*, sampled from castle beach. Control= seawater. One-way ANOVA (*p*= 0.01, seaweed: n= 3, control: n=1) and a post hoc TukeyHSD test (*p*= see Table S2) identified significant differences in the prevalence of the gene obtain from different seaweeds. Statistical similarities indicated by a and b.

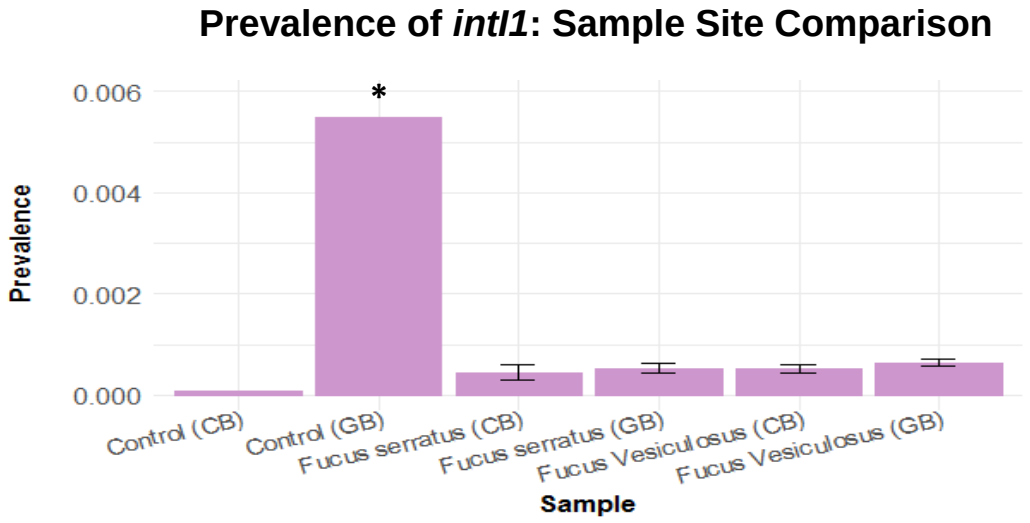


Figure 8

Prevalence of the *intI1* gene in bacterial communities of *Fucus serratus* and *Fucus vesiculosus* sampled from Castle Beach (CB) and The Greenbank Hotel (GB). Control= seawater. One-way ANOVA (*p*= 1.41e-07, seaweed: n= 3, control: n=1) and a post hoc TukeyHSD test (*p*= see Table S2) were used to detect significant differences in the prevalence of the gene between samples. * indicates a significant result.

3.3.2 Metagenomic Characterisation of Seaweed-Associated Communities

In the previous chapter we used culture-based methods to try and isolate potential human pathogens from the surface of different seaweeds. However not all bacteria are culturable (164, 190-192) therefore we attempted to characterise the bacterial communities of different species using metagenomic analysis. We analysed three samples of *A. armata, C. officinalis, F. lumbricalis* and *Ulva lactuca*. Figure 9 and 10 show screenshots of a Krona plot which gives a hierarchical classification of microbiome of one of the *A. armata* samples. Figure 9 gives an overview of contigs which could be classified, the contigs which could not be classified were categorised under 'no hits'. The contigs which could be assigned were initially classified by domain. Figure 10 gives a zoomed in view of the 'Bacteria' section of the Krona plot which uses a multi-layered approach to classify the bacteria present in the community, ranging from phylum to specific species. The Krona plots allowed us to identify and compare possible bacteria present on the different seaweed species, which is summarised in Table 7. Krona plots, bacterial view, for the other *A. armata* samples and *C. officinalis, F. lumbricalis* and *U. lactuca* samples can be found in the supplementary data (Figure S2- Figure S12).

Table 7 gives the average percentage breakdown of the possible bacteria isolated from *A. armata, C. officinalis, F. lumbricalis* and *Ulva lactuca.* Initially, we looked at the percentage of contigs which returned a result. Roughly 80% of the contigs assembled from each sample gave no hits. However, the majority of the successful hits were classed as bacteria, between 13.00 % and 17.91% ± 1.91 % of the root values of the Krona plot. The percentage of hits classified as Eukaryota was <1% for all the seaweed species except *A. armata* (2.3 % ± 1.01%). Roughly 40% of the bacteria isolated from *C. officinalis* and *F. lumbricalis* were classed as *Alphaproteobacteria,* whereas almost 50% the bacteria isolated from *A. armata* was classed under *Gammaproteobacteria.* The bacterial community isolated from *U. lactuca* was more evenly spread throughout the different classes of bacteria compared to the other seaweed species. 33.10 % ± 3.35 % of the bacteria could not be categorised under the five main classes, which were more distinct in the bacterial communities of the other seaweeds. *Betaproteobacteria* made up almost 20% of the *U. lactuca* bacterial community, whereas it only contributed to roughly 5% of the bacterial communities isolated from the other seaweed species.

Between 0.5 % and 3 % of bacteria isolated from the four different seaweeds were classified *Vibrio spp.* The highest percentage of *Vibrio spp* was isolated from *A. armata* (2.10 % ± 0.87 %). Our results suggested the presence of species with similarity to *Vibrio cholerae* and the most common non-cholera *Vibrio* species, *V. parahaemolyticus, V. alginolyticus* and *V. vulnificus* were present on all the seaweed species tested. *E coli* contributed <1 % of the bacteria isolated from *A. armata* and <0.3% isolated from *C. officinalis, F. lumbricalis* and *U. lactuca.* There was no detection of *S. aureus* isolated from any of the seaweed species.

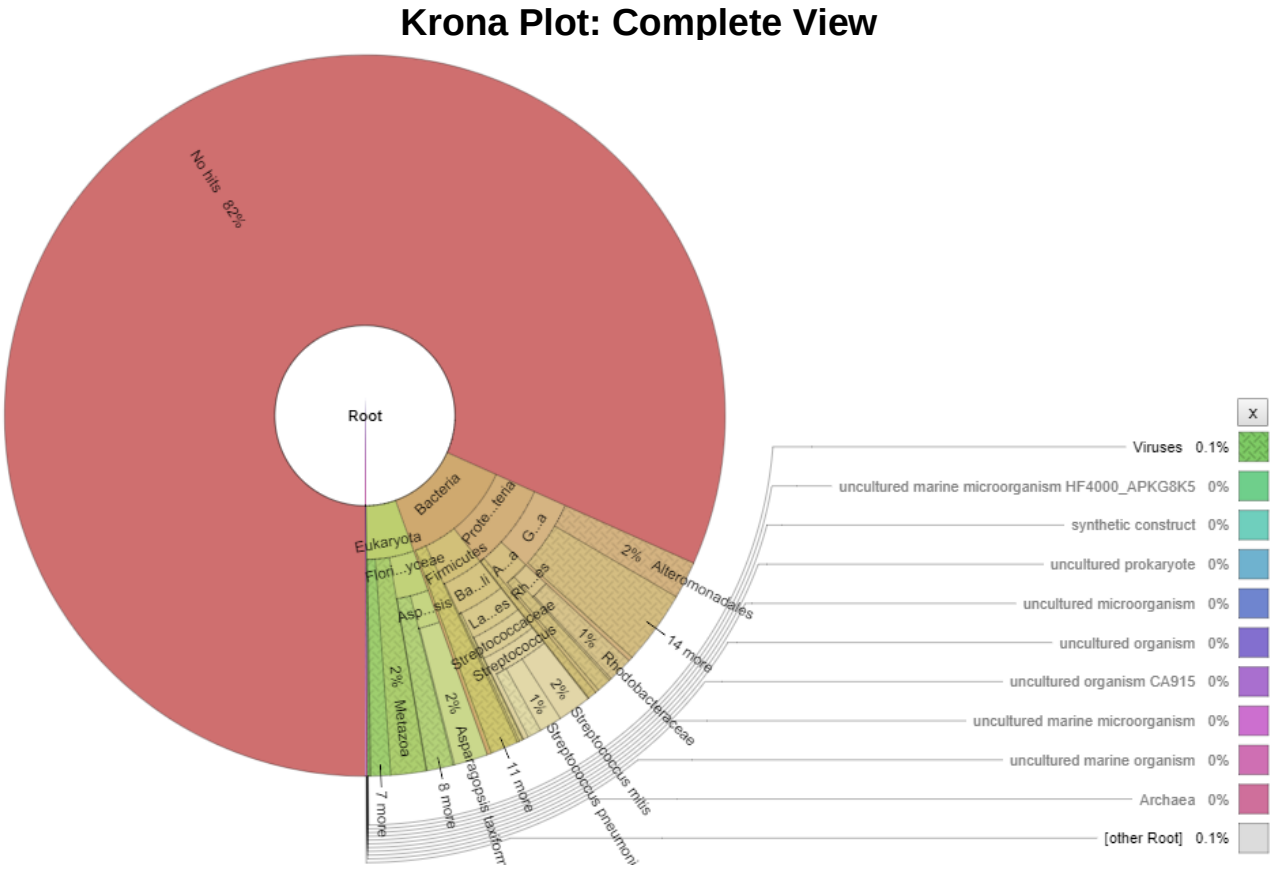


Figure 9

Krona plot displaying an overview of the microbiome isolated from one of the three *Asparagopsis armata* samples presented at the domain level. "No hits" is the percent of contigs which did not return a result

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Krona Plot: ‘Bacteria’ only

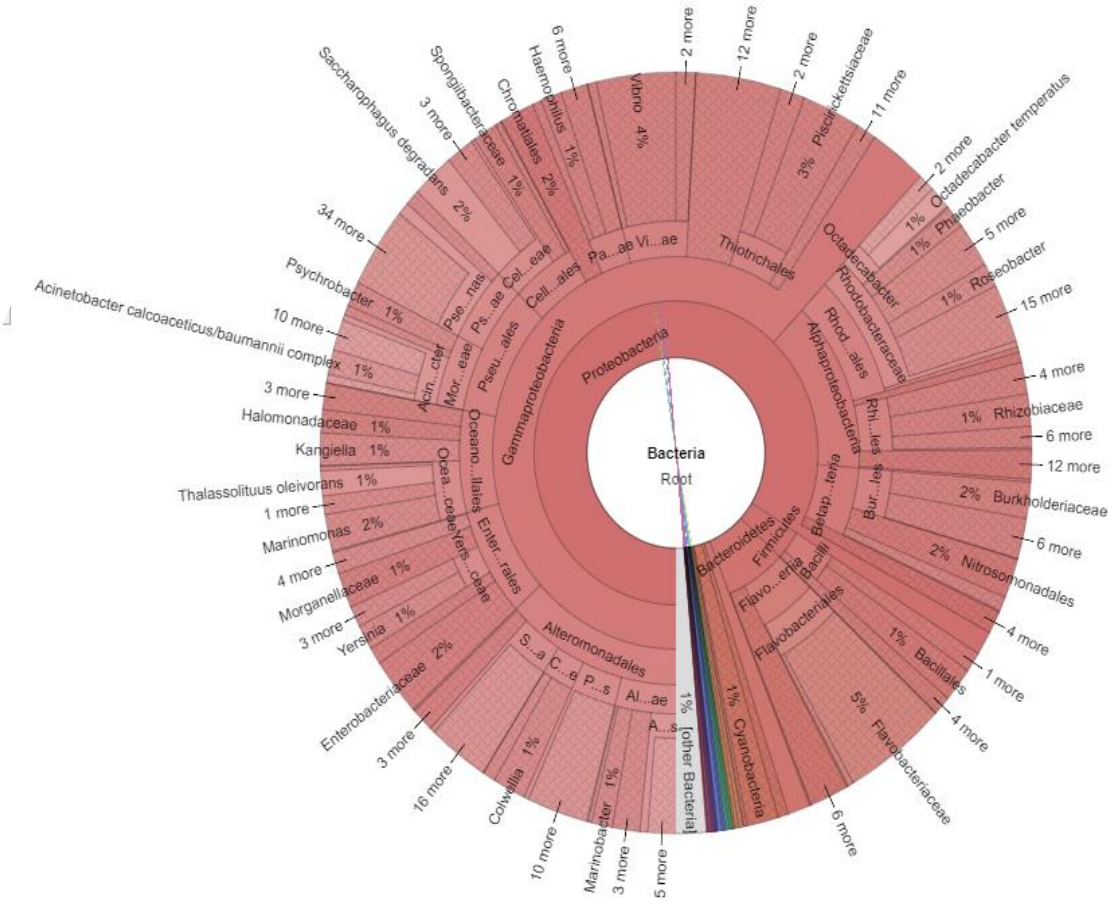

Figure 10

Breakdown of the bacteria isolated from one of the three *Asparagopsis armata* samples ranging from phylum to species.

Table 7. Average Krona plot values (% ± SEM) based on the metagenomic analysis of bacterial communities isolated from three *Asparagopsis armata, Corallina officinalis, Furcellaria lumbricalis* and *Ulva lactuca* samples

		Percentage of Root (% ± SEM)			
		Asparagopsis armata	***Corallina officinalis***	***Furcellaria lumbricalis***	***Ulva lactuca***
Root	No hits	84.67 ± 1.08	81.67 ± 1.91	84.00 ± 1.63	83.00 ± 1.24
	Bacteria	13.00 ± 0.00	17.33 ± 1.91	15.33 ± 1.78	15.67 ± 1.08
	Eukaryota	2.30 ± 1.10	0.57 ± 0.07	0.70 ± 0.05	0.63 ± 0.11
	Percentage of Bacteria (% ± SEM)				
Class	*Gammaproteobacteria*	49.67 ± 5.17	19.00 ± 3.30	20.67 ± 2.84	16.00 ± 2.45
	Alphaproteobacteria	18.00 ± 2.49	41.67 ± 3.92	37.33 ± 4.01	24.67 ± 1.51
	Betaproteobacteria	4.33 ± 0.98	5.00 ± 0.47	6.00 ± 1.41	19.67 ± 3.13
	Bacilli	10.67 ± 7.08	0.90 ± 0.08	2.33 ± 0.27	1.33 ± 0.27
	Flavobacteriia	7.00 ± 2.05	11.33 ± 1.09	18.33 ± 5.19	5.23 ± 2.19
	Other	10.33 ± 0.54	22.10 ± 1.20	15.33 ± 0.98	33.10 ± 3.35
Vibrio	*cholerae*	0.10 ± 0.05	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
	parahaemolyticus	0.13 ± 0.05	0.06 ± 0.00	0.06 ± 0.01	0.05 ± 0.02
	alginolyticus	0.10 ± 0.05	0.03 ± 0.01	0.05 ± 0.01	0.06 ± 0.02
	vulnificus	0.17 ± 0.07	0.07 ± 0.01	0.08 ± 0.01	0.05 ± 0.02
	Other	1.60 ± 0.69	0.69 ± 0.08	1.03 ± 0.26	0.65 ± 0.08
	Total	**2.10 ± 0.87**	**0.90 ± 0.08**	**1.27 ± 0.30**	**0.83 ± 0.13**
	S. aureus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	E. coli	0.80 ± 0.49	0.13 ± 0.02	0.25 ± 0.08	0.27 ± 0.03

3.3.3 Characterisation of ARGs in Seaweed-Associated Communities

To identify the presence ARGs we aligned the Illumina reads and the assembled contigs from all 12 of our samples to the CARD database. We initially looked at the abundance and diversity of ARGs in the bacterial communities associated with *A. armata, C. officinalis, F. lumbricalis* and *U. lactuca*. The abundance of ARGs in each sample was measured by dividing the number of hits returned from BLASTing Illumina reads to the CARD database by the total number of kilobases (KB) in the reads (Figure 11). As contigs are formed from clusters of similar reads it is not possible to use them to quantify the number of ARGs present in a sample. However, analysis of contigs do allow us to identify which ARGs are present in a sample, therefore we used the number of hits returned from aligning contigs with the CARD database over the total number of contig KBs as a measure of ARG diversity (Figure 12). *A. armata* had the highest abundance of ARGs in its bacterial community, followed by *U. lactuca. F. lumbricalis* had the lowest abundance of ARGs, which was shown to be significantly lower than the abundance of ARGs present in *A. armata* bacterial communities (p=0.03, n=3, TukeyHSD). The diversity of ARGs obtained from each seaweed species appear to be relatively similar (all around 0.0075 hits per KB). However, the large error bars, shown in figure 12, suggest the diversity of ARGs varies between samples of the same species. *A. armata* had the largest error bars indicating the diversity of ARGs varied more between those sampled compared to the other seaweed species.

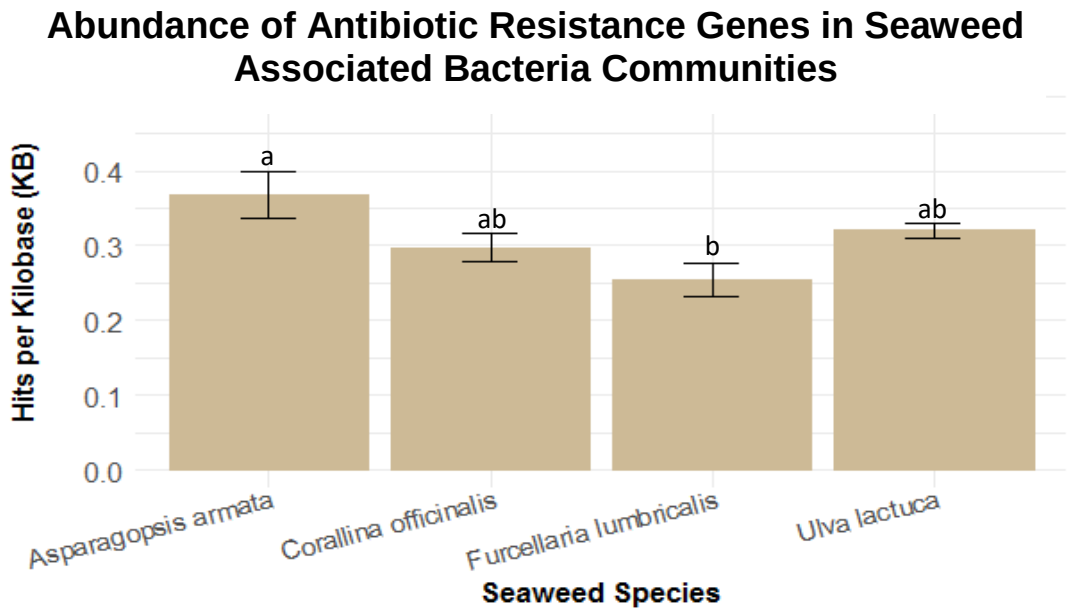

Figure 11

Abundance of antibiotic resistance genes (ARG)) in bacterial communities isolated from *Asparagopsis armata, Corallina officinalis, Furcellaria lumbricalis* and *Ulva lactuca.* Expressed as hits returned from aligning Illumina reads (≥90% amino acid identity, E value ≤1x10^{-5}) over number of Kilo bases One-way ANOVA (*p*= 0.04, n=3) and a post hoc TukeyHSD (*p*= see Table S2) test found a significant difference in the abundance of ARG's associated with different seaweed species. a and b group statistically similar results.

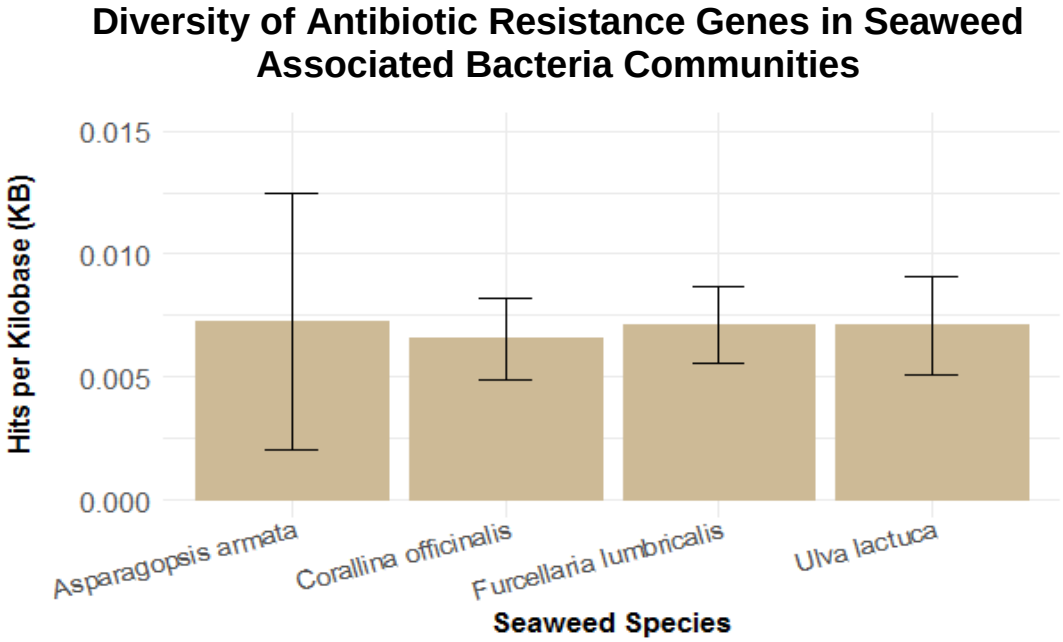

Figure 12

Diversity of antibiotic resistance genes (ARG) in bacterial assoaicted with *Asparagopsis armata, Corallina officinalis, Furcellaria lumbricalis* and *Ulva lactuca.* Presented as hits returned from aligning contigs (≥90% amino acid identity, E value ≤1x10^{-5}) over number of Kilobases One-way ANOVA (*p*= 1.00, n=3) found not signicant difference in ARG diversity.

As well as using contigs to quantitatively analyse the diversity of the ARGs in ours samples we used them to identify the type of resistance genes present. Figure 13 displays the average percentage of hits from three *A. armata, C. officinalis, F. lumbricalis* and *U. lactuca* samples corresponding to genes which confer resistance to specific antimicrobial agents. Resistance to different antimicrobial agents varied between seaweed species. Only one gene had a 100% similarity hit, the gene was present on *A. armata* and conferred resistance to aminocoumarins. Genes conferring resistance aminocoumarins were present in all four seaweed bacterial communities as were genes conferring resistance to elfamycins and fluoroquinolones, however the proportion of those genes differed between species. Over half of the genes obtained from *U. lactuca* corresponded to elfamcyin resistance, whereas only a quarter of the genes isolated from *A. armata* were resistant to that antimicrobial class. *U. lactuca* was the only seaweed species to have resistance to pulvamycins, although the genes conferring resistance to that antimicrobial agents made up 2% of the total number of hits from that species of seaweed. Both *U. lactuca* and *F. lumbricalis* contained gene resistance to thiopeptides. Bacterial DNA isolated from *F. lumbricalis* did not contain genes which conferred resistance to rifampicins. *C. officinalis* was the only seaweed to have resistance to daptomycins, although it was also the only species to not have resistance to enacyloxins. 3% of the hits from the *A. armata* sample corresponded to genes resistant to Tricoslan, those gene were not found to be present on the other seaweeds.

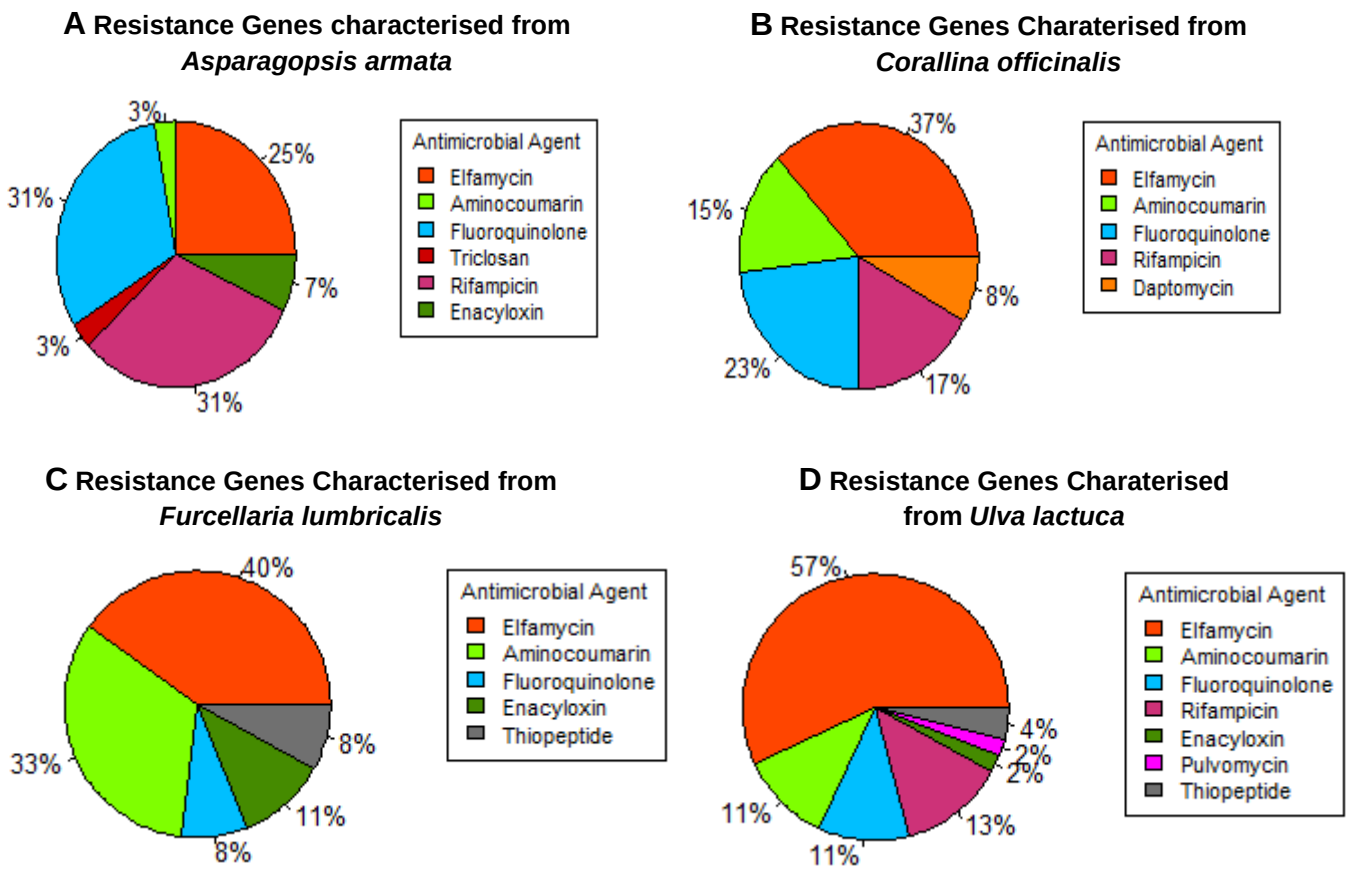

Figure 13

Pie charts showing the average (n=3) percentage of hits which correspond to resistance genes specific to antimicrobial agent present in bacteria isolated from: **A** *Asparagopsis armata*, **B** *Corallina officinalis*, **C** *Furcellaria lumbricalis* and **D** *Ulva lactuca.*

3.4 Discussion

3.4.1 Prevalence of the *intI1* Gene in Seaweed-associated Bacterial Communities

The aims of this chapter were to characterise bacteria associated with seaweed and identify if antibiotic resistance is present in those communities. The *intI1* gene is classed as a biomarker for antibiotic resistance (211) therefore we used PCR and qPCR to detect and quantify this gene in seaweed bacterial communities to assess the levels of antibiotic resistant bacteria. We were able to detect and quantify the prevalence of the *intI1* gene in bacteria obtained from different seaweeds species. The *Fucus* spp. had the highest prevalence of the *intI1* gene making them the best candidates for metagenomic sequencing, however we were unable to isolate a high enough concentration of bacterial DNA hence why

we did not sequence those species. Bacterial DNA isolated from *A. armata* had the lowest level of the gene which corresponds well with the fact there were no antimicrobial resistant bacteria obtained from *A. armata* (Chapter Two). We also compared prevalence of the *intI1* gene in bacterial communities isolated from *Fucus* spp. sampled from a 'clean' site, Castle Beach, and a 'dirty' site, The Greenbank Hotel. Castle Beach has relatively little anthropogenic pollution whereas The Greenbank Hotel Harbour is home to many boats and is polluted by sewage, which is why we have deemed it our 'dirty' site. We found the prevalence of the *intI1* gene was significantly higher in the water sample from the dirty site whereas similar levels were observed for the different seaweed species at both sites. This result would be consistent with a scenario where seaweeds appear to have microbial populations that are not impacted by water quality, so the levels of resistance may be intrinsic rather than effected by anthropogenic pollution.

Although there are no previous studies investigating the presence of the *intI1* gene in seaweed associated bacteria we can compare our results to the prevalence of the gene in other environments. The prevalence of the *intI1* gene in seaweed epiphytic bacteria was similar to the percentage of the gene in bacteria obtained from environments with little antibiotic residue contamination, such as unamended soil (0.0002 % - 0.0036 %) (213, 247) and river water collected upstream from a wastewater treatment plant (248). This result suggests the level of AMR in seaweed microbiomes is low when compared to highly impacted microbial communities in soil, water and sediment.

3.4.2 Characterisation of seaweed epiphytic bacteria

We used metagenomic analysis to characterise the bacteria present on the surface of different seaweed species. However due to time constraints we were unable to compare the composition of the bacterial communities on different samples of the same seaweed species and samples of different species. We used Krona plots to analyse the microbiomes associated with different seaweed samples. The Krona plots showed a high percentage of no hits across all twelve samples. A low percentage of no hits has been previously shown in a study analysing the microbiome of biogas (249), which was attributed to presence of unknown microbes. Although there is a high abundance of unknown microbes in the marine environment (192, 250, 251) this is probably not the sole reason behind the large percent of no hits in our samples, and instead is most likely due to contamination. The Krona plots

revealed that classes of bacteria commonly found on seaweeds (85), including *Alphaproteobacteria, Gammaproteobacteria* and *Bacteroidetes* were present across the four seaweeds species. In Chapter two we were unable to cultivate any *S. aureus* and had very low *E. coli* colonies counts from bacteria obtained from different seaweeds species. Metagenomic analysis revealed there was no *S. aureus* detectable in any of the bacterial communities isolated from the seaweeds, and only very low percentages of *E. coli* were found. We were able to cultivate *Vibrio* spp. and metagenomics revealed species in this genus were present on all the seaweed species. These results are not suggestive of the fact that seaweeds can act as hotspots for human pathogens.

3.4.3 Identification of ARG's in Seaweed Microbiomes

In chapter one we used culture-based techniques to show bacteria isolated from seaweeds can be resistant to conventional antibiotics. In this chapter we will attempt to identify the specific genes behind the observed resistance via metagenomic sequencing. We initially used the number of hits per KB returned from aligning our sequence data with CARD to quantify the abundance and diversity of ARGs in our samples. *A. armata* had the highest abundance of ARGs in its bacterial DNA which is surprising considering the bacteria we isolated from this species showed very little resistance to the antibiotic we tested in chapter two. The diversity of ARGs was relatively similar across the four seaweeds. This could be due to the seaweeds being sampled from the same location and are therefore exposed to the same selective pressures, such as water quality. To test this, we could have used metagenomic analysis to identify ARG's in bacterial communities from the same seaweed species sampled from different locations, however due to budget constraints we were unable to do this. Instead of looking at the number of hits per kilobase we could have extracted 16s data from our sequences, using a programme such as QIIME (252). This would allow us to look at the number of ARGs relative to 16s rRNA (i.e. cell number).

The types of resistance genes present were relatively similar across all the seaweed species suggesting antibiotic resistant bacteria, regardless of resistance profile, will settle on seaweeds independent of species. Metagenomic analysis revealed genes conferring resistance to elfamycins, aminocoumarins and fluoroquinolones were present on all four seaweeds. Elfamycin antibiotics work by inhibiting elongation factor TU interfering with protein synthesis (253). They are not commonly used in the clinic due to a poor

pharmacokinetic profile and solubility (254). There has also only been one documented case of elfamycin resistance genes being present in a sewage treatment plant (255), making it unlikely that the gene to entered the natural environment through anthropogenic pollution. Aminocoumarin antibiotics target DNA gyrases and have a higher affinity for the enzyme than fluoroquinolones (256). However, like elfamycins, they are not often used in the clinic due to poor solubility and high toxicity (257). Both elfamycins and aminocoumarins are natural products produced by Streptomyces (258, 259), species of which were detected in the Krona analysis. This suggests either Streptomyces in seaweed populations might select for resistance mechanisms through antibiotic production or these genes may confer cross resistance to seaweed antimicrobials. This would be interesting to study further. The 3rd group of common resistance genes were fluoroquinolone ARGs. Fluoroquinolones are more widely used and their resistance genes have previously been shown to occur in aquatic environments (260). It is also known that *Shewenella algae* is the progenitor of *qnrA* fluoroquinolone resistance gene (261) and this species is a member of the *Vibrio* which we and previous studies have shown to be common on seaweeds (85, 86). Again, it would be interesting to investigate the fluoroquinolone resistance in the seaweed populations further such as which host species they are associated with and what compounds they confer resistance to. In chapter two, the bacteria isolated from the same seaweed species used for sequencing in this chapter did not appear to be ciprofloxacin resistant, which makes the presence of fluoroquinolone resistance genes surprising. However, we used extracts to test resistance in chapter two which may not be ecologically relevant. There were no ARGs conferring resistance to tetracycline or chloramphenicol, which previous studies have shown to be present in the marine environment (221-225). However, water samples in those studies were taken from or near aquaculture sites which use antibiotics such as oxytetracycline and chloramphenicol thus driving bacteria to develop resistance to those antibiotics. It would be interesting to sample seaweeds from those sites and use metagenomics to determine whether the bacterial communities settled on those seaweeds contain gene conferring resistant to the antibiotics used there.

Chapter 4: Concluding Remarks

From our results we conclude that seaweeds, or the associated microbial communities, may select for bacteria which are resistant to antibiotics. However those bacteria do not differ depending on seaweed species and they are not locally adapted to the seaweed host species. It also appears that abundance and diversity of antimicrobial resistant bacteria are intrinsic to the seaweed host rather than being influenced by anthropogenic pollution.

Supplementary Data

Table S1. Minimal Inhibitory Concentrations of clinical *S. aureus* strains.

Strain	Location	MIC (mg/L)			
		Tet	Ery	Chlor	Kan
51	Royal Cornwall Hospital, UK	-	-	-	-
52	Royal Cornwall Hospital, UK	-	-	-	-
53	Royal Cornwall Hospital, UK	-	-	-	-
57	Royal Cornwall Hospital, UK	5	-	-	-
58	Royal Cornwall Hospital, UK	-	5	-	-
59	Royal Cornwall Hospital, UK	-	-	10	
60	Royal Cornwall Hospital, UK	-	-	-	50
2343	University of Bath, UK	1	1	8	-
2424	University of Bath, UK	1	0.5	8	-
2458	University of Bath, UK	1	8	8	-
2564	University of Bath, UK	1	1	8	-
2636	University of Bath, UK	1	2	8	-
3344	University of Bath, UK	1	8	4	-
3526	University of Bath, UK	1	8	4	-
3729	University of Bath, UK	1	1	4	-
3737	University of Bath, UK	16	1	8	-
3935	University of Bath, UK	1	1	8	-
4244	University of Bath, UK	16	8	8	-

- unknown. Tet= Tetracycline, Ery= Erythromycin, Chlor= Chloramphenicol, Kan= Kanamycin.

Table S2. Summary of all results from one-way ANOVA and post hoc TukeyHSD tests. * marks a significant different between samples. – not significantly different

Samples we are testing significance between	Sample Size (n)	ANOVA (*p*)	TukeyHSD (*p*)	Sig?
Table 3. Sept 2016 Yellow *Vibrio* Colony Counts				
Cystoseira tamariscifolia-Cystoseira baccata	5	<2x10-16	0.00	*
Fucus serratus -Cystoseira baccata	5	<2x10-16	0.83	-
Fucus vesiculosus-Cystoseira baccata	5	<2x10-16	0.22	-

Himanthalia elongata-Cystoseira baccata	5	<2x10-16	0.09	-
Sargassum muticum-Cystoseira baccata	5	<2x10-16	0.02	*
Ulva lactuca-Cystoseira baccata	5	<2x10-16	0.74	-
Fucus serratus -Cystoseira tamariscifolia	5	<2x10-16	0.00	*
Fucus vesiculosus-Cystoseira tamariscifolia	5	<2x10-16	0.00	*
Himanthalia elongata-Cystoseira tamariscifolia	5	<2x10-16	0.00	*
Sargassum muticum-Cystoseira tamariscifolia	5	<2x10-16	0.00	*
Ulva lactuca-Cystoseira tamariscifolia	5	<2x10-16	0.00	*
Fucus vesiculosus-Fucus serratus	5	<2x10-16	0.93	-
Himanthalia elongata-Fucus serratus	5	<2x10-16	0.72	-
Sargassum muticum-Fucus serratus	5	<2x10-16	0.00	*
Ulva lactuca-Fucus serratus	5	<2x10-16	1.00	-
Himanthalia elongata-Fucus vesiculosus	5	<2x10-16	1.00	-
Sargassum muticum-Fucus vesiculosus	5	<2x10-16	0.00	*
Ulva lactuca-Fucus vesiculosus	5	<2x10-16	0.97	-
Sargassum muticum-Himanthalia elongata	5	<2x10-16	0.00	*
Ulva lactuca-Himanthalia elongata	5	<2x10-16	0.81	-
Ulva lactuca-Sargassum muticum	5	<2x10-16	0.00	*
Table 3. Sept 2016 Green *Vibrio* Colony Counts				
Cystoseira tamariscifolia-Cystoseira baccata	5	1.13x10-13	0.00	*
Fucus serratus -Cystoseira baccata	5	1.13x10-13	0.38	-
Fucus vesiculosus-Cystoseira baccata	5	1.13x10-13	0.00	*
Himanthalia elongata-Cystoseira baccata	5	1.13x10-13	0.01	*
Sargassum muticum-Cystoseira baccata	5	1.13x10-13	0.05	-
Ulva lactuca-Cystoseira baccata	5	1.13x10-13	0.00	*
Fucus serratus -Cystoseira tamariscifolia	5	1.13x10-13	0.00	*
Fucus vesiculosus-Cystoseira tamariscifolia	5	1.13x10-13	0.00	*
Himanthalia elongata-Cystoseira tamariscifolia	5	1.13x10-13	0.00	*
Sargassum muticum-Cystoseira tamariscifolia	5	1.13x10-13	0.00	*
Ulva lactuca-Cystoseira tamariscifolia	5	1.13x10-13	0.00	*
Fucus vesiculosus-Fucus serratus	5	1.13x10-13	0.00	*

Himanthalia elongata-Fucus serratus	5	1.13x10-13	0.00	*
Sargassum muticum-Fucus serratus	5	1.13x10-13	0.00	*
Ulva lactuca-Fucus serratus	5	1.13x10-13	0.00	*
Himanthalia elongata-Fucus vesiculosus	5	1.13x10-13	1.00	-
Sargassum muticum-Fucus vesiculosus	5	1.13x10-13	0.94	-
Ulva lactuca-Fucus vesiculosus	5	1.13x10-13	1.00	-
Sargassum muticum-Himanthalia elongata	5	1.13x10-13	0.98	-
Ulva lactuca-Himanthalia elongata	5	1.13x10-13	1.00	-
Ulva lactuca-Sargassum muticum	5	1.13x10-13	0.84	-
Table 3. March 2017 Yellow *Vibrio* Colony Counts				
Callilepharis jubata-Asparagopsis armata	5	<2x10-16	0.00	*
Corallina officinalis-Asparagopsis armata	5	<2x10-16	1.00	-
Fucus serratus -Asparagopsis armata	5	<2x10-16	0.00	*
Fucus vesiculosus-Asparagopsis armata	5	<2x10-16	0.00	*
Furcellaria lumbricalis-Asparagopsis armata	5	<2x10-16	0.00	*
Himanthalia elongata-Asparagopsis armata	5	<2x10-16	0.00	*
Polyides rotundus-Asparagopsis armata	5	<2x10-16	0.00	*
Sargassum muticum-Asparagopsis armata	5	<2x10-16	0.00	*
Ulva lactuca-Asparagopsis armata	5	<2x10-16	0.00	*
Corallina officinalis-Callilepharis jubata	5	<2x10-16	0.00	*
Fucus serratus -Callilepharis jubata	5	<2x10-16	0.02	*
Fucus vesiculosus-Callilepharis jubata	5	<2x10-16	0.02	*
Furcellaria lumbricalis-Callilepharis jubata	5	<2x10-16	0.34	-
Himanthalia elongata-Callilepharis jubata	5	<2x10-16	0.00	*
Polyides rotundus-Callilepharis jubata	5	<2x10-16	0.49	-
Sargassum muticum-Callilepharis jubata	5	<2x10-16	0.20	-
Ulva lactuca-Callilepharis jubata	5	<2x10-16	0.01	*
Fucus serratus -Corallina officinalis	5	<2x10-16	0.00	*
Fucus vesiculosus-Corallina officinalis	5	<2x10-16	0.00	*

Furcellaria lumbricalis-Corallina officinalis	5	<2x10-16	0.00	*
Himanthalia elongata-Corallina officinalis	5	<2x10-16	0.00	*
Polyides rotundus-Corallina officinalis	5	<2x10-16	0.00	*
Sargassum muticum-Corallina officinalis	5	<2x10-16	0.00	*
Ulva lactuca-Corallina officinalis	5	<2x10-16	0.00	*
Fucus vesiculosus-Fucus serratus	5	<2x10-16	1.00	-
Furcellaria lumbricalis-Fucus serratus	5	<2x10-16	0.96	-
Himanthalia elongata-Fucus serratus	5	<2x10-16	1.00	-
Polyides rotundus-Fucus serratus	5	<2x10-16	0.87	-
Sargassum muticum-Fucus serratus	5	<2x10-16	0.00	*
Ulva lactuca-Fucus serratus	5	<2x10-16	1.00	-
Furcellaria lumbricalis-Fucus vesiculosus	5	<2x10-16	0.96	-
Himanthalia elongata-Fucus vesiculosus	5	<2x10-16	1.00	-
Polyides rotundus-Fucus vesiculosus	5	<2x10-16	0.88	-
Sargassum muticum-Fucus vesiculosus	5	<2x10-16	0.00	*
Ulva lactuca-Fucus vesiculosus	5	<2x10-16	1.00	-
Himanthalia elongata-Furcellaria lumbricalis	5	<2x10-16	0.58	-
Polyides rotundus-Furcellaria lumbricalis	5	<2x10-16	1.00	-
Sargassum muticum-Furcellaria lumbricalis	5	<2x10-16	0.00	*
Ulva lactuca-Furcellaria lumbricalis	5	<2x10-16	0.76	-
Polyides rotundus-Himanthalia elongata	5	<2x10-16	0.41	-
Sargassum muticum-Himanthalia elongata	5	<2x10-16	0.00	*
Ulva lactuca-Himanthalia elongata	5	<2x10-16	1.00	-
Sargassum muticum-Polyides rotundus	5	<2x10-16	0.00	*
Ulva lactuca-Polyides rotundus	5	<2x10-16	0.60	-
Ulva lactuca-Sargassum muticum	5	<2x10-16	0.00	*
Table 3. March 2017 Green *Vibrio* Colony Counts				
Calliblepharis jubata-Asparagopsis armata	5	<2x10-16	0.00	*
Corallina officinalis-Asparagopsis armata	5	<2x10-16	0.83	-
Fucus serratus -Asparagopsis armata	5	<2x10-16	0.00	*
Fucus vesiculosus-Asparagopsis armata	5	<2x10-16	0.00	*
Furcellaria lumbricalis-Asparagopsis armata	5	<2x10-16	0.00	*

Himanthalia elongata-Asparagopsis armata	5	<2x10-16	0.00	*
Polyides rotundus-Asparagopsis armata	5	<2x10-16	0.00	*
Sargassum muticum-Asparagopsis armata	5	<2x10-16	0.00	*
Ulva lactuca-Asparagopsis armata	5	<2x10-16	0.00	*
Corallina officinalis-Calliblepharis jubata	5	<2x10-16	0.00	*
Fucus serratus -Calliblepharis jubata	5	<2x10-16	0.03	*
Fucus vesiculosus-Calliblepharis jubata	5	<2x10-16	0.01	*
Furcellaria lumbricalis-Calliblepharis jubata	5	<2x10-16	0.34	-
Himanthalia elongata-Calliblepharis jubata	5	<2x10-16	0.00	*
Polyides rotundus-Calliblepharis jubata	5	<2x10-16	0.97	-
Sargassum muticum-Calliblepharis jubata	5	<2x10-16	0.67	-
Ulva lactuca-Calliblepharis jubata	5	<2x10-16	0.01	*
Fucus serratus -Corallina officinalis	5	<2x10-16	0.00	*
Fucus vesiculosus-Corallina officinalis	5	<2x10-16	0.00	*
Furcellaria lumbricalis-Corallina officinalis	5	<2x10-16	0.00	*
Himanthalia elongata-Corallina officinalis	5	<2x10-16	0.00	*
Polyides rotundus-Corallina officinalis	5	<2x10-16	0.00	*
Sargassum muticum-Corallina officinalis	5	<2x10-16	0.00	*
Ulva lactuca-Corallina officinalis	5	<2x10-16	0.00	*
Fucus vesiculosus-Fucus serratus	5	<2x10-16	1.00	-
Furcellaria lumbricalis-Fucus serratus	5	<2x10-16	0.98	-
Himanthalia elongata-Fucus serratus	5	<2x10-16	0.94	-
Polyides rotundus-Fucus serratus	5	<2x10-16	0.41	-
Sargassum muticum-Fucus serratus	5	<2x10-16	0.00	*
Ulva lactuca-Fucus serratus	5	<2x10-16	1.00	-
Furcellaria lumbricalis-Fucus vesiculosus	5	<2x10-16	0.90	-
Himanthalia elongata-Fucus vesiculosus	5	<2x10-16	0.99	-
Polyides rotundus-Fucus vesiculosus	5	<2x10-16	0.22	-
Sargassum muticum-Fucus vesiculosus	5	<2x10-16	0.00	*
Ulva lactuca-Fucus vesiculosus	5	<2x10-16	1.00	-
Himanthalia elongata-Furcellaria lumbricalis	5	<2x10-16	0.34	-
Polyides rotundus-Furcellaria lumbricalis	5	<2x10-16	0.97	-

Sargassum muticum-Furcellaria lumbricalis	5	<2x10-16	0.00	*
Ulva lactuca-Furcellaria lumbricalis	5	<2x10-16	0.79	-
Polyides rotundus-Himanthalia elongata	5	<2x10-16	0.02	*
Sargassum muticum-Himanthalia elongata	5	<2x10-16	0.00	*
Ulva lactuca-Himanthalia elongata	5	<2x10-16	1.00	-
Sargassum muticum-Polyides rotundus	5	<2x10-16	0.09	-
Ulva lactuca-Polyides rotundus	5	<2x10-16	0.14	-
Ulva lactuca-Sargassum muticum	5	<2x10-16	0.00	*
Figure 2A. Green *Vibrio* Resistance to *Asparagopsis armata*				
N/A	8	0.69	N/A	N/A
Figure 2A. Yellow *Vibrio* Resistance to *Asparagopsis armata*				
N/A	7	0.20	N/A	N/A
Figure 2B. Green *Vibrio* Resistance to *Furcellaria lumbricalis*				
N/A	8	0.11	N/A	N/A
Figure 2B. Yellow *Vibrio* Resistance to *Furcellaria lumbricalis*				
N/A	1	0.19	N/A	N/A
Figure 2C. Green *Vibrio* Resistance to *Polyides rotundus*				
N/A	8	0.06	N/A	N/A
Figure 2C. Yellow *Vibrio* Resistance to *Polyides rotundus*				
N/A	7	0.47	N/A	N/A
Figure 2D. Green *Vibrio* Resistance to *Ulva lactuca*				
N/A	7	0.34	N/A	N/A
Figure 2D. Yellow *Vibrio* Resistance to *Ulva lactuca*				
N/A	7	0.22	N/A	N/A
Figure 4A. Antimicrobial Agents effects on Green *Vibrio*				
Ampicillin 4mg/L-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Ampicillin 8mg/L-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Asparagopsis armata-Ampicillin 16mg/L	38	<2x10-16	0.00	*
Azithromycin 2mg/L-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Azithromycin 4mg/L-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Azithromycin 8mg/L-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Calliblepharis jubata-Ampicillin 16mg/L	38	<2x10-16	0.86	-
Chloramphenicol 16mg/L-Ampicillin 16mg/L	38	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Ampicillin 16mg/L	38	<2x10-16	0.00	*

Corallina officinalis-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Cystoseira baccata-Ampicillin 16mg/L	38	<2x10-16	0.00	*
Fucus serratus-Ampicillin 16mg/L	38	<2x10-16	0.00	*
Fucus vesiculosus-Ampicillin 16mg/L	38	<2x10-16	0.00	*
Furcellaria lumbricalis-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Gentamicin 16mg/L-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Gentamicin 2mg/L-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Gentamicin 8mg/L-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Himanthalia elongata-Ampicillin 16mg/L	38	<2x10-16	0.00	*
Polyides rotundus-Ampicillin 16mg/L	38	<2x10-16	0.96	-
Sargassum muticum-Ampicillin 16mg/L	38	<2x10-16	0.00	*
Ulva lactuca-Ampicillin 16mg/L	38	<2x10-16	1.00	-
Ampicillin 8mg/L-Ampicillin 4mg/L	38	<2x10-16	1.00	-
Asparagopsis armata-Ampicillin 4mg/L	38	<2x10-16	0.00	*
Azithromycin 2mg/L-Ampicillin 4mg/L	38	<2x10-16	1.00	-
Azithromycin 4mg/L-Ampicillin 4mg/L	38	<2x10-16	1.00	-
Azithromycin 8mg/L-Ampicillin 4mg/L	38	<2x10-16	0.79	-
Calliblepharis jubata-Ampicillin 4mg/L	38	<2x10-16	0.04	*
Chloramphenicol 16mg/L-Ampicillin 4mg/L	38	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Ampicillin 4mg/L	38	<2x10-16	0.00	*
Corallina officinalis-Ampicillin 4mg/L	38	<2x10-16	1.00	-
Cystoseira baccata-Ampicillin 4mg/L	38	<2x10-16	0.00	*
Fucus serratus-Ampicillin 4mg/L	38	<2x10-16	0.00	*
Fucus vesiculosus-Ampicillin 4mg/L	38	<2x10-16	0.00	*
Furcellaria lumbricalis-Ampicillin 4mg/L	38	<2x10-16	1.00	-
Gentamicin 16mg/L-Ampicillin 4mg/L	38	<2x10-16	1.00	-
Gentamicin 2mg/L-Ampicillin 4mg/L	38	<2x10-16	1.00	-
Gentamicin 8mg/L-Ampicillin 4mg/L	38	<2x10-16	1.00	-
Himanthalia elongata-Ampicillin 4mg/L	38	<2x10-16	0.00	*
Polyides rotundus-Ampicillin 4mg/L	38	<2x10-16	0.09	-
Sargassum muticum-Ampicillin 4mg/L	38	<2x10-16	0.00	*
Ulva lactuca-Ampicillin 4mg/L	38	<2x10-16	1.00	-
Asparagopsis armata-Ampicillin 8mg/L	38	<2x10-16	0.00	*

Azithromycin 2mg/L-Ampicillin 8mg/L	38	<2x10-16	1.00	-
Azithromycin 4mg/L-Ampicillin 8mg/L	38	<2x10-16	1.00	-
Azithromycin 8mg/L-Ampicillin 8mg/L	38	<2x10-16	0.99	-
Calliblepharis jubata-Ampicillin 8mg/L	38	<2x10-16	0.20	-
Chloramphenicol 16mg/L-Ampicillin 8mg/L	38	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Ampicillin 8mg/L	38	<2x10-16	0.00	*
Corallina officinalis-Ampicillin 8mg/L	38	<2x10-16	1.00	-
Cystoseira baccata-Ampicillin 8mg/L	38	<2x10-16	0.00	*
Fucus serratus-Ampicillin 8mg/L	38	<2x10-16	0.00	*
Fucus vesiculosus-Ampicillin 8mg/L	38	<2x10-16	0.00	*
Furcellaria lumbricalis-Ampicillin 8mg/L	38	<2x10-16	1.00	-
Gentamicin 16mg/L-Ampicillin 8mg/L	38	<2x10-16	1.00	-
Gentamicin 2mg/L-Ampicillin 8mg/L	38	<2x10-16	1.00	-
Gentamicin 8mg/L-Ampicillin 8mg/L	38	<2x10-16	1.00	-
Himanthalia elongata-Ampicillin 8mg/L	38	<2x10-16	0.00	*
Polyides rotundus-Ampicillin 8mg/L	38	<2x10-16	0.37	-
Sargassum muticum-Ampicillin 8mg/L	38	<2x10-16	0.00	*
Ulva lactuca-Ampicillin 8mg/L	38	<2x10-16	1.00	-
Azithromycin 2mg/L-Asparagopsis armata	38	<2x10-16	0.00	*
Azithromycin 4mg/L-Asparagopsis armata	38	<2x10-16	0.00	*
Azithromycin 8mg/L-Asparagopsis armata	38	<2x10-16	0.00	*
Calliblepharis jubata-Asparagopsis armata	38	<2x10-16	0.00	*
Chloramphenicol 16mg/L-Asparagopsis armata	38	<2x10-16	1.00	-
Chloramphenicol 8mg/L-Asparagopsis armata	38	<2x10-16	1.00	-
Corallina officinalis-Asparagopsis armata	38	<2x10-16	0.00	*
Cystoseira baccata-Asparagopsis armata	38	<2x10-16	1.00	-
Fucus serratus-Asparagopsis armata	38	<2x10-16	1.00	-
Fucus vesiculosus-Asparagopsis armata	38	<2x10-16	0.98	-
Furcellaria lumbricalis-Asparagopsis armata	38	<2x10-16	0.00	*
Gentamicin 16mg/L-Asparagopsis armata	38	<2x10-16	0.00	*
Gentamicin 2mg/L-Asparagopsis armata	38	<2x10-16	0.00	*

Gentamicin 8mg/L-Asparagopsis armata	38	<2x10-16	0.00	*
Himanthalia elongata-Asparagopsis armata	38	<2x10-16	0.43	-
Polyides rotundus-Asparagopsis armata	38	<2x10-16	0.00	*
Sargassum muticum-Asparagopsis armata	38	<2x10-16	0.03	*
Ulva lactuca-Asparagopsis armata	38	<2x10-16	0.00	*
Azithromycin 4mg/L-Azithromycin 2mg/L	38	<2x10-16	1.00	-
Azithromycin 8mg/L-Azithromycin 2mg/L	38	<2x10-16	0.79	-
Calliblepharis jubata-Azithromycin 2mg/L	38	<2x10-16	0.04	*
Chloramphenicol 16mg/L-Azithromycin 2mg/L	38	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Azithromycin 2mg/L	38	<2x10-16	0.00	*
Corallina officinalis-Azithromycin 2mg/L	38	<2x10-16	1.00	-
Cystoseira baccata-Azithromycin 2mg/L	38	<2x10-16	0.00	*
Fucus serratus-Azithromycin 2mg/L	38	<2x10-16	0.00	*
Fucus vesiculosus-Azithromycin 2mg/L	38	<2x10-16	0.00	*
Furcellaria lumbricalis-Azithromycin 2mg/L	38	<2x10-16	1.00	-
Gentamicin 16mg/L-Azithromycin 2mg/L	38	<2x10-16	1.00	-
Gentamicin 2mg/L-Azithromycin 2mg/L	38	<2x10-16	1.00	-
Gentamicin 8mg/L-Azithromycin 2mg/L	38	<2x10-16	1.00	-
Himanthalia elongata-Azithromycin 2mg/L	38	<2x10-16	0.00	*
Polyides rotundus-Azithromycin 2mg/L	38	<2x10-16	0.09	-
Sargassum muticum-Azithromycin 2mg/L	38	<2x10-16	0.00	*
Ulva lactuca-Azithromycin 2mg/L	38	<2x10-16	1.00	-
Azithromycin 8mg/L-Azithromycin 4mg/L	38	<2x10-16	0.93	-
Calliblepharis jubata-Azithromycin 4mg/L	38	<2x10-16	0.09	-
Chloramphenicol 16mg/L-Azithromycin 4mg/L	38	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Azithromycin 4mg/L	38	<2x10-16	0.00	*
Corallina officinalis-Azithromycin 4mg/L	38	<2x10-16	1.00	-
Cystoseira baccata-Azithromycin 4mg/L	38	<2x10-16	0.00	*
Fucus serratus-Azithromycin 4mg/L	38	<2x10-16	0.00	*
Fucus vesiculosus-Azithromycin 4mg/L	38	<2x10-16	0.00	*

Furcellaria lumbricalis-Azithromycin 4mg/L	38	<2x10-16	1.00	-
Gentamicin 16mg/L-Azithromycin 4mg/L	38	<2x10-16	1.00	-
Gentamicin 2mg/L-Azithromycin 4mg/L	38	<2x10-16	1.00	-
Gentamicin 8mg/L-Azithromycin 4mg/L	38	<2x10-16	1.00	-
Himanthalia elongata-Azithromycin 4mg/L	38	<2x10-16	0.00	*
Polyides rotundus-Azithromycin 4mg/L	38	<2x10-16	0.20	-
Sargassum muticum-Azithromycin 4mg/L	38	<2x10-16	0.00	*
Ulva lactuca-Azithromycin 4mg/L	38	<2x10-16	1.00	-
Calliblepharis jubata-Azithromycin 8mg/L	38	<2x10-16	1.00	-
Chloramphenicol 16mg/L-Azithromycin 8mg/L	38	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Azithromycin 8mg/L	38	<2x10-16	0.00	*
Corallina officinalis-Azithromycin 8mg/L	38	<2x10-16	0.79	-
Cystoseira baccata-Azithromycin 8mg/L	38	<2x10-16	0.00	*
Fucus serratus-Azithromycin 8mg/L	38	<2x10-16	0.00	*
Fucus vesiculosus-Azithromycin 8mg/L	38	<2x10-16	0.00	*
Furcellaria lumbricalis-Azithromycin 8mg/L	38	<2x10-16	1.00	-
Gentamicin 16mg/L-Azithromycin 8mg/L	38	<2x10-16	1.00	-
Gentamicin 2mg/L-Azithromycin 8mg/L	38	<2x10-16	0.79	-
Gentamicin 8mg/L-Azithromycin 8mg/L	38	<2x10-16	0.79	-
Himanthalia elongata-Azithromycin 8mg/L	38	<2x10-16	0.00	*
Polyides rotundus-Azithromycin 8mg/L	38	<2x10-16	1.00	-
Sargassum muticum-Azithromycin 8mg/L	38	<2x10-16	0.00	*
Ulva lactuca-Azithromycin 8mg/L	38	<2x10-16	0.93	-
Chloramphenicol 16mg/L-Calliblepharis jubata	38	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Calliblepharis jubata	38	<2x10-16	0.00	*
Corallina officinalis-Calliblepharis jubata	38	<2x10-16	0.04	*
Cystoseira baccata-Calliblepharis jubata	38	<2x10-16	0.00	*
Fucus serratus-Calliblepharis jubata	38	<2x10-16	0.00	*
Fucus vesiculosus-Calliblepharis jubata	38	<2x10-16	0.00	*
Furcellaria lumbricalis-Calliblepharis jubata	38	<2x10-16	0.82	-

Gentamicin 16mg/L-Calliblepharis jubata	38	<2x10-16	0.67	-
Gentamicin 2mg/L-Calliblepharis jubata	38	<2x10-16	0.04	*
Gentamicin 8mg/L-Calliblepharis jubata	38	<2x10-16	0.04	*
Himanthalia elongata-Calliblepharis jubata	38	<2x10-16	0.00	*
Polyides rotundus-Calliblepharis jubata	38	<2x10-16	1.00	-
Sargassum muticum-Calliblepharis jubata	38	<2x10-16	0.00	*
Ulva lactuca-Calliblepharis jubata	38	<2x10-16	0.09	-
Chloramphenicol 8mg/L-Chloramphenicol 16mg/L	38	<2x10-16	1.00	-
Corallina officinalis-Chloramphenicol 16mg/L	38	<2x10-16	0.00	*
Cystoseira baccata-Chloramphenicol 16mg/L	38	<2x10-16	1.00	-
Fucus serratus-Chloramphenicol 16mg/L	38	<2x10-16	1.00	-
Fucus vesiculosus-Chloramphenicol 16mg/L	38	<2x10-16	0.90	-
Furcellaria lumbricalis-Chloramphenicol 16mg/L	38	<2x10-16	0.00	*
Gentamicin 16mg/L-Chloramphenicol 16mg/L	38	<2x10-16	0.00	*
Gentamicin 2mg/L-Chloramphenicol 16mg/L	38	<2x10-16	0.00	*
Gentamicin 8mg/L-Chloramphenicol 16mg/L	38	<2x10-16	0.00	*
Himanthalia elongata-Chloramphenicol 16mg/L	38	<2x10-16	0.21	-
Polyides rotundus-Chloramphenicol 16mg/L	38	<2x10-16	0.00	*
Sargassum muticum-Chloramphenicol 16mg/L	38	<2x10-16	0.01	*
Ulva lactuca-Chloramphenicol 16mg/L	38	<2x10-16	0.00	*
Corallina officinalis-Chloramphenicol 8mg/L	38	<2x10-16	0.00	*
Cystoseira baccata-Chloramphenicol 8mg/L	38	<2x10-16	1.00	-
Fucus serratus-Chloramphenicol 8mg/L	38	<2x10-16	1.00	-
Fucus vesiculosus-Chloramphenicol 8mg/L	38	<2x10-16	1.00	-
Furcellaria lumbricalis-Chloramphenicol 8mg/L	38	<2x10-16	0.00	*
Gentamicin 16mg/L-Chloramphenicol 8mg/L	38	<2x10-16	0.00	*
Gentamicin 2mg/L-Chloramphenicol 8mg/L	38	<2x10-16	0.00	*
Gentamicin 8mg/L-Chloramphenicol 8mg/L	38	<2x10-16	0.00	*
Himanthalia elongata-Chloramphenicol 8mg/L	38	<2x10-16	0.69	-

Polyides rotundus-Chloramphenicol 8mg/L	38	<2x10-16	0.00	*
Sargassum muticum-Chloramphenicol 8mg/L	38	<2x10-16	0.09	-
Ulva lactuca-Chloramphenicol 8mg/L	38	<2x10-16	0.00	*
Cystoseira baccata-Corallina officinalis	38	<2x10-16	0.00	*
Fucus serratus-Corallina officinalis	38	<2x10-16	0.00	*
Fucus vesiculosus-Corallina officinalis	38	<2x10-16	0.00	*
Furcellaria lumbricalis-Corallina officinalis	38	<2x10-16	1.00	-
Gentamicin 16mg/L-Corallina officinalis	38	<2x10-16	1.00	-
Gentamicin 2mg/L-Corallina officinalis	38	<2x10-16	1.00	-
Gentamicin 8mg/L-Corallina officinalis	38	<2x10-16	1.00	-
Himanthalia elongata-Corallina officinalis	38	<2x10-16	0.00	*
Polyides rotundus-Corallina officinalis	38	<2x10-16	0.09	-
Sargassum muticum-Corallina officinalis	38	<2x10-16	0.00	*
Ulva lactuca-Corallina officinalis	38	<2x10-16	1.00	-
Fucus serratus-Cystoseira baccata	38	<2x10-16	1.00	-
Fucus vesiculosus-Cystoseira baccata	38	<2x10-16	1.00	-
Furcellaria lumbricalis-Cystoseira baccata	38	<2x10-16	0.00	*
Gentamicin 16mg/L-Cystoseira baccata	38	<2x10-16	0.00	*
Gentamicin 2mg/L-Cystoseira baccata	38	<2x10-16	0.00	*
Gentamicin 8mg/L-Cystoseira baccata	38	<2x10-16	0.00	*
Himanthalia elongata-Cystoseira baccata	38	<2x10-16	0.99	-
Polyides rotundus-Cystoseira baccata	38	<2x10-16	0.00	*
Sargassum muticum-Cystoseira baccata	38	<2x10-16	0.57	-
Ulva lactuca-Cystoseira baccata	38	<2x10-16	0.00	*
Fucus vesiculosus-Fucus serratus	38	<2x10-16	1.00	-
Furcellaria lumbricalis-Fucus serratus	38	<2x10-16	0.00	*
Gentamicin 16mg/L-Fucus serratus	38	<2x10-16	0.00	*
Gentamicin 2mg/L-Fucus serratus	38	<2x10-16	0.00	*
Gentamicin 8mg/L-Fucus serratus	38	<2x10-16	0.00	*
Himanthalia elongata-Fucus serratus	38	<2x10-16	0.65	-
Polyides rotundus-Fucus serratus	38	<2x10-16	0.00	*
Sargassum muticum-Fucus serratus	38	<2x10-16	0.08	-
Ulva lactuca-Fucus serratus	38	<2x10-16	0.00	*

Furcellaria lumbricalis-Fucus vesiculosus	38	<2x10-16	0.00	*
Gentamicin 16mg/L-Fucus vesiculosus	38	<2x10-16	0.00	*
Gentamicin 2mg/L-Fucus vesiculosus	38	<2x10-16	0.00	*
Gentamicin 8mg/L-Fucus vesiculosus	38	<2x10-16	0.00	*
Himanthalia elongata-Fucus vesiculosus	38	<2x10-16	1.00	-
Polyides rotundus-Fucus vesiculosus	38	<2x10-16	0.00	*
Sargassum muticum-Fucus vesiculosus	38	<2x10-16	0.92	-
Ulva lactuca-Fucus vesiculosus	38	<2x10-16	0.00	*
Gentamicin 16mg/L-Furcellaria lumbricalis	38	<2x10-16	1.00	-
Gentamicin 2mg/L-Furcellaria lumbricalis	38	<2x10-16	1.00	-
Gentamicin 8mg/L-Furcellaria lumbricalis	38	<2x10-16	1.00	-
Himanthalia elongata-Furcellaria lumbricalis	38	<2x10-16	0.00	*
Polyides rotundus-Furcellaria lumbricalis	38	<2x10-16	0.94	-
Sargassum muticum-Furcellaria lumbricalis	38	<2x10-16	0.00	*
Ulva lactuca-Furcellaria lumbricalis	38	<2x10-16	1.00	-
Gentamicin 2mg/L-Gentamicin 16mg/L	38	<2x10-16	1.00	-
Gentamicin 8mg/L-Gentamicin 16mg/L	38	<2x10-16	1.00	-
Himanthalia elongata-Gentamicin 16mg/L	38	<2x10-16	0.00	*
Polyides rotundus-Gentamicin 16mg/L	38	<2x10-16	0.86	-
Sargassum muticum-Gentamicin 16mg/L	38	<2x10-16	0.00	*
Ulva lactuca-Gentamicin 16mg/L	38	<2x10-16	1.00	-
Gentamicin 8mg/L-Gentamicin 2mg/L	38	<2x10-16	1.00	-
Himanthalia elongata-Gentamicin 2mg/L	38	<2x10-16	0.00	*
Polyides rotundus-Gentamicin 2mg/L	38	<2x10-16	0.09	-
Sargassum muticum-Gentamicin 2mg/L	38	<2x10-16	0.00	*
Ulva lactuca-Gentamicin 2mg/L	38	<2x10-16	1.00	-
Himanthalia elongata-Gentamicin 8mg/L	38	<2x10-16	0.00	*
Polyides rotundus-Gentamicin 8mg/L	38	<2x10-16	0.09	-
Sargassum muticum-Gentamicin 8mg/L	38	<2x10-16	0.00	*
Ulva lactuca-Gentamicin 8mg/L	38	<2x10-16	1.00	-
Polyides rotundus-Himanthalia elongata	38	<2x10-16	0.00	*

Sargassum muticum-Himanthalia elongata	38	<2x10-16	1.00	-
Ulva lactuca-Himanthalia elongata	38	<2x10-16	0.00	*
Sargassum muticum-Polyides rotundus	38	<2x10-16	0.00	*
Ulva lactuca-Polyides rotundus	38	<2x10-16	0.20	-
Ulva lactuca-Sargassum muticum	38	<2x10-16	0.00	*
Figure 4B. Antimicrobial Agents effects on Yellow *Vibrio*				
Ampicillin 4mg/L-Ampicillin 16mg/L	27	<2x10-16	0.99	-
Ampicillin 8mg/L-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Asparagopsis armata-Ampicillin 16mg/L	27	<2x10-16	0.00	*
Azithromycin 2mg/L-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Azithromycin 4mg/L-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Azithromycin 8mg/L-Ampicillin 16mg/L	27	<2x10-16	0.94	-
Calliblepharis jubata-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Chloramphenicol 16mg/L-Ampicillin 16mg/L	27	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Ampicillin 16mg/L	27	<2x10-16	0.00	*
Corallina officinalis-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Cystoseira baccata-Ampicillin 16mg/L	27	<2x10-16	0.00	*
Fucus serratus-Ampicillin 16mg/L	27	<2x10-16	0.00	*
Fucus vesiculosus-Ampicillin 16mg/L	27	<2x10-16	0.00	*
Furcellaria lumbricalis-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Gentamicin 16mg/L-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Gentamicin 2mg/L-Ampicillin 16mg/L	27	<2x10-16	0.99	-
Gentamicin 8mg/L-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Himanthalia elongata-Ampicillin 16mg/L	27	<2x10-16	0.00	*
Polyides rotundus-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Sargassum muticum-Ampicillin 16mg/L	27	<2x10-16	0.00	*
Ulva lactuca-Ampicillin 16mg/L	27	<2x10-16	1.00	-
Ampicillin 8mg/L-Ampicillin 4mg/L	27	<2x10-16	1.00	-
Asparagopsis armata-Ampicillin 4mg/L	27	<2x10-16	0.00	*
Azithromycin 2mg/L-Ampicillin 4mg/L	27	<2x10-16	1.00	-
Azithromycin 4mg/L-Ampicillin 4mg/L	27	<2x10-16	1.00	-
Azithromycin 8mg/L-Ampicillin 4mg/L	27	<2x10-16	0.05	-

Calliblepharis jubata-Ampicillin 4mg/L	27	<2x10-16	0.42	-
Chloramphenicol 16mg/L-Ampicillin 4mg/L	27	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Ampicillin 4mg/L	27	<2x10-16	0.00	*
Corallina officinalis-Ampicillin 4mg/L	27	<2x10-16	1.00	-
Cystoseira baccata-Ampicillin 4mg/L	27	<2x10-16	0.00	*
Fucus serratus-Ampicillin 4mg/L	27	<2x10-16	0.00	*
Fucus vesiculosus-Ampicillin 4mg/L	27	<2x10-16	0.00	*
Furcellaria lumbricalis-Ampicillin 4mg/L	27	<2x10-16	0.58	-
Gentamicin 16mg/L-Ampicillin 4mg/L	27	<2x10-16	1.00	-
Gentamicin 2mg/L-Ampicillin 4mg/L	27	<2x10-16	1.00	-
Gentamicin 8mg/L-Ampicillin 4mg/L	27	<2x10-16	1.00	-
Himanthalia elongata-Ampicillin 4mg/L	27	<2x10-16	0.00	*
Polyides rotundus-Ampicillin 4mg/L	27	<2x10-16	0.67	-
Sargassum muticum-Ampicillin 4mg/L	27	<2x10-16	0.00	*
Ulva lactuca-Ampicillin 4mg/L	27	<2x10-16	1.00	-
Asparagopsis armata-Ampicillin 8mg/L	27	<2x10-16	0.00	*
Azithromycin 2mg/L-Ampicillin 8mg/L	27	<2x10-16	1.00	-
Azithromycin 4mg/L-Ampicillin 8mg/L	27	<2x10-16	1.00	-
Azithromycin 8mg/L-Ampicillin 8mg/L	27	<2x10-16	0.44	-
Calliblepharis jubata-Ampicillin 8mg/L	27	<2x10-16	0.96	-
Chloramphenicol 16mg/L-Ampicillin 8mg/L	27	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Ampicillin 8mg/L	27	<2x10-16	0.00	*
Corallina officinalis-Ampicillin 8mg/L	27	<2x10-16	1.00	-
Cystoseira baccata-Ampicillin 8mg/L	27	<2x10-16	0.00	*
Fucus serratus-Ampicillin 8mg/L	27	<2x10-16	0.00	*
Fucus vesiculosus-Ampicillin 8mg/L	27	<2x10-16	0.00	*
Furcellaria lumbricalis-Ampicillin 8mg/L	27	<2x10-16	0.99	-
Gentamicin 16mg/L-Ampicillin 8mg/L	27	<2x10-16	1.00	-
Gentamicin 2mg/L-Ampicillin 8mg/L	27	<2x10-16	1.00	-
Gentamicin 8mg/L-Ampicillin 8mg/L	27	<2x10-16	1.00	-
Himanthalia elongata-Ampicillin 8mg/L	27	<2x10-16	0.00	*
Polyides rotundus-Ampicillin 8mg/L	27	<2x10-16	0.99	-
Sargassum muticum-Ampicillin 8mg/L	27	<2x10-16	0.00	*
Ulva lactuca-Ampicillin 8mg/L	27	<2x10-16	1.00	-
Azithromycin 2mg/L-Asparagopsis armata	27	<2x10-16	0.00	*

Azithromycin 4mg/L-Asparagopsis armata	27	<2x10-16	0.00	*
Azithromycin 8mg/L-Asparagopsis armata	27	<2x10-16	0.00	*
Calliblepharis jubata-Asparagopsis armata	27	<2x10-16	0.00	*
Chloramphenicol 16mg/L-Asparagopsis armata	27	<2x10-16	1.00	-
Chloramphenicol 8mg/L-Asparagopsis armata	27	<2x10-16	1.00	-
Corallina officinalis-Asparagopsis armata	27	<2x10-16	0.00	*
Cystoseira baccata-Asparagopsis armata	27	<2x10-16	1.00	-
Fucus serratus-Asparagopsis armata	27	<2x10-16	1.00	-
Fucus vesiculosus-Asparagopsis armata	27	<2x10-16	1.00	-
Furcellaria lumbricalis-Asparagopsis armata	27	<2x10-16	0.00	*
Gentamicin 16mg/L-Asparagopsis armata	27	<2x10-16	0.00	*
Gentamicin 2mg/L-Asparagopsis armata	27	<2x10-16	0.00	*
Gentamicin 8mg/L-Asparagopsis armata	27	<2x10-16	0.00	*
Himanthalia elongata-Asparagopsis armata	27	<2x10-16	1.00	-
Polyides rotundus-Asparagopsis armata	27	<2x10-16	0.00	*
Sargassum muticum-Asparagopsis armata	27	<2x10-16	0.40	-
Ulva lactuca-Asparagopsis armata	27	<2x10-16	0.00	*
Azithromycin 4mg/L-Azithromycin 2mg/L	27	<2x10-16	1.00	-
Azithromycin 8mg/L-Azithromycin 2mg/L	27	<2x10-16	0.13	-
Calliblepharis jubata-Azithromycin 2mg/L	27	<2x10-16	0.67	-
Chloramphenicol 16mg/L-Azithromycin 2mg/L	27	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Azithromycin 2mg/L	27	<2x10-16	0.00	*
Corallina officinalis-Azithromycin 2mg/L	27	<2x10-16	1.00	-
Cystoseira baccata-Azithromycin 2mg/L	27	<2x10-16	0.00	*
Fucus serratus-Azithromycin 2mg/L	27	<2x10-16	0.00	*
Fucus vesiculosus-Azithromycin 2mg/L	27	<2x10-16	0.00	*
Furcellaria lumbricalis-Azithromycin 2mg/L	27	<2x10-16	0.81	-
Gentamicin 16mg/L-Azithromycin 2mg/L	27	<2x10-16	1.00	-
Gentamicin 2mg/L-Azithromycin 2mg/L	27	<2x10-16	1.00	-

Gentamicin 8mg/L-Azithromycin 2mg/L	27	<2x10-16	1.00	-
Himanthalia elongata-Azithromycin 2mg/L	27	<2x10-16	0.00	*
Polyides rotundus-Azithromycin 2mg/L	27	<2x10-16	0.87	-
Sargassum muticum-Azithromycin 2mg/L	27	<2x10-16	0.00	*
Ulva lactuca-Azithromycin 2mg/L	27	<2x10-16	1.00	-
Azithromycin 8mg/L-Azithromycin 4mg/L	27	<2x10-16	0.16	-
Calliblepharis jubata-Azithromycin 4mg/L	27	<2x10-16	0.73	-
Chloramphenicol 16mg/L-Azithromycin 4mg/L	27	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Azithromycin 4mg/L	27	<2x10-16	0.00	*
Corallina officinalis-Azithromycin 4mg/L	27	<2x10-16	1.00	-
Cystoseira baccata-Azithromycin 4mg/L	27	<2x10-16	0.00	*
Fucus serratus-Azithromycin 4mg/L	27	<2x10-16	0.00	*
Fucus vesiculosus-Azithromycin 4mg/L	27	<2x10-16	0.00	*
Furcellaria lumbricalis-Azithromycin 4mg/L	27	<2x10-16	0.86	-
Gentamicin 16mg/L-Azithromycin 4mg/L	27	<2x10-16	1.00	-
Gentamicin 2mg/L-Azithromycin 4mg/L	27	<2x10-16	1.00	-
Gentamicin 8mg/L-Azithromycin 4mg/L	27	<2x10-16	1.00	-
Himanthalia elongata-Azithromycin 4mg/L	27	<2x10-16	0.00	*
Polyides rotundus-Azithromycin 4mg/L	27	<2x10-16	0.91	-
Sargassum muticum-Azithromycin 4mg/L	27	<2x10-16	0.00	*
Ulva lactuca-Azithromycin 4mg/L	27	<2x10-16	1.00	-
Calliblepharis jubata-Azithromycin 8mg/L	27	<2x10-16	1.00	-
Chloramphenicol 16mg/L-Azithromycin 8mg/L	27	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Azithromycin 8mg/L	27	<2x10-16	0.01	*
Corallina officinalis-Azithromycin 8mg/L	27	<2x10-16	0.10	-
Cystoseira baccata-Azithromycin 8mg/L	27	<2x10-16	0.00	*
Fucus serratus-Azithromycin 8mg/L	27	<2x10-16	0.00	*
Fucus vesiculosus-Azithromycin 8mg/L	27	<2x10-16	0.00	*
Furcellaria lumbricalis-Azithromycin 8mg/L	27	<2x10-16	1.00	-
Gentamicin 16mg/L-Azithromycin 8mg/L	27	<2x10-16	0.83	-

Gentamicin 2mg/L-Azithromycin 8mg/L	27	<2x10-16	0.05	-
Gentamicin 8mg/L-Azithromycin 8mg/L	27	<2x10-16	0.23	-
Himanthalia elongata-Azithromycin 8mg/L	27	<2x10-16	0.01	*
Polyides rotundus-Azithromycin 8mg/L	27	<2x10-16	1.00	-
Sargassum muticum-Azithromycin 8mg/L	27	<2x10-16	0.49	-
Ulva lactuca-Azithromycin 8mg/L	27	<2x10-16	0.17	-
Chloramphenicol 16mg/L-Calliblepharis jubata	27	<2x10-16	0.00	*
Chloramphenicol 8mg/L-Calliblepharis jubata	27	<2x10-16	0.00	*
Corallina officinalis-Calliblepharis jubata	27	<2x10-16	0.60	-
Cystoseira baccata-Calliblepharis jubata	27	<2x10-16	0.00	*
Fucus serratus-Calliblepharis jubata	27	<2x10-16	0.00	*
Fucus vesiculosus-Calliblepharis jubata	27	<2x10-16	0.00	*
Furcellaria lumbricalis-Calliblepharis jubata	27	<2x10-16	1.00	-
Gentamicin 16mg/L-Calliblepharis jubata	27	<2x10-16	1.00	-
Gentamicin 2mg/L-Calliblepharis jubata	27	<2x10-16	0.42	-
Gentamicin 8mg/L-Calliblepharis jubata	27	<2x10-16	0.83	-
Himanthalia elongata-Calliblepharis jubata	27	<2x10-16	0.00	*
Polyides rotundus-Calliblepharis jubata	27	<2x10-16	1.00	-
Sargassum muticum-Calliblepharis jubata	27	<2x10-16	0.06	-
Ulva lactuca-Calliblepharis jubata	27	<2x10-16	0.75	-
Chloramphenicol 8mg/L-Chloramphenicol 16mg/L	27	<2x10-16	1.00	-
Corallina officinalis-Chloramphenicol 16mg/L	27	<2x10-16	0.00	*
Cystoseira baccata-Chloramphenicol 16mg/L	27	<2x10-16	1.00	-
Fucus serratus-Chloramphenicol 16mg/L	27	<2x10-16	1.00	-
Fucus vesiculosus-Chloramphenicol 16mg/L	27	<2x10-16	1.00	-
Furcellaria lumbricalis-Chloramphenicol 16mg/L	27	<2x10-16	0.00	*
Gentamicin 16mg/L-Chloramphenicol 16mg/L	27	<2x10-16	0.00	*
Gentamicin 2mg/L-Chloramphenicol 16mg/L	27	<2x10-16	0.00	*
Gentamicin 8mg/L-Chloramphenicol 16mg/L	27	<2x10-16	0.00	*
Himanthalia elongata-Chloramphenicol 16mg/L	27	<2x10-16	1.00	-

Polyides rotundus-Chloramphenicol 16mg/L	27	<2x10-16	0.00	*
Sargassum muticum-Chloramphenicol 16mg/L	27	<2x10-16	0.87	-
Ulva lactuca-Chloramphenicol 16mg/L	27	<2x10-16	0.00	*
Corallina officinalis-Chloramphenicol 8mg/L	27	<2x10-16	0.00	*
Cystoseira baccata-Chloramphenicol 8mg/L	27	<2x10-16	0.98	-
Fucus serratus-Chloramphenicol 8mg/L	27	<2x10-16	1.00	-
Fucus vesiculosus-Chloramphenicol 8mg/L	27	<2x10-16	1.00	-
Furcellaria lumbricalis-Chloramphenicol 8mg/L	27	<2x10-16	0.00	*
Gentamicin 16mg/L-Chloramphenicol 8mg/L	27	<2x10-16	0.00	*
Gentamicin 2mg/L-Chloramphenicol 8mg/L	27	<2x10-16	0.00	*
Gentamicin 8mg/L-Chloramphenicol 8mg/L	27	<2x10-16	0.00	*
Himanthalia elongata-Chloramphenicol 8mg/L	27	<2x10-16	1.00	-
Polyides rotundus-Chloramphenicol 8mg/L	27	<2x10-16	0.00	*
Sargassum muticum-Chloramphenicol 8mg/L	27	<2x10-16	1.00	-
Ulva lactuca-Chloramphenicol 8mg/L	27	<2x10-16	0.00	*
Cystoseira baccata-Corallina officinalis	27	<2x10-16	0.00	*
Fucus serratus-Corallina officinalis	27	<2x10-16	0.00	*
Fucus vesiculosus-Corallina officinalis	27	<2x10-16	0.00	*
Furcellaria lumbricalis-Corallina officinalis	27	<2x10-16	0.75	-
Gentamicin 16mg/L-Corallina officinalis	27	<2x10-16	1.00	-
Gentamicin 2mg/L-Corallina officinalis	27	<2x10-16	1.00	-
Gentamicin 8mg/L-Corallina officinalis	27	<2x10-16	1.00	-
Himanthalia elongata-Corallina officinalis	27	<2x10-16	0.00	*
Polyides rotundus-Corallina officinalis	27	<2x10-16	0.83	-
Sargassum muticum-Corallina officinalis	27	<2x10-16	0.00	*
Ulva lactuca-Corallina officinalis	27	<2x10-16	1.00	-
Fucus serratus-Cystoseira baccata	27	<2x10-16	1.00	-
Fucus vesiculosus-Cystoseira baccata	27	<2x10-16	1.00	-
Furcellaria lumbricalis-Cystoseira baccata	27	<2x10-16	0.00	*
Gentamicin 16mg/L-Cystoseira baccata	27	<2x10-16	0.00	*

Gentamicin 2mg/L-Cystoseira baccata	27	<2x10-16	0.00	*
Gentamicin 8mg/L-Cystoseira baccata	27	<2x10-16	0.00	*
Himanthalia elongata-Cystoseira baccata	27	<2x10-16	0.98	-
Polyides rotundus-Cystoseira baccata	27	<2x10-16	0.00	*
Sargassum muticum-Cystoseira baccata	27	<2x10-16	0.16	-
Ulva lactuca-Cystoseira baccata	27	<2x10-16	0.00	*
Fucus vesiculosus-Fucus serratus	27	<2x10-16	1.00	-
Furcellaria lumbricalis-Fucus serratus	27	<2x10-16	0.00	*
Gentamicin 16mg/L-Fucus serratus	27	<2x10-16	0.00	*
Gentamicin 2mg/L-Fucus serratus	27	<2x10-16	0.00	*
Gentamicin 8mg/L-Fucus serratus	27	<2x10-16	0.00	*
Himanthalia elongata-Fucus serratus	27	<2x10-16	1.00	-
Polyides rotundus-Fucus serratus	27	<2x10-16	0.00	*
Sargassum muticum-Fucus serratus	27	<2x10-16	0.53	-
Ulva lactuca-Fucus serratus	27	<2x10-16	0.00	*
Furcellaria lumbricalis-Fucus vesiculosus	27	<2x10-16	0.00	*
Gentamicin 16mg/L-Fucus vesiculosus	27	<2x10-16	0.00	*
Gentamicin 2mg/L-Fucus vesiculosus	27	<2x10-16	0.00	*
Gentamicin 8mg/L-Fucus vesiculosus	27	<2x10-16	0.00	*
Himanthalia elongata-Fucus vesiculosus	27	<2x10-16	1.00	-
Polyides rotundus-Fucus vesiculosus	27	<2x10-16	0.00	*
Sargassum muticum-Fucus vesiculosus	27	<2x10-16	0.46	-
Ulva lactuca-Fucus vesiculosus	27	<2x10-16	0.00	*
Gentamicin 16mg/L-Furcellaria lumbricalis	27	<2x10-16	1.00	-
Gentamicin 2mg/L-Furcellaria lumbricalis	27	<2x10-16	0.58	-
Gentamicin 8mg/L-Furcellaria lumbricalis	27	<2x10-16	0.92	-
Himanthalia elongata-Furcellaria lumbricalis	27	<2x10-16	0.00	*
Polyides rotundus-Furcellaria lumbricalis	27	<2x10-16	1.00	-
Sargassum muticum-Furcellaria lumbricalis	27	<2x10-16	0.03	*
Ulva lactuca-Furcellaria lumbricalis	27	<2x10-16	0.87	-
Gentamicin 2mg/L-Gentamicin 16mg/L	27	<2x10-16	1.00	-
Gentamicin 8mg/L-Gentamicin 16mg/L	27	<2x10-16	1.00	-

Himanthalia elongata-Gentamicin 16mg/L	27	<2x10-16	0.00	*
Polyides rotundus-Gentamicin 16mg/L	27	<2x10-16	1.00	-
Sargassum muticum-Gentamicin 16mg/L	27	<2x10-16	0.00	*
Ulva lactuca-Gentamicin 16mg/L	27	<2x10-16	1.00	-
Gentamicin 8mg/L-Gentamicin 2mg/L	27	<2x10-16	1.00	-
Himanthalia elongata-Gentamicin 2mg/L	27	<2x10-16	0.00	*
Polyides rotundus-Gentamicin 2mg/L	27	<2x10-16	0.67	-
Sargassum muticum-Gentamicin 2mg/L	27	<2x10-16	0.00	*
Ulva lactuca-Gentamicin 2mg/L	27	<2x10-16	1.00	-
Himanthalia elongata-Gentamicin 8mg/L	27	<2x10-16	0.00	*
Polyides rotundus-Gentamicin 8mg/L	27	<2x10-16	0.96	-
Sargassum muticum-Gentamicin 8mg/L	27	<2x10-16	0.00	*
Ulva lactuca-Gentamicin 8mg/L	27	<2x10-16	1.00	-
Polyides rotundus-Himanthalia elongata	27	<2x10-16	0.00	*
Sargassum muticum-Himanthalia elongata	27	<2x10-16	1.00	-
Ulva lactuca-Himanthalia elongata	27	<2x10-16	0.00	*
Sargassum muticum-Polyides rotundus	27	<2x10-16	0.02	*
Ulva lactuca-Polyides rotundus	27	<2x10-16	0.92	-
Ulva lactuca-Sargassum muticum	27	<2x10-16	0.00	*
Figure 5. Antimicrobial Agents effects on *S. aureus*				
Chloramphenicol 20-Chloramphenicol 10	3	<2x10-16	0.00	*
Chloramphenicol 5-Chloramphenicol 10	3	<2x10-16	0.06	-
Erythromycin 10-Chloramphenicol 10	3	<2x10-16	0.00	*
Erythromycin 2.5-Chloramphenicol 10	3	<2x10-16	0.00	*
Erythromycin 5-Chloramphenicol 10	3	<2x10-16	0.00	*
Fucoxanthin 1600-Chloramphenicol 10	3	<2x10-16	0.06	-
Fucoxanthin 800-Chloramphenicol 10	3	<2x10-16	0.06	-
Kanamycin 100-Chloramphenicol 10	3	<2x10-16	0.00	*
Kanamycin 25-Chloramphenicol 10	3	<2x10-16	0.00	*
Kanamycin 50-Chloramphenicol 10	3	<2x10-16	0.00	*
Tetracycline 10-Chloramphenicol 10	3	<2x10-16	0.00	*
Tetracycline 2.5-Chloramphenicol 10	3	<2x10-16	0.00	*
Tetracycline 5-Chloramphenicol 10	3	<2x10-16	0.00	*

Chloramphenicol 5-Chloramphenicol 20	3	<2x10-16	0.00			*
Erythromycin 10-Chloramphenicol 20	3	<2x10-16	1.00			-
Erythromycin 2.5-Chloramphenicol 20	3	<2x10-16	0.53			-
Erythromycin 5-Chloramphenicol 20	3	<2x10-16	1.00			-
Fucoxanthin 1600-Chloramphenicol 20	3	<2x10-16	0.00			*
Fucoxanthin 800-Chloramphenicol 20	3	<2x10-16	0.00			*
Kanamycin 100-Chloramphenicol 20	3	<2x10-16	0.00			*
Kanamycin 25-Chloramphenicol 20	3	<2x10-16	0.00			*
Kanamycin 50-Chloramphenicol 20	3	<2x10-16	0.00			*
Tetracycline 10-Chloramphenicol 20			3	<2x10-16	0.00	*
Tetracycline 2.5-Chloramphenicol 20			3	<2x10-16	0.00	*
Tetracycline 5-Chloramphenicol 20			3	<2x10-16	0.00	*
Erythromycin 10-Chloramphenicol 5			3	<2x10-16	0.00	*
Erythromycin 2.5-Chloramphenicol 5			3	<2x10-16	0.00	*
Erythromycin 5-Chloramphenicol 5			3	<2x10-16	0.00	*
Fucoxanthin 1600-Chloramphenicol 5			3	<2x10-16	1.00	-
Fucoxanthin 800-Chloramphenicol 5			3	<2x10-16	1.00	-
Kanamycin 100-Chloramphenicol 5			3	<2x10-16	0.00	*
Kanamycin 25-Chloramphenicol 5			3	<2x10-16	0.00	*
Kanamycin 50-Chloramphenicol 5			3	<2x10-16	0.00	*
Tetracycline 10-Chloramphenicol 5			3	<2x10-16	0.00	*
Tetracycline 2.5-Chloramphenicol 5			3	<2x10-16	0.00	*
Tetracycline 5-Chloramphenicol 5			3	<2x10-16	0.00	*
Erythromycin 2.5-Erythromycin 10			3	<2x10-16	0.53	-
Erythromycin 5-Erythromycin 10			3	<2x10-16	1.00	-
Fucoxanthin 1600-Erythromycin 10			3	<2x10-16	0.00	*
Fucoxanthin 800-Erythromycin 10			3	<2x10-16	0.00	*
Kanamycin 100-Erythromycin 10			3	<2x10-16	0.00	*
Kanamycin 25-Erythromycin 10			3	<2x10-16	0.00	*
Kanamycin 50-Erythromycin 10			3	<2x10-16	0.00	*
Tetracycline 10-Erythromycin 10			3	<2x10-16	0.00	*
Tetracycline 2.5-Erythromycin 10			3	<2x10-16	0.00	*
Tetracycline 5-Erythromycin 10			3	<2x10-16	0.00	*
Erythromycin 5-Erythromycin 2.5			3	<2x10-16	0.53	-
Fucoxanthin 1600-Erythromycin 2.5			3	<2x10-16	0.00	*
Fucoxanthin 800-Erythromycin 2.5			3	<2x10-16	0.00	*
Kanamycin 100-Erythromycin 2.5			3	<2x10-16	0.00	*
Kanamycin 25-Erythromycin 2.5			3	<2x10-16	0.00	*
Kanamycin 50-Erythromycin 2.5			3	<2x10-16	0.00	*
Tetracycline 10-Erythromycin 2.5			3	<2x10-16	0.00	*
Tetracycline 2.5-Erythromycin 2.5			3	<2x10-16	0.00	*
Tetracycline 5-Erythromycin 2.5			3	<2x10-16	0.00	*
Fucoxanthin 1600-Erythromycin 5			3	<2x10-16	0.00	*
Fucoxanthin 800-Erythromycin 5			3	<2x10-16	0.00	*
Kanamycin 100-Erythromycin 5			3	<2x10-16	0.00	*
Kanamycin 25-Erythromycin 5			3	<2x10-16	0.00	*
Kanamycin 50-Erythromycin 5			3	<2x10-16	0.00	*
Tetracycline 10-Erythromycin 5			3	<2x10-16	0.00	*
Tetracycline 2.5-Erythromycin 5			3	<2x10-16	0.00	*
Tetracycline 5-Erythromycin 5			3	<2x10-16	0.00	*

Fucoxanthin 800-Fucoxanthin 1600	3	<2x10-16	1.00	-
Kanamycin 100-Fucoxanthin 1600	3	<2x10-16	0.00	*
Kanamycin 25-Fucoxanthin 1600	3	<2x10-16	0.00	*
Kanamycin 50-Fucoxanthin 1600	3	<2x10-16	0.00	*
Tetracycline 10-Fucoxanthin 1600	3	<2x10-16	0.00	*
Tetracycline 2.5-Fucoxanthin 1600	3	<2x10-16	0.00	*
Tetracycline 5-Fucoxanthin 1600	3	<2x10-16	0.00	*
Kanamycin 100-Fucoxanthin 800	3	<2x10-16	0.00	*
Kanamycin 25-Fucoxanthin 800	3	<2x10-16	0.00	*
Kanamycin 50-Fucoxanthin 800	3	<2x10-16	0.00	*
Tetracycline 10-Fucoxanthin 800	3	<2x10-16	0.00	*
Tetracycline 2.5-Fucoxanthin 800	3	<2x10-16	0.00	*
Tetracycline 5-Fucoxanthin 800	3	<2x10-16	0.00	*
Kanamycin 25-Kanamycin 100	3	<2x10-16	1.00	-
Kanamycin 50-Kanamycin 100	3	<2x10-16	1.00	-
Tetracycline 10-Kanamycin 100	3	<2x10-16	0.06	-
Tetracycline 2.5-Kanamycin 100	3	<2x10-16	0.00	*
Tetracycline 5-Kanamycin 100	3	<2x10-16	0.00	*
Kanamycin 50-Kanamycin 25	3	<2x10-16	1.00	-
Tetracycline 10-Kanamycin 25	3	<2x10-16	0.06	-
Tetracycline 2.5-Kanamycin 25	3	<2x10-16	0.00	*
Tetracycline 5-Kanamycin 25	3	<2x10-16	0.00	*
Tetracycline 10-Kanamycin 50	3	<2x10-16	0.06	-
Tetracycline 2.5-Kanamycin 50	3	<2x10-16	0.00	*
Tetracycline 5-Kanamycin 50	3	<2x10-16	0.00	*
Tetracycline 2.5-Tetracycline 10	3	<2x10-16	0.00	*
Tetracycline 5-Tetracycline 10	3	<2x10-16	0.00	*
Tetracycline 5-Tetracycline 2.5	3	<2x10-16	0.00	*
Chloramphenicol 20-Chloramphenicol 10	3	<2x10-16	0.00	*
Chloramphenicol 5-Chloramphenicol 10	3	<2x10-16	0.06	-
Erythromycin 10-Chloramphenicol 10	3	<2x10-16	0.00	*
Erythromycin 2.5-Chloramphenicol 10	3	<2x10-16	0.00	*
Erythromycin 5-Chloramphenicol 10	3	<2x10-16	0.00	*
Fucoxanthin 1600-Chloramphenicol 10	3	<2x10-16	0.06	-
Fucoxanthin 800-Chloramphenicol 10	3	<2x10-16	0.06	-
Kanamycin 100-Chloramphenicol 10	3	<2x10-16	0.00	*
Kanamycin 25-Chloramphenicol 10	3	<2x10-16	0.00	*
Kanamycin 50-Chloramphenicol 10	3	<2x10-16	0.00	*
Tetracycline 10-Chloramphenicol 10	3	<2x10-16	0.00	*
Tetracycline 2.5-Chloramphenicol 10	3	<2x10-16	0.00	*
Tetracycline 5-Chloramphenicol 10	3	<2x10-16	0.00	*
Chloramphenicol 5-Chloramphenicol 20	3	<2x10-16	0.00	*
Erythromycin 10-Chloramphenicol 20	3	<2x10-16	1.00	-
Erythromycin 2.5-Chloramphenicol 20	3	<2x10-16	0.53	-
Erythromycin 5-Chloramphenicol 20	3	<2x10-16	1.00	-
Fucoxanthin 1600-Chloramphenicol 20	3	<2x10-16	0.00	*
Fucoxanthin 800-Chloramphenicol 20	3	<2x10-16	0.00	*
Kanamycin 100-Chloramphenicol 20	3	<2x10-16	0.00	*
Kanamycin 25-Chloramphenicol 20	3	<2x10-16	0.00	*
Kanamycin 50-Chloramphenicol 20	3	<2x10-16	0.00	*
Tetracycline 10-Chloramphenicol 20	3	<2x10-16	0.00	*
Tetracycline 2.5-Chloramphenicol 20	3	<2x10-16	0.00	*
Tetracycline 5-Chloramphenicol 20	3	<2x10-16	0.00	*
Erythromycin 10-Chloramphenicol 5	3	<2x10-16	0.00	*
Erythromycin 2.5-Chloramphenicol 5	3	<2x10-16	0.00	*

Erythromycin 5-Chloramphenicol 5	3	<2x10-16	0.00	*
Fucoxanthin 1600-Chloramphenicol 5	3	<2x10-16	1.00	-
Fucoxanthin 800-Chloramphenicol 5	3	<2x10-16	1.00	-
Kanamycin 100-Chloramphenicol 5	3	<2x10-16	0.00	*
Kanamycin 25-Chloramphenicol 5	3	<2x10-16	0.00	*
Kanamycin 50-Chloramphenicol 5	3	<2x10-16	0.00	*
Tetracycline 10-Chloramphenicol 5	3	<2x10-16	0.00	*
Tetracycline 2.5-Chloramphenicol 5	3	<2x10-16	0.00	*
Tetracycline 5-Chloramphenicol 5	3	<2x10-16	0.00	*
Erythromycin 2.5-Erythromycin 10	3	<2x10-16	0.53	-
Erythromycin 5-Erythromycin 10	3	<2x10-16	1.00	-
Fucoxanthin 1600-Erythromycin 10	3	<2x10-16	0.00	*
Fucoxanthin 800-Erythromycin 10	3	<2x10-16	0.00	*
Kanamycin 100-Erythromycin 10	3	<2x10-16	0.00	*
Kanamycin 25-Erythromycin 10	3	<2x10-16	0.00	*
Kanamycin 50-Erythromycin 10	3	<2x10-16	0.00	*
Tetracycline 10-Erythromycin 10	3	<2x10-16	0.00	*
Tetracycline 2.5-Erythromycin 10	3	<2x10-16	0.00	*
Tetracycline 5-Erythromycin 10	3	<2x10-16	0.00	*
Erythromycin 5-Erythromycin 2.5	3	<2x10-16	0.53	-
Fucoxanthin 1600-Erythromycin 2.5	3	<2x10-16	0.00	*
Fucoxanthin 800-Erythromycin 2.5	3	<2x10-16	0.00	*
Kanamycin 100-Erythromycin 2.5	3	<2x10-16	0.00	*
Kanamycin 25-Erythromycin 2.5	3	<2x10-16	0.00	*
Kanamycin 50-Erythromycin 2.5	3	<2x10-16	0.00	*
Tetracycline 10-Erythromycin 2.5	3	<2x10-16	0.00	*
Tetracycline 2.5-Erythromycin 2.5	3	<2x10-16	0.00	*
Tetracycline 5-Erythromycin 2.5	3	<2x10-16	0.00	*
Fucoxanthin 1600-Erythromycin 5	3	<2x10-16	0.00	*
Fucoxanthin 800-Erythromycin 5	3	<2x10-16	0.00	*
Kanamycin 100-Erythromycin 5	3	<2x10-16	0.00	*
Kanamycin 25-Erythromycin 5	3	<2x10-16	0.00	*
Kanamycin 50-Erythromycin 5	3	<2x10-16	0.00	*
Tetracycline 10-Erythromycin 5	3	<2x10-16	0.00	*
Tetracycline 2.5-Erythromycin 5	3	<2x10-16	0.00	*
Tetracycline 5-Erythromycin 5	3	<2x10-16	0.00	*
Fucoxanthin 800-Fucoxanthin 1600	3	<2x10-16	1.00	-
Kanamycin 100-Fucoxanthin 1600	3	<2x10-16	0.00	*
Kanamycin 25-Fucoxanthin 1600	3	<2x10-16	0.00	*
Kanamycin 50-Fucoxanthin 1600	3	<2x10-16	0.00	*
Tetracycline 10-Fucoxanthin 1600	3	<2x10-16	0.00	*
Tetracycline 2.5-Fucoxanthin 1600	3	<2x10-16	0.00	*
Tetracycline 5-Fucoxanthin 1600	3	<2x10-16	0.00	*
Kanamycin 100-Fucoxanthin 800	3	<2x10-16	0.00	*
Kanamycin 25-Fucoxanthin 800	3	<2x10-16	0.00	*
Kanamycin 50-Fucoxanthin 800	3	<2x10-16	0.00	*
Tetracycline 10-Fucoxanthin 800	3	<2x10-16	0.00	*
Tetracycline 2.5-Fucoxanthin 800	3	<2x10-16	0.00	*
Tetracycline 5-Fucoxanthin 800	3	<2x10-16	0.00	*
Kanamycin 25-Kanamycin 100	3	<2x10-16	1.00	-
Kanamycin 50-Kanamycin 100	3	<2x10-16	1.00	-
Tetracycline 10-Kanamycin 100	3	<2x10-16	0.06	-
Tetracycline 2.5-Kanamycin 100	3	<2x10-16	0.00	*
Tetracycline 5-Kanamycin 100	3	<2x10-16	0.00	*
Kanamycin 50-Kanamycin 25	3	<2x10-16	1.00	-

Tetracycline 10-Kanamycin 25	3	<2x10-16	0.06	-
Tetracycline 2.5-Kanamycin 25	3	<2x10-16	0.00	*
Tetracycline 5-Kanamycin 25	3	<2x10-16	0.00	*
Tetracycline 10-Kanamycin 50	3	<2x10-16	0.06	-
Tetracycline 2.5-Kanamycin 50	3	<2x10-16	0.00	*
Tetracycline 5-Kanamycin 50	3	<2x10-16	0.00	*
Tetracycline 2.5-Tetracycline 10	3	<2x10-16	0.00	*
Tetracycline 5-Tetracycline 10	3	<2x10-16	0.00	*
Tetracycline 5-Tetracycline 2.5	3	<2x10-16	0.00	*
Figure 6A. Antibiotic Resistant Bacteria Isolated from *Corallina officinalis*				
Chorlamphenicol 2-Ampicillin 2	6	2.48E-08	0.31	-
Ciprofloxacin 2-Ampicillin 2	6	2.48E-08	0.39	-
Gentamicin 16-Ampicillin 2	6	2.48E-08	1.00	-
Tetracycline 24-Ampicillin 2	6	2.48E-08	0.00	*
Ciprofloxacin 2-Chorlamphenicol 2	6	2.48E-08	1.00	-
Gentamicin 16-Chorlamphenicol 2	6	2.48E-08	0.34	-
Tetracycline 24-Chorlamphenicol 2	6	2.48E-08	0.00	*
Gentamicin 16-Ciprofloxacin 2	6	2.48E-08	0.42	-
Tetracycline 24-Ciprofloxacin 2	6	2.48E-08	0.00	*
Tetracycline 24-Gentamicin 16	6	2.48E-08	0.00	*
Figure 6B. Antibiotic Resistant Bacteria Isolated from *Furcellaria lumbricalis*				
Chorlamphenicol 2-Ampicillin 2	6	1.83E-11	0.00	*
Ciprofloxacin 2-Ampicillin 2	6	1.83E-11	0.00	*
Gentamicin 16-Ampicillin 2	6	1.83E-11	1.00	-
Tetracycline 24-Ampicillin 2	6	1.83E-11	0.00	*
Ciprofloxacin 2-Chorlamphenicol 2	6	1.83E-11	1.00	-
Gentamicin 16-Chorlamphenicol 2	6	1.83E-11	0.00	*
Tetracycline 24-Chorlamphenicol 2	6	1.83E-11	0.00	*
Gentamicin 16-Ciprofloxacin 2	6	1.83E-11	0.00	*
Tetracycline 24-Ciprofloxacin 2	6	1.83E-11	0.00	*
Tetracycline 24-Gentamicin 16	6	1.83E-11	0.00	*
Figure 6C. Antibiotic Resistant Bacteria Isolated from *Ulva lactuca*				
Chorlamphenicol 2-Ampicillin 2	6	1.33E-05	0.03	*
Ciprofloxacin 2-Ampicillin 2	6	1.33E-05	0.05	-
Gentamicin 16-Ampicillin 2	6	1.33E-05	0.14	-
Tetracycline 24-Ampicillin 2	6	1.33E-05	0.10	-
Ciprofloxacin 2-Chorlamphenicol 2	6	1.33E-05	1.00	-
Gentamicin 16-Chorlamphenicol 2	6	1.33E-05	0.96	-
Tetracycline 24-Chorlamphenicol 2	6	1.33E-05	0.00	
Gentamicin 16-Ciprofloxacin 2	6	1.33E-05	0.99	-
Tetracycline 24-Ciprofloxacin 2	6	1.33E-05	0.00	*
Tetracycline 24-Gentamicin 16	6	1.33E-05	0.00	*
Figure 6. Resistance to Ampicillin (2mg/L)				
Furcellaria lumbricalis-Corallina officinalis	6	0.01	0.94	-
Ulva lactuca-Corallina officinalis	6	0.01	0.01	*
Ulva lactuca-Furcellaria lumbricalis	6	0.01	0.03	*
Figure 6. Resistance to Chloramphenicol (2 mg/L)				
N/A	6	0.09	N/A	N/A
Figure 6. Resistance to Ciprofloxacin (2 mg/L)				

N/A	6	0.30	N/A	N/A
Figure 6. Resistance to Gentamicin (16 mg/L)				
N/A	6	0.30	N/A	N/A
Figure 6. Resistance to Tetracycline (24 mg/L)				
Furcellaria lumbricalis-Corallina officinalis	6	0.04	0.64	-
Ulva lactuca-Corallina officinalis	6	0.04	0.18	-
Ulva lactuca-Furcellaria lumbricalis	6	0.04	0.03	*
Figure 7. Prevalence of *intI1* Species Comparison				
Control-Asparagopsis armata	3	0.01	1.00	-
Corallina officinalis-Asparagopsis armata	3	0.01	1.00	-
Fucus serratus-Asparagopsis armata	3	0.01	0.05	-
Fucus Vesiculosus -Asparagopsis armata	3	0.01	0.02	*
Furcellaria lumbricalis-Asparagopsis armata	3	0.01	0.27	-
Ulva lactuca-Asparagopsis armata	3	0.01	1.00	-
Corallina officinalis-Control	3	0.01	1.00	-
Fucus serratus-Control	3	0.01	0.39	-
Fucus Vesiculosus -Control	3	0.01	0.24	-
Furcellaria lumbricalis-Control	3	0.01	0.76	-
Ulva lactuca-Control	3	0.01	1.00	-
Fucus serratus-Corallina officinalis	3	0.01	0.11	-
Fucus Vesiculosus -Corallina officinalis	3	0.01	0.05	-
Furcellaria lumbricalis-Corallina officinalis	3	0.01	0.46	-
Ulva lactuca-Corallina officinalis	3	0.01	1.00	-
Fucus Vesiculosus -Fucus serratus	3	0.01	1.00	-
Furcellaria lumbricalis-Fucus serratus	3	0.01	0.99	-
Ulva lactuca-Fucus serratus	3	0.01	0.08	-
Furcellaria lumbricalis-Fucus Vesiculosus	3	0.01	0.87	-
Ulva lactuca-Fucus Vesiculosus	3	0.01	0.03	*
Ulva lactuca-Furcellaria lumbricalis	3	0.01	0.37	-
Figure 8. Prevalence of *intI1* Site Comparison				
Control (GB)-Control (CB)	3	1.41E-07	0.00	*
Fucus serratus (CB)-Control (CB)	3	1.41E-07	0.56	-
Fucus serratus (GB)-Control (CB)	3	1.41E-07	0.37	-
Fucus Vesiculosus (CB)-Control (CB)	3	1.41E-07	0.41	-
Fucus Vesiculosus (GB)-Control (CB)	3	1.41E-07	0.20	-
Fucus serratus (CB)-Control (GB)	3	1.41E-07	0.00	*
Fucus serratus (GB)-Control (GB)	3	1.41E-07	0.00	*
Fucus Vesiculosus (CB)-Control (GB)	3	1.41E-07	0.00	*
Fucus Vesiculosus (GB)-Control (GB)	3	1.41E-07	0.00	*
Fucus serratus (GB)-Fucus serratus (CB)	3	1.41E-07	0.99	-
Fucus Vesiculosus (CB)-Fucus serratus (CB)	3	1.41E-07	1.00	-
Fucus Vesiculosus (GB)-Fucus serratus (CB)	3	1.41E-07	0.79	-
Fucus Vesiculosus (CB)-Fucus serratus (GB)	3	1.41E-07	1.00	-
Fucus Vesiculosus (GB)-Fucus serratus (GB)	3	1.41E-07	0.97	-
Fucus Vesiculosus (GB)-Fucus Vesiculosus (CB)	3	1.41E-07	0.95	-
Figure 11. Abundance of ARG's				
Corallina officinalis-Asparagopsis armata	3	0.04	0.18	-
Furcellaria lumbricalis-Asparagopsis armata	3	0.04	0.03	*
Ulva lactuca-Asparagopsis armata	3	0.04	0.46	-
Furcellaria lumbricalis-Corallina officinalis	3	0.04	0.54	-

Ulva lactuca-Corallina officinalis	3	0.04	0.88	-
Ulva lactuca-Furcellaria lumbricalis	3	0.04	0.22	-
Figure 12. Diversity of ARG's				
N/A	3	1.00	N/A	N/A

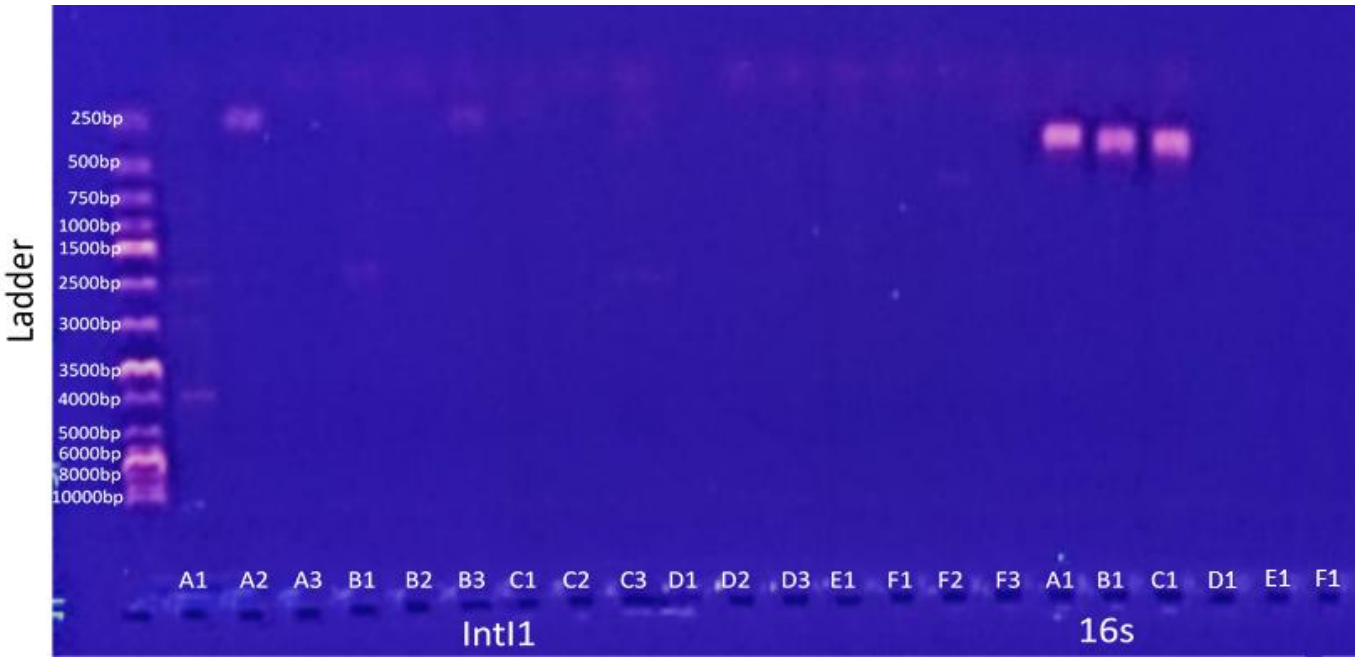

Figure S1

PCR plate visualising the expression of IntI1 and 16s genes in bacteria isolated from: A: Ulva lactuca B: Fucus vesiculosus C: Fucus serratus D: Himanthalia elongate and E: Cystoseira baccata.

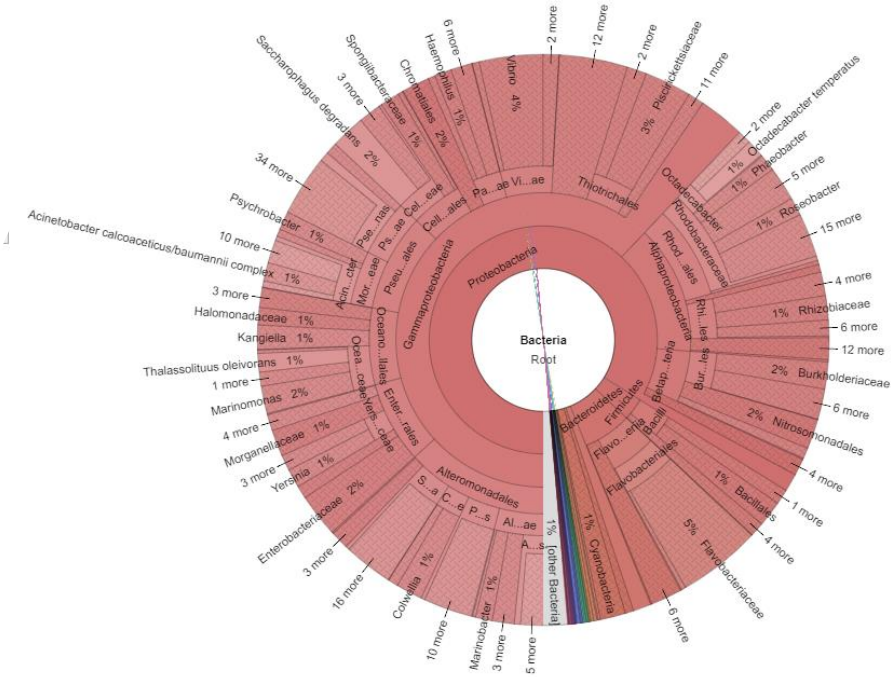

Figure S2

Breakdown of the bacteria isolated from sample two (*Asparagopsis armata)* samples ranging from phylum to species.

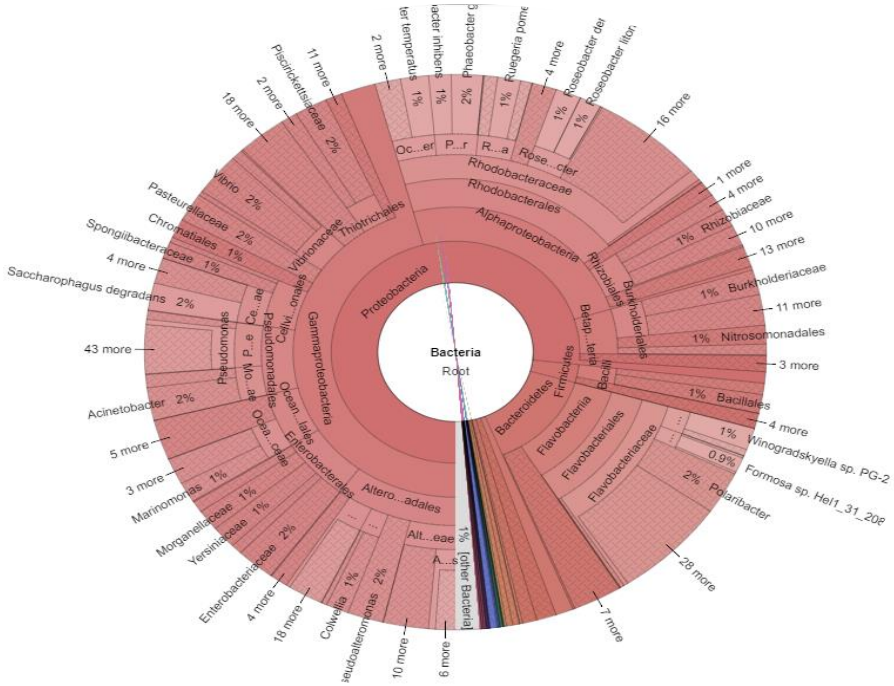

Figure S3

Breakdown of the bacteria isolated from sample three (*Asparagopsis armata)* samples ranging from phylum to species.

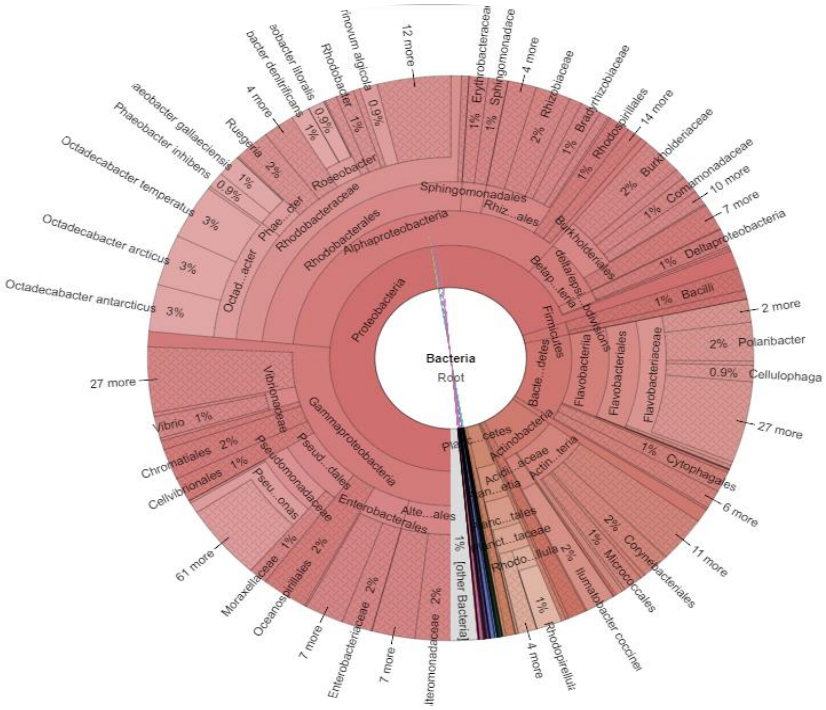

Figure S4

Breakdown of the bacteria isolated from sample four (*Corallina officinalis)* samples ranging from phylum to species.

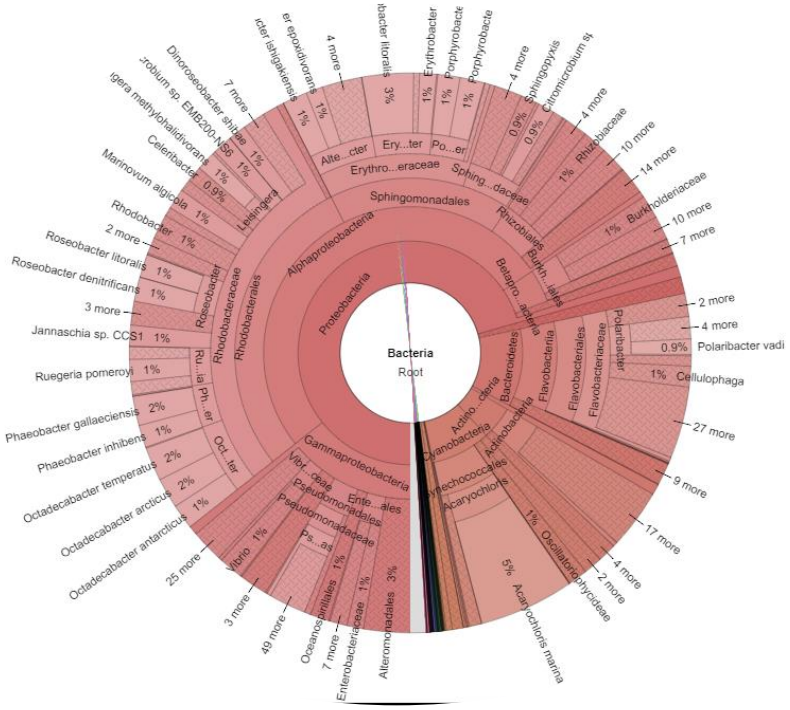

Figure S5

Breakdown of the bacteria isolated from sample five (*Corallina officinalis)* samples ranging from phylum to species.

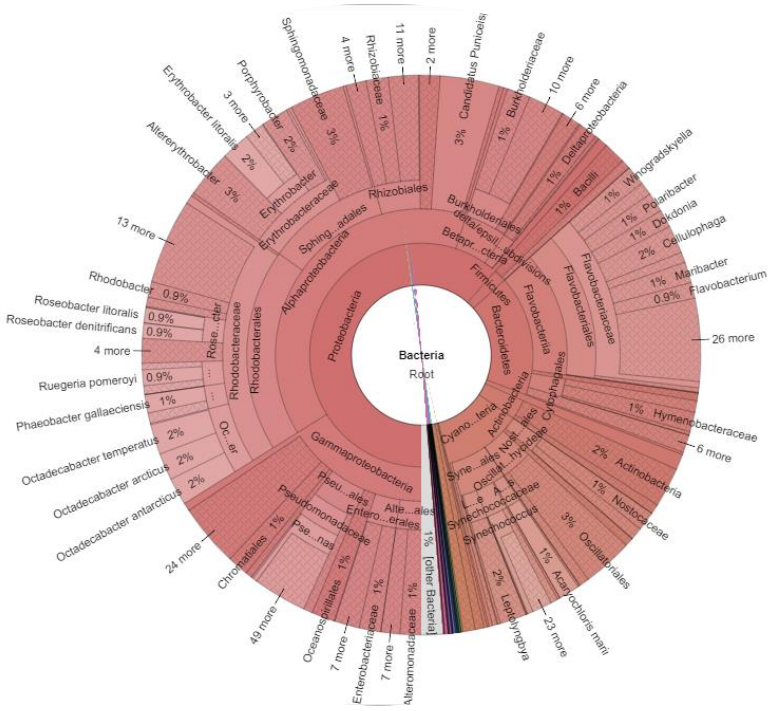

Figure S6

Breakdown of the bacteria isolated from sample six (*Corallina officinalis)* samples ranging from phylum to species.

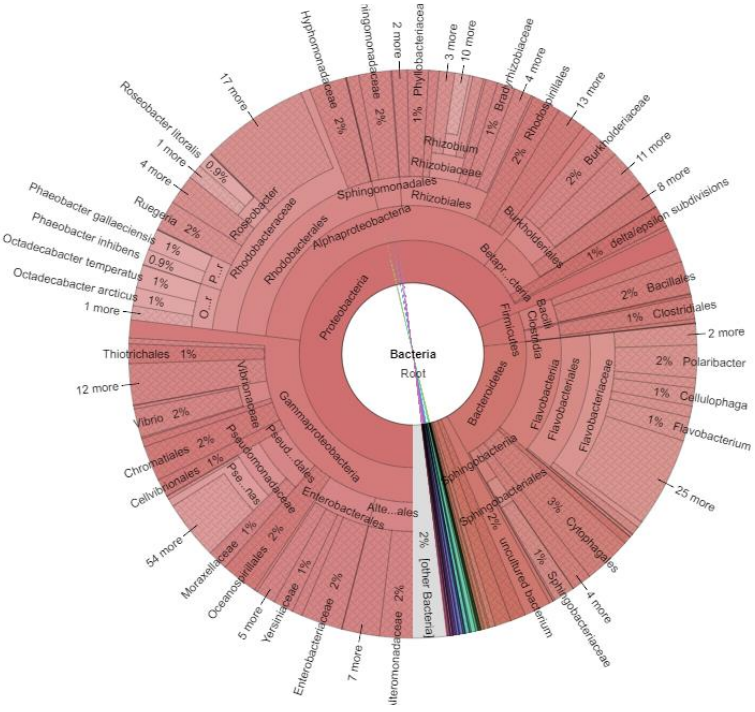

Figure S7

Breakdown of the bacteria isolated from sample seven (*Ulva lactuca)* samples ranging from phylum to species.

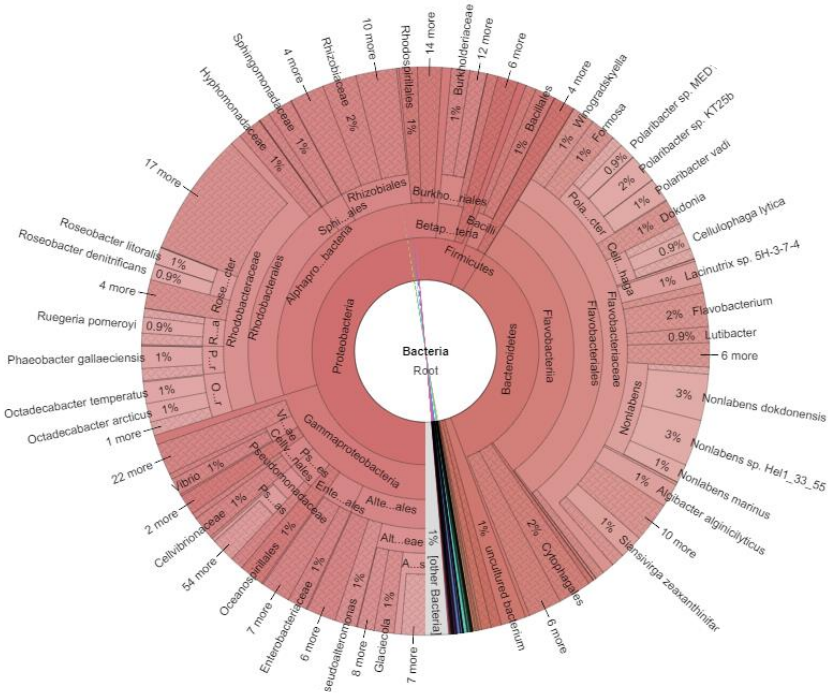

Figure S8

Breakdown of the bacteria isolated from sample eight (*Ulva lactuca)* samples ranging from phylum to species.

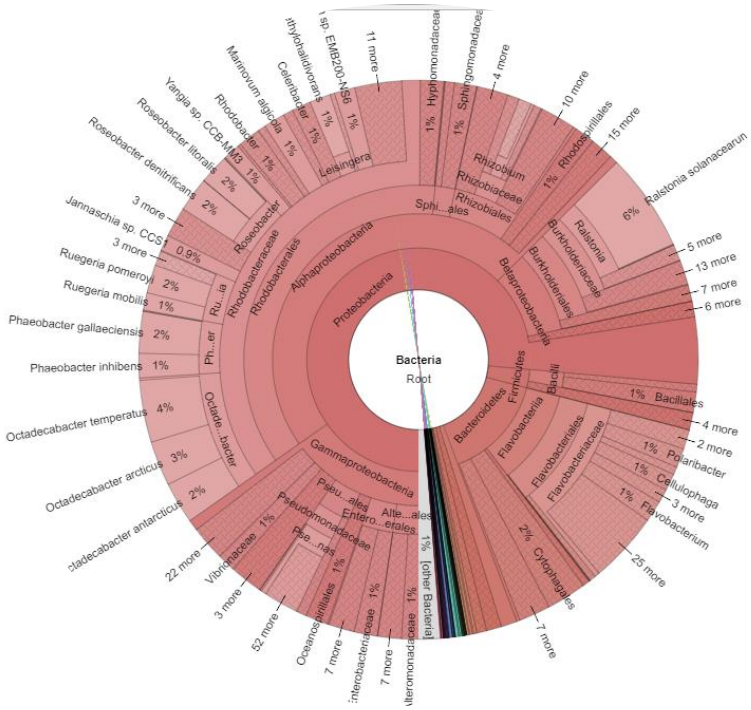

Figure S9

Breakdown of the bacteria isolated from sample nine (*Ulva lactuca)* samples ranging from phylum to species.

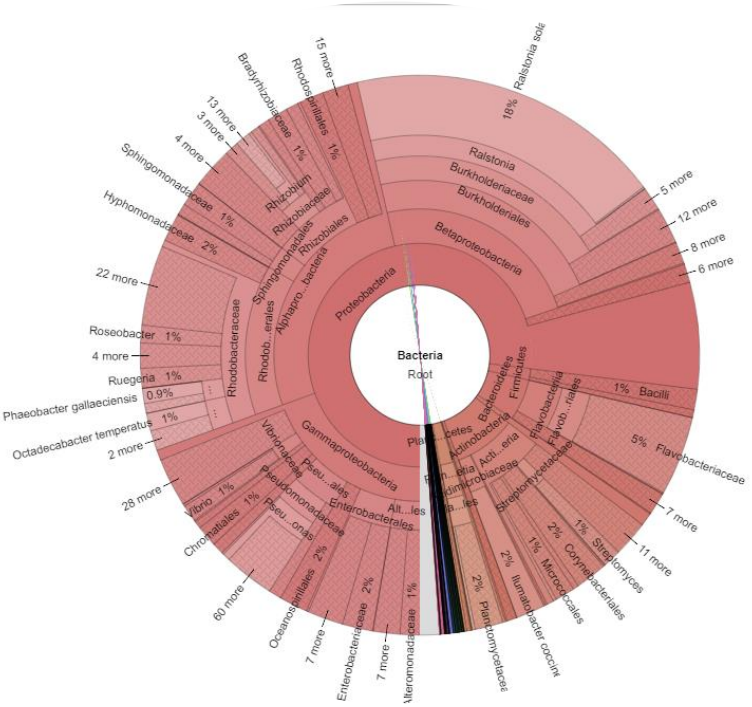

Figure S10

Breakdown of the bacteria isolated from sample ten (*Furcellaria lumbricalis)* samples ranging from phylum to species.

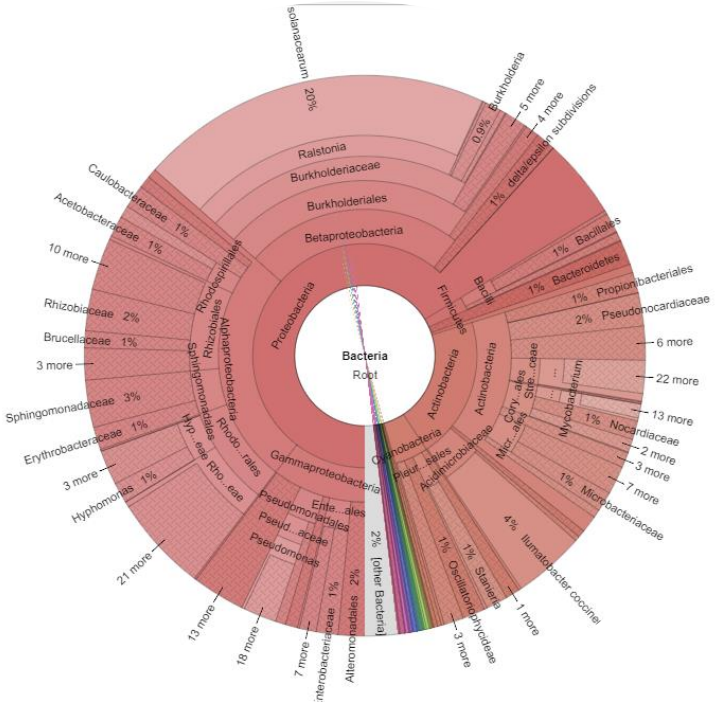

Figure S11

Breakdown of the bacteria isolated from sample eleven (*Furcellaria lumbricalis)* samples ranging from phylum to species.

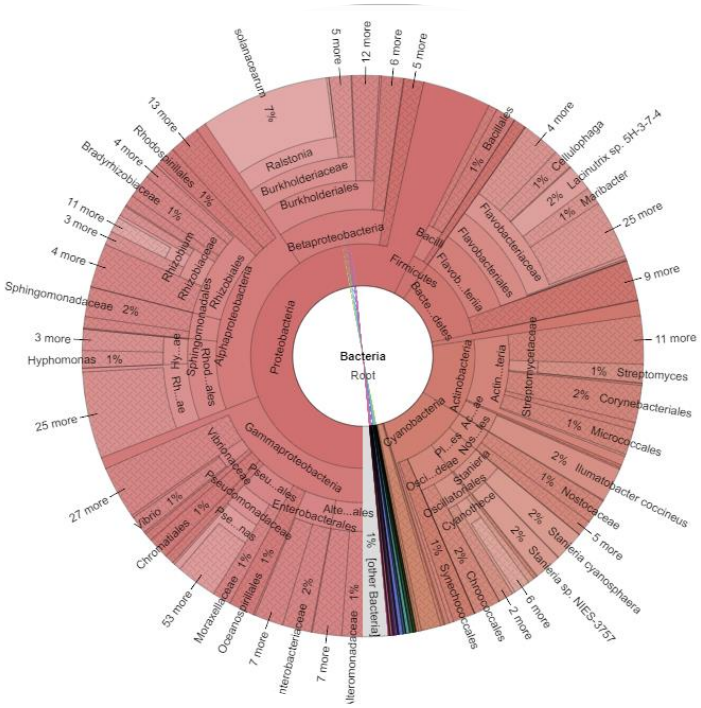

Figure S12

Breakdown of the bacteria isolated from sample twelve (*Furcellaria lumbricalis)* samples ranging from phylum to species.

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