Marine macroalgae and their associated microbiomes as a source of antimicrobial chemical diversity

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Short Running Title: Antimicrobials from macroalgae and their microbiomes

Abstract

Antimicrobial resistance (AMR) represents one of the major health threats faced by humanity over the next few years. To prevent a global epidemic of antimicrobial-resistant infections, the discovery of new antimicrobials and antibiotics, better anti-infection strategies and diagnostics, and changes to our current use of antibiotics have all become of paramount importance. Numerous studies investigating the bioactivities of seaweed extracts as well as their secondary and primary metabolites highlight the vast biochemical diversity of seaweeds, with new modes of action making them ideal sources for the discovery of novel antimicrobial bioactive compounds of pharmaceutical interest. In recent years, researchers have focused on characterizing the endophytic and epiphytic microbiomes of various algal species in an attempt to elucidate host-microbe interactions as well as to understand the function of microbial communities. Although environmental and host-associated factors crucially shape microbial composition, microbial mutualistic and obligate symbionts are often found to play a fundamental role in regulating many aspects of host fitness involving ecophysiology and metabolism. In particular, algal "core" epiphytic bacterial communities play an important role in the protection of surfaces from biofouling, pathogens and grazers through the production of bioactive metabolites. Together, marine macroalgae and their associated microbiomes represent unique biological systems offering great potential for the isolation and identification of novel compounds and strategies to contrast the rise and dissemination of AMR.

Key words: Algae, antimicrobials, antimicrobial resistance, bacteria, biofilms, epiphytes, marine, microbiome, pathogens, resistance, seaweeds

Introduction

The emergence of antimicrobial resistance (AMR) in bacteria is an ancient natural process (D'Costa *et al.,* 2011) resulting from the perpetual selection of new traits evolving as a result of mutation (Livermore, 2002), gradual increases in tolerance to sub-lethal concentrations of biocides (Scenihr, 2009) and horizontal gene transfer through transformation, transduction, recombination and conjugation events (Furuya & Lowy, 2006). Despite the undeniable contribution of antibiotic use to the development of a much healthier modern society, the release of large quantities of antibiotics into the environment as a result of their manufacture at an industrial global scale for use in the clinical setting and for agriculture and animal care has increased the selective pressure on bacterial human pathogens (Busetti *et al.,* 2014). As a result, in the clinical setting the link between antibiotic use and the generation and dissemination of resistant and multi-resistant strains is well established (Hawkey, 2008; Wellington *et al.,* 2013). The world faces an emerging epidemic of antibiotic-resistant infections, the second-leading cause of premature death worldwide (Spellberg *et al.,* 2008). Without effective solutions to confront AMR, by 2050, 10 million lives a year and more than 100 trillion USD of economic output world-wide could be at risk due to the rise of drug-resistant infections (O'Neill, 2014). This article reviews the role of microbial biofilms in infection and the acquisition of resistance, examines the processes involved in the development and maintenance of microbial biofilms with a particular focus on the role of quorum sensing, discusses antimicrobial bioactives obtained from marine organisms, and reviews the current state of knowledge of marine macroalgae and their associated microbiomes as a source of antimicrobial chemical diversity

Biofilms

Biofilms, consortia of surface-attached microbial cells immersed in a self-secreted extracellular polymeric matrix (Costerton *et al*., 1978; Donlan, 2002), constitute the principal form of microbial growth in almost all natural and pathogenic environments and a widespread survival strategy amongst microorganisms (O'Toole, 2011; Nett *et al.,* 2012). The National Institutes of Health (NIH) estimates that up to 80% of all human infections implicate microbial biofilms (Davies, 2003; Wu *et al.,* 2015). In fact, biofilm aetiology has been described as the root cause of a majority of chronic and recurrent human infections and in almost all device-associated infections (Wu *et al.,* 2015; Justin & Melander, 2009; Harrison *et al.,* 2010; Hoiby *et al.,* 2011). Microbial biofilms favour both spontaneous mutation and vertical evolution of resistance genes (Savage *et al.,* 2013) as well as the intra- and inter-specific transmission and exchange of genetic components like plasmids harbouring resistance genes through horizontal gene transfer mechanisms (such as transformation,

conjugation and transduction) and the consequent dissemination of resistance genes (Appelbaum, 2007). For example, within biofilms, bacteria have been shown to use transposable elements to acquire resistance and develop multi-resistance (Ready *et al.,* 2002). The emergence of multiple-antibiotic-resistant strains amongst pathogens normally present in the hospital environment is of particular concern and over a decade ago hospital-acquired infections were estimated to be responsible for an additional annual health care cost of £986 million in England and Wales alone (Plowman, 2000, Plowman *et al.,* 2001). In light of the role of bacterial biofilms in infection and dissemination of AMR, the isolation and characterization of novel antibiofilm bioactives as well as the identification of novel therapeutic approaches is crucial.

Within biofilms, both Gram positive and Gram negative bacteria use quorum sensing (QS), a type of cell-to-cell communication based on the release and detection of small signalling compounds, to coordinate multicellular behaviour and control a wide variety of physiological activities (Papenfort & Bassler, 2016). Some bacterial species use QS to coordinate the transcription and translation of unrelated genetic loci. For instance, the opportunistic human pathogen *Pseudomonas aeruginosa* uses two hierarchically organized LuxI/LuxR type homologue pairs generally used by some Gram negative bacteria to produce and respond to acyl homoserine lactones, LasI/LasR and Rh1I/Rh1R, to control 170-400 genes via a complex network (Hentzer *et al.,* 2002; Schuster *et al.,* 2003; Wagner *et al.,* 2003; Parsek & Greenberg, 2005). The synchronized synthesis and release of gene products with substantially different functions suggests that QS is an adaptative response evolved to cope with conditions of high population density, for instance those found in associations with plant and metazoan hosts (Swift *et al.,* 2001).

Biofilms in the marine environment

In marine environments all unprotected submerged surfaces are rapidly colonized by a succession of marine organisms in a process known as biofouling (Callow & Callow, 2002). Biofouling begins with the adsorption of dissolved organic matter by newly available surfaces and these “conditioned” surfaces are then rapidly colonized by prokaryotes and unicellular eukaryotes to form microbial biofilms (microfouling). In the marine environment, this stage is followed by macrofouling, the recruitment of invertebrate larvae and algal spores (Callow & Callow, 2002). Marine biofilms typically grow as diverse multi-species communities (Mueller *et al.,* 2006). In the photic zone they are usually dominated by phototrophic microalgal consortia (Rao *et al.,* 1997) and represent a crucial carbon source for other trophic levels, affecting mass transfer processes at the ecosystem level.

Marine microbial biofilms play a crucial role in regulating the colonisation of surfaces by marine microorganisms, invertebrates and algae and in some cases might be responsible for inducing cellular metamorphosis in some larval types (Dobretsov *et al*., 2006). For example, tetrabromopyrrole, a compound produced by a *Pseudoalteromonas* bacterium, causes larval metamorphosis of the coral *Acropora millepora* (Tebben *et al.,* 2011). The complexity of the modulations of these phenomena is paralleled by the extreme diversity in the distribution and composition of biological and chemical species found in marine microbial biofilms. Experiments using monospecific biofilms (Dobretsov *et al*., 2006; Wieczorek & Todd, 1998; Qian *et al.,* 2007) have shown an influence on the activity of the marine flora ascribable to the synthesis and release of antimicrobial compounds and a range of stimulatory signalling molecules that mostly remain to be isolated and characterized (Bowman, 2007).

Bioactive compounds

A "bioactive compound" can be defined as a secondary metabolite which at low concentrations exerts either beneficial or harmful effects on living organisms and is therefore of interest for potential industrial or medical applications (Rangel-Huerta *et al.*, 2015) .Of the more than 1 million natural products that have been discovered from both terrestrial and marine living organisms 20-25% have shown antimicrobial, antifungal, anti-protozoan, anti-nematode, anticancer, antiviral or anti-inflammatory properties (Bérdy, 2005; Penesyan *et al*., 2010 ; Newman & Cragg, 2106). The diversity of natural compounds can be ascribed to the process of natural selection that has driven the evolution of molecules best suited to perform their biological activities (Koehn & Carter, 2005).

Natural products, chemicals produced by living organisms, are a traditional source of pharmacologically active compounds (Molinski *et al.,* 2009), and continue to be a major inspiration for the majority of US Food and Drug Administration (FDA)-approved agents and for drug discovery and design. In fact, more than 60% of small molecule agents approved for use as drugs can be traced back to natural products such as aspirin (willow/birch), morphine (poppy), penicillin (fungus), Lovastatin (fungus), Adriamycin/dauxorubicin (bacterium) and Taxol™ (yew tree).

Although the first indication of the presence in seawater of bacteria with an inhibitory effect against human pathogens such as *Vibrio cholerae* and *Bacillus anthracis* has been attributed to De Giaxa (1889; see Balcazar *et al*., 2007), the "modern" study of bioactives of marine origin emerged more than 70 years ago with the pioneering work of the Italian microbiologist Giuseppe Brotzu (Professor of Hygiene at the University of Cagliari, Italy). In 1945 Brotzu grew cultures from seawater samples collected near a sewage outlet in Sardinia

(Mediterranean Sea) and tested isolates for antibiotic activity. Strong inhibitory activity by the fungus *Cephalosporium acremonium* against a broad range of pathogens led to the discovery of the cephalosporin family of antibiotics (Bo, 2000). Rosenfield & Zobell (1947) carried out the first large-scale systematic study on the antibiotic activity of marine organisms against *B. anthracis*. Spongothymidine and spongouridine extracted and identified from the Caribbean sponge *Tethya crypta* (Bergmann & Feeney, 1950, 1951) were natural nucleoside analogues, structurally similar to the nucleosides of nucleic acids, but containing arabinose rather than the typical ribose. More importantly, these marine-derived compounds displayed unexpected antiviral activities and became the basis for the synthesis of several antiviral and anticancer drugs including AZT (zidovudine; Fowler *et al.,* 2016), commercially known as Retrovir® (GlaxoSmithKline), the first drug for the treatment of HIV, and Acyclovir (sold as Zovirax®; Han *et al.,* 2017), used to treat infections caused by the herpes simplex virus. Vidarabine®, also known as Ara-A, is a synthetic purine nucleoside analogue derived from the marine bacterium *Streptomyces antibioticus* isolated from *T. crypta* sponges (Agrawal *et al*, 2016), used typically as an opthalmic ointment for the treatment of acute herpes keratoconjunctivitis (Akkaya & Ozkurt, 2016) and recurrent superficial keratitis caused by HSV-1 and HSV-2.

Today marine ecosystems still largely constitute an untapped resource for pharmaceutical and biotechnological biodiscovery. In the marine environment, whereas submerged non-living surfaces rapidly become macrofouled, the living surfaces of organisms are comparatively free from macrofouling and are covered with a thin film of epibiotic bacteria (Armstrong *et al.,* 2001). This is in part ascribable to metabolites effective as antifouling compounds and to the surface characteristics of marine organisms. Marine macroalgae (seaweeds) are known to utilize a plethora of secondary metabolites to defend themselves from herbivores and bacterial colonization of their exposed surfaces. For example, halogenated furanones produced by the red alga *Delisea pulchra* display antibiofilm effects against *Bacillus subtilis* (Ren *et al*., 2002), *Escherichia coli* (Ren *et al*., 2001) and *Pseudomonas aeruginosa* (Hentzer *et al.,* 2002).

Microbes growing on the surface of a host can also contribute to the host's overall antifouling strategy. For example, epibiotic bacteria that colonize the surface of some crustacean larvae synthesize simple antimicrobial molecules that can defend the larvae from fungal infections (Gil-Turnes *et al*., 1989). Bacteria isolated from the surface of a tunicate and grown as biofilms hindered the attachment of barnacle and tunicate larvae (Holmstrom *et al*., 1992). Moreover, the presence of epiphytic bacteria on the surface of seaweeds has been shown to be important for proper development, with atypical morphology observed in axenic culture (e.g. Marshall *et al.,* 2006; Wichard *et al*., 2015), suggesting that seaweeds and their

epiphytic microbiome collaborate as a unified functional entity or holobiont (reviewed by Egan *et al*., 2012).

Quorum sensing inhibition as a novel strategy to attenuate bacterial virulence

An emerging approach designed to attenuate bacterial virulence (i.e. the ability to cause damage to living organisms via the production of virulence factors such as enzymes and toxins) and limit the emergence of pathogenic traits relies on interfering with cell-to-cell communication, processes now commonly termed "quorum quenching" and "quorum sensing inhibition" (QSI). In fact, the inability to co-ordinate communal behaviours can prevent bacterial pathogens from escaping or overcoming host immune responses and establishing an infection (Rasmussen & Givskov, 2006; Hentzer *et al.,* 2003). Moreover, the ability to switch off virulence gene expression exogenously (Brackman *et al.,* 2011) offers a novel strategy for the treatment or prevention of infection (Camara *et al*., 2002). Overall the use of QSIs represents an "antivirulence" strategy relying on the exploitation of small compounds with the capacity of disarming pathogens thereby rendering them harmless within their host by targeting precise factors (such as toxin function and delivery, virulence gene regulation, or cell adhesion) necessary for the establishment of an infection (Mellbye & Schuster, 2011). In certain species of bacteria, disruption of QS has been shown to affect biofilm formation (Irie & Parsek, 2008) and differentiation (Hardie & Heurlier, 2008), often rendering the biofilm more susceptible to treatment with biocides and antibiotics (Brackman & Coenye, 2015). For example, acylated homoserine lactone (AHL) QS mutants of *Burkholderia cenocepacia* and *P. aeruginosa* form flatter, less structured biofilm (Diggle *et al*., 2007) and are drastically impaired in their ability to maintain cells within the biofilm (Huber *et al.,* 2001; Tomlin *et al.,* 2005; Yang *et al.,* 2009). Of relevance from a strategic therapeutic perspective, QSI-based treatments have been shown to increase the susceptibility of bacterial biofilms to antibiotics both *in vitro* and *in vivo.* For example, a significantly greater percentage of infected wax moth *Galleria mellonella* larvae and *C. elegans* survived infection by *P. aeruginosa* and *B. cenocepacia* following combined treatment with antibiotic and QS inhibitors, compared to treatment with an antibiotic alone (Brackman *et al.,* 2011).

Paradoxically, the strong selective pressure imposed by the use of antibiotics in the clinical setting makes this environment a fertile ground for the generation and spread of resistant and multiresistant strains with a consequent rise in morbidity and mortality due to hospital-acquired infections (Hawkey, 2008). Since QS is not directly involved in essential processes such as cell division, one can reason that its inhibition will not generate a severe selective pressure likely to result in the development of resistance (Rasmussen & Givskov, 2006; Sperandio, 2007; Kendall & Sperandio, 2007). In fact, the impairment of QS results in a disruption of the signalling systems responsible for the synthesis and secretion of a number

of virulence factors. Although it is reasonable to conclude that resistance to QS would be selected *in vivo* during infection, when QS is involved in colonization, systemic spread and immune evasion (Defoirdt *et al*., 2010), a broad-spectrum combinatorial approach relying on the use of conventional antibiotics in combination with QSIs as an anti-virulence approach would diminish the chance of this event considerably. In a study investigating the vertical evolution of QSI resistance as well as the fitness conferred during bacterial social interaction, Mellbye & Schuster (2011) co-cultured wild type *Pseudomonas aeruginosa* together with QS mutants (mimicking a QSI-sensitive phenotype) in minimal medium containing either bovine serum albumin (BSA) or adenosine as a sole carbon source. Whereas BSA degradation requires extracellular proteases thus providing a social benefit, adenosine is metabolized intracellularly providing a benefit for the individual. QSI-sensitive mimics were found to retard the growth of wild-type QSI-resistant mimics when grown in BSA (public nutrient acquisition) indicating QSI resistance is unlikely to spread, especially during infection (Mellbye & Schuster, 2011).

QSI targets

Marine organisms have proven to be a rich source of natural compounds exhibiting quorum sensing inhibitory activity (Dobretsov *et al*., 2009, 2011; Saurav *et al*., 2017). In a study examining the inhibition of marine biofouling by QSI, of 78 bioactives tested from compound libraries derived from marine organisms including sponges, seaweeds, fungi, bacteria, tunicates and cyanobacteria,, more than half of them displayed QSI activity (Dobretsov *et al.,* 2011). In particular, the compounds hymenialdisin, demethoxy encecalin, microcolins A and B and kojic acid were found to inhibit the QS responses of the LuxR based reporter strains induced by N-3-oxo-hexanoyl-L-homoserine lactone at micromolar concentrations.

The three components of the Gram negative AHL system are (1) the signal molecule generator, (2) the signal molecule itself and (3) the signal molecule receptor, representing the key targets of QSI for an anti-pathogenic drug approach (Rasmussen & Givskov, 2006).

(1) In AHL-based Gram negative QS, an inactivation of the LuxI-type synthase would interrupt the synthesis of the relative AHL signal meaning that a significant threshold concentration could not be reached, with failure to activate the downstream genes responsible for virulence. *In vitro,* a few substrate analogues have been found to actively block the production of AHL. For example, analogues of *S*-adenosyl-*L*-methionine (SAM) have proven to be potent inhibitors of AHL synthase in *P. aeruginosa* (Rasmussen & Givskov, 2006). This has yet to be tested *in vivo* and remains the least investigated method of interfering with QS.

(2) The signalling molecule itself constitutes another target to inhibit QS. The three principal strategies to de-activate a signalling molecule are metabolic, chemical and enzymatic degradation or inactivation. An alkaline pH causes the homoserine lactone ring

(Fig. 1) to open (Yates *et al.,* 2002). For example, when a plant recognizes colonization by the pathogen *Erwinia carotovora,* which uses AHL-based QS to regulate the synthesis of virulence factors, the plant actively causes alkalinization at the site of attack resulting in lactonolysis. In addition to pH, several other factors including temperature and the length of the acyl side chain influence the opening of the lactone ring. An increase in temperature will accelerate the rate at which the ring opens, whereas the longer the side chain the slower will be the lactonolysis.

AHL lactonases are enzymes that catalyse the ring opening reaction of the lactone ring (Rasmussen & Givskov, 2006). Several *Bacillus* species are known to produce the lactonase enzyme AiiA (Dong *et al.,* 2000), which is specific for the degradation of AHLs. Homologues of AiiA have also been found in other members of the *Bacillus genus* as well as members of the genera *Pseudomonas, Arthrobacter* and *Klebsiella* (Rasmussen & Givskov, 2006). This form of inactivation is reversible when the pH is acidic. Moreover, when the AiiA gene was heterologously expressed in *P. aeruginosa* PAO1 a significant inhibition of virulence gene production and swarming motility was achieved (Reimmann *et al.,* 2002). Similarly, when cloned and expressed in *Burkholderia* species the AiiA gene coding for the lactonase enzyme significantly reduced virulence in this pathogen (Ulrich 2004; Wopperer *et al.,* 2006). AHL acylases are another class of enzymes that can deactivate the Gram negative signalling molecule by cleaving the *N*-acyl bond of AHLs. Production of acylases has been reported in numerous genera of bacteria including *Ralstonia*, pseudomonads, and a *Streptomyces* (Lin *et al.,* 2003). Bacteria such as *Variovorax paradoxus* and *P. aeruginosa* produce amino acylases responsible for the cleavage of the peptide bond of the signal molecule (Rasmussen & Givskov, 2006) and can use the products of this metabolism as their sole source of energy. It has been hypothesized that *P. aeruginosa* creates its own AHL-acylases to regulate its own QS system, possibly to evade detection during initial infection of a host (Sio *et al.,* 2006).

(3) In AHL-based QS, the LuxR transcription factor responsible for the regulation of downstream QS-dependent pathways represents another valid target for QSI. The use of small AHL analogues to prevent LuxR activation has proven a successful strategy to target LuxR type transcription factors (Suga & Smith, 2003). These analogues can displace the original AHL and cause activation of the LuxR-type protein, acting as competitive agonists (Schaefer *et al.,* 1996). Synthetic analogues are developed in one of three ways: substitution in the acyl side chain leaving the ring unchanged; substitution and alteration to the lactone ring while the side chain remains unchanged; or extensive modification to both the side chain and lactone ring (Rasmussen & Givskov, 2006).

Algal compounds – promising leads for the treatment of biofilm-related infections

Macroalgal bioactives such as sulphated polysaccharides and kahalalides have long been recognized for medical applications (Smit, 2004) and interest in them remains high (e.g. Barbosa *et al*., 2014). However, to date, only a few lead compounds and their synthetic derivatives have progressed to animal trials (e.g. Wu *et al.,* 2004).

Seaweeds rely on the coating/secretion of secondary metabolites (toxins and broad spectrum antimicrobials and antivirals) for protection against micro- and macro-colonizing organisms (Hentzer *et al.,* 2003). For example, several halogenated furanone compounds isolated from the red seaweed *Delisea pulchra* (Givskov *et al.,* 1996) are released at its surface at concentrations capable of inhibiting both prokaryotic and eukaryotic colonization (Steinberg *et al*., 2002). These compounds were shown to be QSI-active against a broad range of bacteria (Hentzer *et al.,* 2002; Givskov *et al.,* 1996). The furanones produced by *Delisea* accelerate the turnover of the LuxR transcription factor inhibiting QS-dependent gene expression in Gram negative bacteria (Manefield *et al.,* 2002) and the capacity to synthesize such compounds is likely to have evolved as an antifouling strategy to preserve the surface of algal fronds from colonization by Gram negative marine bacteria. However, as they are brominated, their application in humans is limited, making it necessary to search for QSI from other natural sources (Zhu & Sun, 2008). Overall, macroalgae have yielded more than 3,000 natural products, accounting for approximately 20% of marine natural compounds (Amsler, 2008).

Red seaweeds (Rhodophyta)

Research on red seaweeds has discovered the majority of macroalgal secondary metabolites accounting for more than 1500 bioactives (Maschek & Baker, 2008)). With the exception of phlorotannins, which are unique to brown algae, red seaweeds synthesize all major classes of algal natural products (Blunt *et al.,* 2016). Red algae primarily synthesize isoprenoid and acetogenin derivatives, as well as amino acid, shikimate and nucleic acid derivatives (Amsler, 2008). Halogenated compounds underpin red algal chemistry, with over 90% of compounds reported to contain bromine or chlorine.

The genus *Laurencia* (Rhodomelaceae, Ceramiales) has been the subject of nearly 50% of the publications on red algal chemistry, producing a plethora of halogenated sesquiterpenes and C15 acetogenins, as well as higher terpenes (Davis & Vasanthi, 2011). *Laurencia* species occur widely on temperate and tropical coasts and are recognized as a rich source of novel secondary metabolites (Cabrita *et al*., 2010). Several of them display promising antimicrobial activity against a range of bacteria. For example, an unidentified species of *Laurencia* from Malaysia exerted potent antimicrobial activity against a range of marine bacteria; two halogenated C15 acetogenin compounds, elatol and iso-obtusol, were isolated from this alga and structurally elucidated based on spectroscopic data, confirming the potential of these

compounds as a source of pharmaceutically relevant bioactives (Vairappan *et al.,* 2001). In extracts from *L. majuscula*, elatol inhibited six bacterial species, with significant antimicrobial activities against *Staphylococcus epidermis*, *Klebsiella pneumonia* and *Salmonella sp.*. Iso-obtusol, a polyhalogenated sesquiterpene produced by *Laurencia obtusa,* was found to display antimicrobial activity against several bacteria, and proved particularly active against *K. pneumonia* and *Salmonella* sp. (Vairappan, 2003). Interestingly, the antimicrobial activity of elatol and iso-obtusol was found to be equal or better than conventional antibiotics against *K. pneumonia* and *Salmonella* sp. through a bacteriostatic mode of action (Vairappan, 2003). Subsequently, Vairappan *et al.* (2010) discovered a novel brominated diterpene, 10-acetoxyangasiol, as well as four previously known metabolites, aplysidiol, cupalaurenol, 1-methyl-2,3,5-tribromoindole, and chamigrane epoxide in *Laurencia* sp. These compounds displayed strong antimicrobial activity against clinically relevant bacteria including *Staphylococcus aureus*, *Streptococcus pyogenes, Salmonella sp.* and *Vibrio cholera*.

Members of the order Bonnemaisoniales also produce a diverse array of secondary halogenated metabolites displaying antimicrobial activity (Nash *et al*., 2005). *Delisea*, *Asparagopsis*, *Bonnemaisonia* and *Ptilonia* all synthesize a group of linear halogenated ketones and branched lactones. Amongst these, the fimbrolides, a group of halogenated furanones (Fig. 2) from *Delisea pulchra* from southeastern Australia, show QSI activity against a range of bacteria, functioning as an intracellular signal antagonist as well as accelerating LuxR turnover (Rasmussen *et al.,* 2000; Manefield *et al.,* 2002), and hence providing an antifouling defence (Kjelleberg & Steinberg, 2001). From a screen of 39 macroalgae, *Asparagopsis taxiformis* extracts were shown to inhibit QS in *C. violaceum* CV026 bioreporter assays (Jha *et al*., 2013). Based on Ion Cyclotron Resonance Fourier Transformation Mass Spectrometry analysis of the QSI-active fraction, the authors proposed that the compound responsible for the QSI activity was 2-dodecanoyloxyethanesulfonate **(**Fig. 6; Jha *et al*., 2013).

Bonnemaisonia hamifera (Figs 7, 8) is native to Japan, was introduced into the North Atlantic Ocean prior to 1890 (Maggs & Stegenga, 1998) and is now widely distributed there. *B. hamifera* has a heteromorphic life cycle, alternating between a diploid filamentous "*Trailliella"* tetrasporophyte and a haploid gametophyte (Breeman *et al*., 1988). Like *Delisea pulchra, B. hamifera* produces an assortment of mono- and poly-halogenated bioactives including 2-heptanones, 2-heptanols, acetates and acids, some of which display antimicrobial activity (Siuda *et al.,* 1975; Jacobsen & Madsen, 1978; McConnell & Fenical, 1979, Nylund *et al*., 2013; Enge *et al*., 2013).

One of the main secondary metabolites, 1,1,3,3-tetrabromo-2-heptanone (Fig. 11), stored in specialized gland cells in the *Trailliella* phase, has an ecologically relevant role as an

antifouling agent against bacterial surface colonization. Natural surface concentrations (3.6 μg cm^{-2}) of 1,1,3,3-tetrabromo-2-heptanone applied to artificial panels significantly reduced the number of settled bacteria (Nylund *et al.,* 2008). Moreover, organic extracts of *B. hamifera* show broad-spectrum antimicrobial activity at ecologically relevant concentrations (Nylund *et al.,* 2005, 2008, 2013) confirming the potential of this species as a novel source of marine-derived antibiofilm compounds active against human pathogens. The compound also acts as a chemical grazing deterrent (Enge *et al*., 2013), which is metabolically expensive to produce but protects the seaweed against bacteria as well as grazers (Nylund *et al*., 2013).

It is interesting to note that several of these members of the Bonnemaisoniales found in Europe and containing halogenated compounds such as bromophenols (Paul *et al*., 2006) are aliens. These compounds undoubtedly contribute to their invasive potential by deterring grazing and allowing the establishment of high biomass (Enge *et al*., 2013). This is a clear indication that alien species are worth targeting in the search for new bioactives. QSI compounds have also been described from a few non-invasive red algae, such as *Ahnfeltiopsis flabelliformis* (Gigartinales) from Korea which has been shown to produce three AHL inhibitory compounds, floridoside (Fig. 3), betonicine (Fig. 4) and isethionic acid **(**Fig. 5) (Kim *et al*., 2007).

Brown seaweeds (Phaeophyceae)

Brown algae have also yielded a rich chemical diversity with more than 1,140 reported secondary metabolites. The most studied and representative bioactives of the brown seaweeds comprise diterpenes, phlorotannins, and small C11 acetogenins, all with very little halogenation (Blunt *et al.,* 2007). Phlorotannins are distinguishing compounds of brown algae, with a wide range of activities of pharmacological interest including antimicrobial (Eom *et al*., 2012), antiviral (Ahn *et al.,* 2004), antidiabetic (Lee & Jeon, 2013; Kang *et al.,* 2013), anti-inflammatory (Sugiura *et al.,* 2013), anti-allergic (Sugiura *et al.,* 2009), anti-cancer (Lee *et al.,* 2012), and anti-neurodegenerative diseases (Myung *et al.,* 2005, Sathya *et al.,* 2013; Jung *et al.,* 2009; Heo *et al.,* 2012) especially against Alzheimer's disease (Yoon *et al.,* 2008; Yoon *et al.,* 2009i Ahn *et al.,* 2012). The ecological role of phlorotannins in brown seaweeds appears to include defence against epiphytes (Nakajima *et al*., 2016), as well as grazing deterrence (McClintock & Baker, 2001).

Although many studies examining brown algal chemistry have focused on *Dictyota* (Dictyotaceae) and its wealth of terpenes (>250) (Munro & Blunt, 2005), several other genera display activities of pharmacological relevance. For example carotenoids from several brown algae have a wide range of bioactivities (Peng *et al.,* 2011). The meroditerpenoid methoxybifurcarenone isolated from *Cystoseira tamariscifolia* displays antifungal activity

against three plant pathogenic fungi and antibacterial activity against *Agrobacterium tumefaciens* and *E. coli* (Bennamara *et al.,* 1999).

Halidrys siliquosa (family Sargassaceae) is a large temperate macroalga growing up to 120 cm long in rock pools and sometimes as forests in the shallow subtidal zone. The bioactive potential of *H. siliquosa* was identified over four decades ago. Hornsey & Hide (1974, 1976) screened crude extracts of *H. siliquosa* against a series of opportunistic human pathogens and discovered antimicrobial activity against *Staphylococcus aureus, E. coli, Bacillus subtilis, Streptococcus pyogenes* and *Proteus.* Culioli *et al.* (2008) reported the antifouling activity of meroditerpenoids isolated from this species and identified nine tetraprenyltoluquinol-related metabolites exhibiting antifouling properties and inhibiting the growth of the marine bacteria *Cobetia marina, Marinobacterium stanieri, Vibrio fischeri, Pseudoalteromonas haloplanktis.* Non-cytotoxic concentrations of these meroditerpenoids were found to prevent the settlement of cyprids of *Balanus amphitrite*. *H. siliquosa* crude extract was active against the parasites *Trypanosoma brucei rhodesiense, T. cruzi* and *Leishmania donovani* and the bacterium *Mycobacterium tubercolosis* (Spavieri *et al.,* 2010) highlighting the potential of this alga for the treatment of mycobacterial and protozoal infections.

Busetti *et al.* (2015) reported antimicrobial and antibiofilm activity of methanolic extracts of *H. siliquosa* against clinically relevant human pathogens of the genera *Staphylococcus, Streptococcus, Enterococcus, Pseudomonas, Proteus, Stenotrophomonas*, and *Chromobacterium*. Biofilms of *S. aureus* MRSA ATCC 33593 and *S. aureus* MRSA NCTC 10442 were found to be susceptible to *H. siliquosa* extract which achieved minimum biofilm eradication concentration (MBEC) values of 1.25 mg ml^{-1} and 5 mg ml^{-1} respectively. Active extracts showed no toxicity against wax moth (*G. mellonella*) larvae across a wide range of concentrations (Busetti *et al.,* 2015). The activity of *H. siliquosa* methanolic extracts against the emerging pathogen *Stenotrophomonas maltophilia* suggests the production of bioactives with the potential to be used in a treatment strategy for cystic fibrosis as well as therapies for *Staphylococcus* biofilm-related infections. Moreover, the promising range of activities displayed by *H. siliquosa* organic extracts against clinically relevant, antibiotic-resistant, human pathogens highlight this alga as a candidate for further studies focused on the isolation of antibiofilm compounds and antimicrobials for the treatment of infections involving multi-resistant pathogenic strains.

Macroalgal microbiomes as a source of novel bioactives of pharmaceutical relevance

In recent years, several studies characterizing algal epiphytic bacterial communities (Figs 12-13) have highlighted the presence of "core microbial species" in mutualistic or obligate association with their host (Singh *et al*., 2015). In particular, several bacterial epiphytes have been reported to produce bioactive compounds that can protect macroalgal surfaces from biofouling (Dobretsov & Qian, 2002). However, whereas several concerted studies have focused on characterizing the composition of the human microbiomes as well as deciphering the physiological significance of the host-microbe interactions underlying the mutualistic relationships therein, in seaweeds the microbiomes and the significance of their functional relationship with their hosts remain largely unexplored. The advent of culture-independent, DNA-based, metagenomic and transcriptomic methods has provided powerful new tools for the characterization of host-associated microbiomes as well as for the elucidation of the many, complex, yet often fundamental processes involved in host–microbe interactions, providing future studies the tools to investigate the functional microbiome involved in the often complex life cycles of macroalgae (Singh & Reddy, 2016). The discoveries deriving from such studies could assist in promoting fitness and productivity in macroalgal species of commercial interest through the modulation of a functionally active microbiome as well as providing enormous potential for the discovery of novel antibiofilm or QSI compounds of clinical relevance.

For example, the epiphytic bacterium *Pseudoalteromonas tunicata* isolated from the surface of *Ulva lactuca* can hinder biofilm formation of competing Gram negative microbes through the synthesis of pigmented substances that inhibit LuxR-dependent transcriptional control through a similar mode of action to the furanones (McLean *et al.,* 2004). *Halobacillus salinus*, a marine Gram positive bacterium isolated from a seagrass, synthesizes and releases QSI bioactives active against Gram negative strains (Teasdale *et al.,* 2009) through competitive binding (Teasdale *et al.,* 2009). These examples indicate that QS inhibition represents a natural, widespread, antifouling strategy evolved by marine organisms making marine ecosystems an ideal source for the discovery of QS inhibitors with potentially clinically relevant antibiofilm activity.

In a recent study, an isolate belonging to the *Pseudoalteromonas* genus obtained from the algal fronds of the red seaweed *Plocamium maggsiae* displayed potent QSI activity against acyl homoserine lactone-based reporter strains (Busetti *et al*., 2014). The isolate's filter-sterilized supernatant significantly diminished biofilm biomass both during biofilm formation as well as in pre-established, mature *P. aeruginosa* PAO1 biofilms causing a 0.97-log reduction and a 2-log reduction in PAO1 biofilm viable counts in the biofilm formation and eradication assays. The crude organic extract obtained from this isolate displayed a minimum inhibitory concentration (MIC) of 2 mg ml^{-1} against PAO1 but failed to produce a minimum bactericidal concentration (MBC) confirming the lack of antimicrobial activity in

the extract at the concentrations tested. Sub-MIC concentrations of the crude organic extract were found to significantly reduce the quorum sensing (QS)-dependent production of the two virulence factors pyoverdin and pyocyanin in *P. aeruginosa* PAO1 without affecting growth. A combinatorial approach using tobramycin and the crude organic extract at 1 mg ml^{-1} against planktonic *P. aeruginosa* PAO1 increased the effectiveness of tobramycin by ten times, lowering its MIC against this pathogen from 0.75 to 0.075 mg ml^{-1} (Busetti *et al*., 2014). The results of this study confirm the efficacy of combinatorial strategies combining current antibiotic treatment with (non-antibiotic) QSI compounds derived from algal microbial epiphytes to improve the efficacy of current antibiotic treatments.

Future perspectives

The imminent global health threat of antimicrobial resistance with the realistic prospect of mankind entering a 'post-antibiotic era' has driven research into innovative therapeutic strategies relying on different targets and approaches for the treatment of microbial infections. The gradual elucidation of widespread bacterial communication (QS) systems regulated by small diffusible signal molecules as a means to coordinate group behaviours has revolutionized our classical conception of bacteria as unicellular and thus independent in nature. Targeting complex social behaviours, which include virulence and pathogenicity, regulated by chemical intra- and inter-species signal molecules which allow them to coordinate their behaviour at a community level, represents a novel target for non-antibiotic anti-infective chemotherapy.

Marine organisms are known to produce a variety of QSIs that can thwart biofilm development of competing species (McClean & Winson *et al.,* 1997; Bauer & Robinson 2002; Saurav *et al*., 2017), representing an important resource for the isolation of novel "antipathogenic" antibiofilm compounds. Bacteria from algal microbiomes remain a relatively untapped source of novel candidate compounds displaying QSI activity with the potential to attenuate biofilm formation, virulence factor production or increase the antimicrobial susceptibility of clinically important pathogenic bacteria in the constant fight against emergence of multi-resistant microorganisms (Saurav *et al*., 2017).

As in many other discovery and development programs in marine bioactives, there are a multitude of challenges associated with the biodiscovery and commercialization of macroalgal compounds as pharmaceutical agents. These include accessibility to the biodiversity, efficient screening, sustainable supply, variability in the spectrum and quantities of bioactives produced (due to factors such as seasonality and geographic distribution), elucidation of the mechanism of action, suitable pharmacokinetics/ pharmacodynamic parameters and ultimately costs associated with sustainable aquaculture and processing. Despite this, a significant body of early-stage biodiscovery research highlights marine

macroalgae as promising sources of novel antimicrobials, antibiofilm compounds, antivirals, anticancer, antimicrobial, anti-inflammatory and neuroprotective agents.

Several studies have validated approaches that combine regular antibiotic agents with non-antibiotic compounds, such as QSIs, to enhance the effectiveness of present treatments, but have not yet moved to clinical trials. Drawing inspiration from nature, future studies could focus on evaluating the combinatorial effects of algal secondary metabolites with those produced by the core members of their bacterial microbiomes in an attempt to mimic the complex natural chemical mechanisms underlying the mutualistic symbiotic relationships in their environments.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

All authors contributed to the manuscript. A. Busetti prepared the first draft; A. Busetti, C.A. Maggs and B.F. Gilmore reviewed, revised and updated the manuscript.

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Figure legends

Figs 1-6. Molecular structures of acyl homoserine lactones and quorum sensing inhibitors isolated from marine algae. **Fig. 1.** General structure of acyl homoserine lactones. **Fig. 2.** Halogenated furanones. **Fig. 3.** Floridoside. **Fig. 4.** Betonicine. **Fig. 5.** Isethionic acid. **Fig. 6.** 2-dodecanoyloxyethanesulfonate. Figure adapted from Saurav *et al*. (2017).

Figs 7-10. The red algae *Bonnemaisonia hamifera* and *Bonnemaisonia asparagoides* display strong antimicrobial activity against AHL quorum sensing bioreporter strain *Chromobacterium violaceum*. Algal samples were overlaid with *C. violaceum* in 0.5% agar prior to incubation. **Fig. 7**. *B. hamifera* washed in ddH_2O. **Fig. 8**. *B. hamifera* pre-washed in 70% ethanol; QSI activity not altered by ethanol wash. **Fig. 9**. *B. asparagoides* washed in ddH_2O. **Fig. 10**. *B. asparagoides* washed in 70% ethanol, exhibiting significant loss of QSI activity which has been extracted by the ethanol wash.

Fig. 11. Structure of 1,1,3,3-tetrabromo-2-heptanone, a poly-brominated 2-heptanone produced by *Bonnemaisonia hamifera* displaying antifouling properties.

Figs 12-13. SEM of the epiphytic microbial colonisation of *Halidrys siliquosa* algal fronds. **Fig. 12.** Diatom embedded amongst diverse prokaryotes. **Fig. 13.** Three-dimensional structure of microbial biofilm.

Marine macroalgae and their associated microbiomes as a source of antimicrobial chemical diversity

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Short Running Title: Antimicrobials from macroalgae and their microbiomes

Abstract

Antimicrobial resistance (AMR) represents one of the major health threats faced by humanity ~~in the coming decades~~ over the next few years. To prevent a global epidemic of antimicrobial-resistant infections, the discovery of new antimicrobials and antibiotics, better anti-infection strategies and diagnostics, and changes to our current use of antibiotics have all become of paramount importance. Numerous studies investigating the bioactivities of seaweed extracts as well as their secondary and primary metabolites highlight the vast biochemical ~~virtuosity~~ diversity of seaweeds, with new modes of action making them ideal sources for the discovery of novel antimicrobial bioactive compounds of pharmaceutical interest. In recent years, researchers have focused on characterizing the endophytic and epiphytic microbiomes of various algal species in an attempt to elucidate host-microbe interactions as well as to understand the function of microbial communities. Although environmental and host-associated factors crucially shape microbial composition, microbial mutualistic and obligate symbionts are often found to play a ~~crucial~~ fundamental role in regulating many aspects of host fitness involving ecophysiology and metabolism. In particular, algal "core" epiphytic bacterial communities play an important role in the protection of surfaces from biofouling, pathogens and grazers through the production of bioactive metabolites. Together, marine macroalgae and their associated microbiomes represent unique biological systems offering great potential for the isolation and identification of novel compounds and strategies to contrast the rise and dissemination of AMR.

Key words: Algae, antimicrobials, antimicrobial resistance, bacteria, biofilms, epiphytes, marine, microbiome, pathogens, resistance, seaweeds

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Introduction

The emergence of antimicrobial resistance (AMR) in bacteria is an ancient natural process (D'Costa *et al.,* 2011) resulting from the perpetual selection of new traits evolving as a result of mutation (Livermore, 2002), gradual increases in tolerance to sub-lethal concentrations of biocides (Scenihr, 2009) and horizontal gene transfer through transformation, transduction, recombination and conjugation events (Furuya & Lowy, 2006). Despite the undeniable contribution of antibiotic use to the development of a much healthier modern society, the release of large quantities of antibiotics into the environment as a result of their manufacture ~~of antibiotics~~ at an industrial global scale for use in the clinical setting and for agriculture and animal care ~~and the consequent release of large quantities of antibiotics into the environment,~~ has ~~accentuated~~~~heightened~~increased the selective pressure on bacterial human pathogens (Busetti *et al.,* 2014). As a result, in the ~~nosocomial~~ clinical ~~environment~~ setting ~~an evident clear relationship~~the ~~between~~ link between~~ing~~ antibiotic~~microbial~~ use ~~and~~and the generation~~emergence~~ and dissemination of resistant and multi-resistant strains is ~~now evident~~well established (Hawkey, 2008; Wellington *et al.,* 2013). The world faces an emerging epidemic of antibiotic-resistant infections, the second-leading cause of premature death worldwide (Spellberg *et al.,* 2008)~~, causing an estimated 700,000 deaths per year~~. ~~A 2014 review commissioned by the then British Prime Minister, David Cameron, highlighted that without~~ Without effective solutions to confront AMR, by 2050, 10 million lives a year and more than 100 trillion US$ of economic output world-wide ~~will~~ could be at risk due to the rise of drug-resistant infections (O'Neill, 2014). ~~In this article, we~~ review This article reviews the role of microbial biofilms in infection and the acquisition of resistance, examines the processes involved in the development and maintenance of microbial biofilms with a particular focus on the role of quorum sensing, discusses antimicrobial bioactives obtained from marine organisms, and reviews the current state of knowledge of marine macroalgae and their associated microbiomes as a source of antimicrobial chemical diversity.

Biofilms

Biofilms, ~~communities~~ consortia of surface ~~associated~~ attached microbial cells ~~embedded~~ immersed in a self-secreted extracellular polymeric matrix (Costerton *et al.*, 1978; Donlan, 2002), ~~represent~~ constitute the ~~predominant~~ principal ~~mode~~ form of microbial growth in almost all natural and pathogenic environments and a widespread survival strategy amongst microorganisms (O'Toole, 2011; Nett *et al.,* 2012). The National Institutes of Health (NIH) estimates that up to 80% of all human infections implicate~~involve~~ microbial biofilms (Davies, 2003; Wu *et al.,* 2015). In fact, biofilm aetiology has been described as the root cause of a

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majority of chronic and recurrent human infections and in almost all device-associated infections (Wu *et al.,* 2015; Justin & Melander, 2009; Harrison *et al.,* 2010; Hoiby *et al.,* 2011). Microbial biofilms provide constitute a favourable propitious environment ambientfavour both for spontaneous mutation and vertical evolution of resistance genes (Savage *et al.,* 2013) as well as foras the intra- and inter-specific transmission and exchange of genetic elements components like plasmids harbouring resistance genes through horizontal gene transfer mechanisms (such as transformation, conjugation and transduction) and the consequent dissemination of resistance genes (Appelbaum, 2007). For example, within biofilms, bacteria have been shown to use transposable elements to acquire resistance and develop multi-resistance (Ready *et al.,* 2002). The emergence of multiple-antibiotic-resistant strains amongst pathogens normally present in the nosocomial hospital environment is of particular concern and over a decade ago hospital-acquired infections were estimated to be responsible for an additional annual health care cost of £986 million in England and Wales alone (Plowman, 2000, Plowman *et al.,* 2001). In light of the role of bacterial biofilms in infection and dissemination of AMR, the discovery isolation and characterisation of novel antibiofilm compoundsbioactives and as well as the identification of novel therapeutic strategiesapproaches is crucial.

Within biofilms, both Gram positive and Gram negative bacteria use quorum sensing (QS), a form type of cell-to-cell communication based on the release and detection of small signalling moleculescompounds, to coordinate multicellular behaviour and regulatecontrol a wide varietydiverse array of physiological activities (Papenfort & Bassler, 2016). Some bacterial species use QS to coordinate the expression transcription and translation of unlinked unrelated genetic loci. For exampleinstance, in the opportunistic human pathogen *Pseudomonas aeruginosa* uses two hierarchically organised LuxI/LuxR type homologue pairs generally used by some Gram negative bacteria to produce and respond to acyl homoserine lactones. LasI/LasR and Rh1I/Rh1R, to, regulatecontrol 170-400 genes via a complex network (Hentzer *et al.,* 2002; Schuster *et al.,* 2003; Wagner *et al.,* 2003; Parsek & Greenberg, 2005). The coordinated synchronized production synthesis and release of multiple proteinsgene products of diversewith substantially different functions suggests that QS is an adaptational adaptative responsed evolved to cope with conditions of high population density, such as for instance those encounteredfound in associations with plant and animal metazoan hosts (Swift *et al.,* 2001).

Biofilms in the marine environment

In marine aquatic environments all unprotected submerged surfaces are rapidly colonized by a succession of marine organisms in a process known as biofouling (Callow & Callow, 2002). Biofouling begins with the adsorption of dissolved organic matter by newly available surfaces

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and these "conditioned" surfaces are then rapidly colonized by prokaryotes and unicellular eukaryotes to form microbial biofilms (microfouling). In the marine environment, this stage is followed by macrofouling, the recruitment of invertebrate larvae and algal spores (Callow & Callow, 2002). Marine biofilms typically grow as diverse multi-species communities (Mueller *et al.*, 2006). In the photic zone they are usually dominated by phototrophic microalgal consortia (Rao *et al.*, 1997) and represent a crucial carbon source for other trophic levels, affecting mass transfer processes at the ecosystem level.

Marine microbial biofilms play a crucial role in regulating the colonisation of surfaces by marine microorganisms, invertebrates and algae and in some cases might be responsible for inducing cellular metamorphosis in some larval types ~~Marine microbial biofilms play a crucial role in regulating the settlement of a variety of marine microorganisms, invertebrates and algae and may promote cellular metamorphosis~~ (Dobretsov *et al*., 2006). For example, tetrabromopyrrole, a compound produced by a *Pseudoalteromonas* bacterium, causes larval metamorphosis of the coral *Acropora millepora* (Tebben *et al.*, 2011). The complexity of the modulations of these phenomena is paralleled by the extreme diversity in the distribution and composition of biological and chemical species found in marine microbial biofilms. Experiments using monospecific biofilms (Dobretsov *et al*., 2006; Wieczorek & Todd, 1998; Qian *et al.*, 2007) have shown an influence on the activity of the marine flora ascribable to the ~~production~~ synthesis and release of antimicrobial~~biotic~~ compounds and ~~as well as~~ a range of stimulatory ~~chemical cues~~ signalling molecules that mostly remain ~~so far mostly~~ ~~in great part~~ to be isolated and ~~un~~characterized (Bowman, 2007).

Bioactive compounds

A "bioactive compound" can be defined ~~is~~ as a secondary metabolite which at low concentrations ~~is either beneficial or harmful to~~exerts either beneficial or harmful effects on living organisms and is therefore of interest for potential industrial or medical applications (Rangel-Huerta *et al.*, 2015~~needs reference~~) . ~~More~~ Bérdy (~~2005~~) ~~reported that m~~Of the more than 1 million natural products ~~have~~ that have been discovered from both terrestrial and marine living organisms ~~(Table 1; Bérdy, 2005)~~, of which 20-25% ~~have~~ have shown antimicrobial, antifungal, anti-protozoan, anti-nematode, anticancer, antiviral or anti-inflammatory properties (Bérdy, 2005; Penesyan *et al.*, 2010 ; Newman ~~and~~& Cragg, 2106). The diversity of natural compounds can be ascribed to the process of natural selection that has driven the evolution of molecules best suited to perform their biological activities (Koehn & Carter, 2005).

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Natural products, chemicals produced by living organisms, are a traditional source of pharmacologically active compounds (Molinski *et al.,* 2009), and continue to be a major ~~source of~~ inspiration for the majority of US ~~FDA~~Food and Drug Administration (FDA)-approved agents and for drug discovery and design. In fact, more than 60% of small molecule agents approved for use as drugs can be traced back to natural products such as aspirin (willow/birch), morphine (poppy), penicillin (fungus), Lovastatin (fungus), Adriamycin/dauxorubicin (bacterium) and Taxol™ (yew tree).

Although the first indication of the presence in seawater of bacteria with an inhibitory effect against human pathogens such as *Vibrio cholerae* and *Bacillus anthracis* has been attributed to De Giaxa (1889; see Balcazar *et al.*, 2007), the "modern" study of bioactives of marine origin emerged more than 70 years ago with the pioneering work of the Italian microbiologist Giuseppe Brotzu (Professor of Hygiene at the University of Cagliari, Italy). In 1945 Brotzu grew cultures from seawater samples collected near a sewage outlet in Sardinia (Mediterranean Sea) and tested isolates for antibiotic activity. Strong inhibitory activity by the fungus *Cephalosporium acremonium* against a broad range of pathogens led to the discovery of the cephalosporin family of antibiotics (Bo, 2000). Rosenfield & Zobell (1947) carried out the first large-scale systematic study on the antibiotic activity of marine organisms against *B. anthracis*. Spongothymidine and spongouridine extracted and identified from the Caribbean sponge *Tethya crypta* (Bergmann & Feeney, 1950, 1951) were natural nucleoside analogues, structurally similar to the nucleosides of nucleic acids, but containing arabinose rather than the typical ribose. More importantly, these marine-derived compounds displayed unexpected antiviral activities and became the basis for the synthesis of several antiviral and anticancer drugs including AZT (zidovudine; Fowler *et al.,* 2016), commercially known as Retrovir® (GlaxoSmithKline), the first drug for the treatment of HIV, and Acyclovir (sold as Zovirax®; Han *et al.,* 2017), used to treat infections caused by the herpes simplex virus. Vidarabine®, also known as Ara-A, is a synthetic purine nucleoside analogue derived from the marine bacterium *Streptomyces antibioticus* isolated from *T. crypta* sponges (Agrawal *et al*, 2016), used typically as an opthalmic ointment for the treatment of acute herpes keratoconjunctivitis (Akkaya & Ozkurt, 2016) and recurrent superficial keratitis caused by HSV-1 and HSV-2.

Today marine ecosystems still largely constitute an untapped resource for pharmaceutical and biotechnological biodiscovery. In the marine environment, whereas submerged non-living surfaces rapidly become macrofouled, the living surfaces of ~~the majority of marine~~ organisms ~~remain~~ are ~~relatively~~comparatively free from macrofouling and are covered with a thin film of epibiotic bacteria (Armstrong *et al.,* 2001). This is in part ascribable to metabolites effective as antifouling compounds and to the surface characteristics of marine organisms. Marine macroalgae (seaweeds) are known to utilize a plethora of

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secondary metabolites to defend themselves from herbivores and bacterial colonization of their exposed surfaces. For example, halogenated furanones produced by the red alga *Delisea pulchra* display antibiofilm effects against *Bacillus subtilis* (Ren *et al*., 2002), *Escherichia coli* (Ren *et al*., 2001) and *Pseudomonas aeruginosa* (Hentzer *et al.*, 2002).

Microbes growing on the surface of a host can also contribute to the host's overall antifouling strategy. For example, epibiotic bacteria ~~found on~~that colonis~~z~~e the surface of ~~larvae of~~ some crustacean larvae~~s~~ ~~produce~~ synthes~~i~~ize simple ~~antibacterial~~ antimicrobial ~~compounds~~ molecules that can ~~protect~~defend the larvae from fungal infection~~s~~ (Gil-Turnes *et al*., 1989). Bacteria isolated from the surface of a tunicate and grown as biofilms ~~prevented~~ hindered the ~~settlement~~ attachment of barnacle and tunicate larvae (Holmstrom *et al*., 1992). Moreover, the presence of epiphytic bacteria on the surface of seaweeds has been shown to be important for proper development, with atypical morphology observed in axenic culture (e.g. Marshall *et al.*, 2006; Wichard *et al*., 2015), suggesting that ~~macroalgae~~ seaweeds and their epiphytic ~~bacteria~~ microbiome ~~interact~~collaborate as a unified functional entity or holobiont (reviewed by Egan *et al*., 2012).

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Quorum sensing inhibition as a novel strategy to attenuate bacterial virulence

An emerging approach designed to attenuate bacterial virulence (i.e. the ability to cause damage to living organisms via the production of virulence factors such as enzymes and toxins) and limit the emergence of pathogenic traits relies on interfering with cell-to-cell communication, processes now commonly termed "quorum quenching" and "quorum sensing inhibition" (QSI). ~~.~~ In fact, the inability to co-ordinate communal behaviours can prevent bacterial pathogens from escaping or overcoming host immune responses ~~so that bacteria fail to adapt to the host environment and do not~~and establish~~ing~~ an infection (Rasmussen & Givskov, 2006; Hentzer *et al.*, 2003). Moreover, the ability to switch off virulence gene expression exogenously (Brackman *et al*., 2011) offers a novel strategy for the treatment or prevention of infection (Camara *et al*., 2002). Overall the use of QSIs ~~can be considered~~represents an "antivirulence" ~~approach~~ strategy ~~based~~ relying on the ~~use~~ exploitation of small ~~molecules~~ compounds with the capacity~~capable~~ of disarming pathogens thereby rendering them harmless within their host by targeting ~~specific~~precise factors (such as toxin function and delivery, virulence gene regulation, or cell adhesion) necessary for the ~~successful~~efficacious establishment of an infection~~, such as toxin function, toxin delivery, virulence gene regulation, or cell adhesion~~ (Mellbye & Schuster, 2011). In certain species of bacteria, disruption of QS has been shown to affect biofilm formation (Irie & Parsek, 2008) and differentiation (Hardie & Heurlier, 2008), often rendering the biofilm more susceptible to treatment with biocides and antibiotics (Brackman & Coenye, 2015). For example, acylated homoserine lactone (AHL) QS mutants of *Burkholderia cenocepacia* and *P. aeruginosa* form

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flatter, less structured biofilm (Diggle *et al*., 2007) and are drastically impaired in their ability to maintain cells within the biofilm (Huber *et al.,* 2001; Tomlin *et al.,* 2005; Yang *et al.,* 2009). Of relevance from a strategic therapeutic perspective, QSI-based treatments have been shown to increase the susceptibility of bacterial biofilms to antibiotics both *in vitro* and *in vivo*. For example, a significantly higher greater percentage of infected wax moth *Galleria mellonella* larvae and *C. elegans* survived infection by *P. aeruginosa* and *B. cenocepacia* following combined treatment with antibiotic and QS inhibitors, compared to treatment with an antibiotic alone (Brackman *et al.,* 2011).

Paradoxically, tThe strong selective pressure imposed by the use of antibiotics antimicrobial use is particularly evident in the nosocomial clinicalnosocomialclinical environment setting makes this environment a fertile ground forwhere clear relationships between antimicrobial use and the emergence generation and spread of resistant and multiresistant strains can be seen with a consequent rise in morbidity and mortality due to hospital-acquired infections (Hawkey, 2008). Since QS is not directly involved in essential processes, such as growth of the bacteriacell division, one can reason that its inhibition will not generate a harshsevere selective pressure apt likely to produce result in the development of resistance (Rasmussen & Givskov, 2006; Sperandio, 2007; Kendall & Sperandio, 2007). In fact, the impairment of it has been shown that by inhibiting QS results in a disruption of the signalling systems controlling the production and releaseresponsible for the synthesis and secretion of a number of virulence factors is achieved. Although it is reasonable to conclude that resistance to QS It has been hypothesized that the emergence of QS resistance would be selected *in vivo* during infection, when QS is involved inever QS promotes colonization, systemic spread, andor immune evasion (Defoirdt *et al*., 2010). , although a broad-spectrum combinatorial approach relying on the use of conventional antibiotics in combination with QSIs as an anti-virulence approach would diminish the chance of this event considerably.Such antimicrobial/anti-virulence approach lessens the likelihood of this considerably. In a study investigating the vertical evolution of QSI resistance as well as the fitness conferred during bacterial social interaction, Mellbye and Schuster (Mellbye & Schuster (, 2011) co-cultured wild type *Pseudomonas aeruginosa* together with QS mutants (mimicking a QSI-sensitive phenotype) in minimal medium containing either bovine serum albumin (BSA) or adenosine as a sole carbon source. Whereas BSA degradation requires extracellular proteases thus providing a social benefit, adenosine is metabolized intracellularly providing a benefit for the individual. QSI-sensitive mimics were found to retard the growth of wild-type QSI-resistant mimics when grown in BSA (public nutrient acquisition) indicating QSI resistance is unlikely to spread, especially during infection (QSI-sensitive mimics) behaved as social cheaters delaying population growth and preventing enrichment of wild-type co-operators (QSI-

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resistant mimics) only when nutrient acquisition was public (extracellular), suggesting that QSI resistance is unlikely to spread (Mellbye & Schuster, 2011).

QSI targets

Marine organisms have proven to be a rich source A large number of natural compounds isolated from a variety of marine organisms have been shown to possessingexhibiting quorum sensing inhibitory activity (Dobretsov *et al*., 2009, 2011; Saurav *et al*., 2017). In a study examining the inhibition of marine biofouling by bacterial QSI, of 78 natural bioactivesproducts tested from chemical compound libraries containing compounds derived from marine organisms including (sponges, algaeseaweeds, fungi, bacteria, tunicates and cyanobacteria,) and terrestrial plants screened, more than half of them displayed QSI activity (Dobretsov *et al*., 2011). In particular, the compounds hymenialdisin, demethoxy enecalin, microcolins A and B and kojic acid were found to inhibit at micromolar concentrations inhibitedthe QS responses of the LuxR based reporter strains induced by N-3-oxo-hexanoyl-L-homoserine lactone at micromolar concentrations.

The three components of the Gram negative AHL system are (1) the signal molecule generator, (2) the signal molecule itself and (3) the signal molecule receptor, representing the key targets of QSI for an anti-pathogenic drug approach (Rasmussen & Givskov, 2006).

(1) In AHL-based Gram negative QS, an inactivation of the LuxI-type synthase would block interrupt the production synthesis of the relative AHL signal molecule meaning that a significant threshold concentration could not be reached, with failure to activate the downstream genes responsible for virulence. *In vitro,* a few substrate analogues have been found to actively block the production of AHL. For example, analogues of *S*-adenosyl-*L*-methionine (SAM) have proven to be potent inhibitors of AHL synthase in *P. aeruginosa* (Rasmussen & Givskov, 2006). This has yet to be tested *in vivo* and remains the least investigated method of interfering with QS.

(2) The signalling molecule itself constitutes another target to inhibit QS. QS can be inhibited by targeting the signal molecule itself. The three principalmain strategies to de-activate a signalling molecule are metabolic, chemical and enzymatic degradation or inactivation. An alkaline pH causes the homoserine lactone ring (Fig. 1) to open (Yates *et al.,* 2002). For example, wWhen a plant recognizes coloniszation by the pathogen *Erwinia carotovora,* which uses AHL-based QS to control regulate the expression synthesis of virulence factors, the plant actively causes alkalinization at the site of attack resulting in lactonolysis. In addition to pH, several other factors including temperature and the length of the acyl side chain influence the opening of the lactone ring. An increase in temperature will accelerate the rate at which the ring opens, whereas the longer the side chain the slower will be the lactonolysis.

AHL lactonases are enzymes that catalyse the ring opening reaction of the lactone ring (Rasmussen & Givskov, 2006). Several *Bacillus* species have been foundare known to produce the lactonase enzyme AiiA (Dong *et al.*, 2000), which is specific for the degradation of AHLs. Homologues of AiiA have also been found in other members of the *Bacillus genus* as well as members of the genera bacterial species including *Pseudomonas, aeruginosa, Arthrobacter* species and *Klebsiella pneumonia* (Rasmussen & Givskov, 2006). This form of inactivation is reversible when the pH is acidic. Moreover, when the AiiA gene was heterologously expressed in expression of AHL lactonase in the human pathogen *P. aeruginosa* PAO1 resulted in largea significant decreases inhibition ofin virulence gene expression production and swarming motility was achieved (Reimmann *et al.*, 2002). Similarly, when cloned and expressed in Expression of AHL lactonase in *Burkholderia* species the AiiA gene coding for the lactonase enzyme significantly reducedcan also attenuate virulence in this pathogen (Ulrich 2004; Wopperer *et al.*, 2006). AHL acylases are another class of enzymes that can deactivate the Gram negative signalling molecule by cleavinge the *N*-acyl bond of AHLs. Production of acylases has have been found reported in in several different types ofnumerous genera of bacteria including *Ralstonia* strain XJ12B, pseudomonads, and a *Streptomyces* species (Lin *et al.*, 2003). Bacteria such as *Variovorax paradoxus* and *P. aeruginosa* produce amino acylases responsible for the cleavage of the peptide bond of the signal molecule (Rasmussen & Givskov, 2006) and can use the products of this metabolism as their sole source of energy. Interestingly, it It has been hypothesized that *P. aeruginosa* creates its own AHL-acylases to regulate its own QS system, possibly to evade detection during initial infection of a host (Sio *et al.*, 2006).

(3) In AHL -based QS, The third target for QSI is the LuxR transcription factor responsible for the regulation of downstream QS-dependent pathways represents another valid target for QSI. The use of small AHL analogues to prevent LuxR activation has proven a successful strategy to target LuxR type transcription factors Small AHL analogues have been used to block the activation of LuxR (Suga & Smith, 2003). These analogues can displace the original AHL and cause activation of the LuxR-type protein, acting as competitive agonists (Schaefer *et al.*, 1996). Synthetic analogues are developed in one of three ways: substitution in the acyl side chain leaving the ring unchanged; substitution and alteration to the lactone ring while the side chain remains unchanged; or extensive modification to both the side chain and lactone ring (Rasmussen & Givskov, 2006).

Algal compounds -- promising leads for the treatment of biofilm-related infections

Macroalgal bioactives such as sulphated polysaccharides and kahalalides have long been recognized for medical applications (Smit, 2004) and interest in them remains high (e.g.

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Barbosa *et al*., 2014). However, to date, only a few lead compounds and their synthetic derivatives have progressed to animal trials (e.g. Wu *et al*., 2004). ~~despite the many purported clinical applications for seaweed preparations, to date only a few small clinical trials have been conducted by a limited number of researchers~~.

Seaweeds rely on the coating/secretion of secondary metabolites (toxins and broad spectrum antimicrobials and antivirals) for protection against micro- and macro-colonizing organisms (Hentzer *et al*., 2003). For example, several halogenated furanone compounds isolated from the red seaweed *Delisea pulchra* (Givskov *et al*., 1996) are released at its surface at concentrations capable of inhibiting both prokaryotic and eukaryotic colonization (Steinberg *et al*., 2002). These compounds were shown to be QSI, active against a broad range of bacteria (Hentzer *et al*., 2002; Givskov *et al*., 1996). ~~Their production may have evolved in response to the negative impacts of AHL-dependent colonization of the surface of the algae by marine bacteria.~~ These furanones produced by *Delisea* accelerate the turnover of the LuxR transcription factor inhibiting ~~effectively antagonize AHL~~QS-dependent gene expression ~~through accelerated degradation of the transcriptional activator~~in Gram negative bacteria (Manefield *et al*., 2002) and the capacity to synthesize such compounds is likely to have evolved as an antifouling strategy to preserve the surface of algal fronds from ~~the~~ colonization by Gram negative marine bacteria. However, as they are brominated, their application in humans is limited, making it necessary to search for QSI from other natural sources (Zhu & Sun, 2008). Overall, macroalgae have yielded more than 3,000 natural products, accounting for approximately 20% of marine natural compounds (Amsler, 2008~~, Table 1~~).

Red seaweeds (Rhodophyta)

Research on red seaweeds has discovered the majority of macroalgal secondary metabolites accounting for more than 1500 bioactives (Maschek, J.A. & Baker, B.J., 2008) ~~Alex to add reference~~). With the exception of phlorotannins, which are unique to brown algae, red seaweeds synthesize all major classes of algal natural products (Blunt *et al*., 2016). Red algae primarily synthesize isoprenoid and acetogenin derivatives, as well as amino acid, shikimate and nucleic acid derivatives (Amsler, 2008). Halogenated compounds underpin red algal chemistry, with over 90% of compounds reported to contain bromine or chlorine.

The genus *Laurencia* (Rhodomelaceae, Ceramiales) ~~accounts~~ has been the subject of ~~for~~ nearly 50% ~~half~~ of the ~~reports~~ publications on red algal chemistry, producing a plethora of halogenated sesquiterpenes and C15 acetogenins ~~characterized by the presence of halogen atoms in their chemical structures~~, as well as ~~along with a few~~ higher terpenes (~~C20 and greater~~; Davis & Vasanthi, 2011). *Laurencia* species occur widely on temperate and tropical coasts and are recognized as a rich source of novel secondary metabolites (Cabrita *et al*.,

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2010). Several of them display promising antimicrobial activity against a range of bacteria. ~~An~~ For example, an unidentified species of *Laurencia* from Malaysia exerted potent antimicrobial activity against a range of marine bacteria; two halogenated C15 acetogenin compounds, elatol and iso-obtusol, were isolated from this alga and structurally elucidated based on spectroscopic data, confirming the potential of these compounds as a source of pharmaceutically relevant bioactives (Vairappan *et al.*, 2001). In extracts from *L. ~~majusculemajuscula~~*, elatol inhibited six bacterial species, with significant ~~antibacterial~~ antimicrobial activities against *Staphylococcus epidermis*, *Klebsiella pneumonia* and *Salmonella sp.*; ~~while i~~Iso-obtusol, a polyhalogenated sesquiterpene produced by *Laurencia obtusa*, was found to ~~exhibited antibacterial~~display antimicrobial activity against ~~four~~ several bacteria, and proved particularly active against~~with significant activity against~~ *K. pneumonia* and *Salmonella* sp. (Vairappan, 2003). Interestingly, the antimicrobial activity of ~~Interestingly,~~ elatol and iso-obtusol was found to be equal or better ~~showed equal or better antibacterial activity~~ than ~~tested commercial~~conventional antibiotics ~~while iso-obtusol displayed similar potency to commercial antibiotics~~ against *K. pneumonia* and *Salmonella* sp. ~~T~~through ~~sp. Both compounds had~~ a bacteriostatic mode of action ~~against the tested bacteria~~ (Vairappan, 2003). Subsequently, Vairappan *et al.* (2010) ~~found~~ discovered a novel brominated diterpene, 10-acetoxyangasiol, as well as four previously known metabolites, aplysidiol, cupalaurenol, 1-methyl-2,3,5-tribromoindole, and chamigrane epoxide in *Laurencia* sp. ~~Isolated~~ These compounds~~metabolites exhibited potent~~displayed strong ~~antibacterial~~ antimicrobial activit~~ies~~y against clinically relevant bacteria including ~~*Staphylococcus aureus*, *Staphylococcus sp.*, *Streptococcus pyogenes, Salmonella sp.* and *Vibrio cholera*~~.

Members of the order Bonnemaisoniales also produce a diverse array of secondary halogenated metabolites displaying antimicrobial activity (Nash *et al.*, 2005). *Delisea*, *Asparagopsis*, *Bonnemaisonia* and *Ptilonia* all synthesize a group of linear halogenated ketones and branched lactones. Amongst these, the fimbrolides, a group of halogenated furanones (Fig. 2) from *Delisea pulchra* from southeastern Australia, show QSI activity against a range of bacteria, functioning as an intracellular signal antagonist as well as accelerating LuxR turnover (Rasmussen *et al.*, 2000; Manefield *et al.*, 2002), and hence providing an antifouling defence (Kjelleberg & Steinberg, 2001). From a screen of 39 macroalgae, *Asparagopsis taxiformis* extracts were shown to inhibit QS in *C. violaceum* CV026 bioreporter assays (Jha *et al.*, 2013). ~~The authors proposed, B~~based on Ion Cyclotron Resonance Fourier Transformation Mass Spectrometry ~~(ICR-FT/MS)~~ analysis of the QSI-active fraction, the authors proposed that the compound responsible for the QSI activity was 2-dodecanoyloxyethanesulfonate (Fig. 6; Jha *et al.*, 2013).

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Bonnemaisonia hamifera (Figs 7, 8) is native to Japan, was introduced into the North Atlantic Ocean prior to 1890 (Maggs & Stegenga, 1998) and is now widely distributed there. *B. hamifera* has a heteromorphic life cycle, alternating between a diploid filamentous "*Trailliella*" tetrasporophyte and a haploid gametophyte (Breeman *et al*., 1988). Like *Delisea pulchra, B. hamifera* produces an assortment of mono- and poly-halogenated ~~compounds~~ bioactives including 2-heptanones, 2-heptanols, acetates and acids, some of which display antimicrobial activity (Siuda *et al.,* 1975; Jacobsen & Madsen, 1978; McConnell & Fenical, 1979, Nylund *et al*., 2013; Enge *et al*., 2013).

One of the main secondary metabolites, 1,1,3,3-tetrabromo-2-heptanone (Fig. 11), stored in specialized gland cells in the *Trailliella* phase, has an ecologically relevant role as an antifouling agent against bacterial surface colonization. Natural surface concentrations (3.6 µg cm^{-2}) of 1,1,3,3-tetrabromo-2-heptanone applied to artificial panels significantly reduced the number of settled bacteria (Nylund *et al.,* 2008). Moreover, organic~~crude~~ extracts of *B. hamifera* show broad-spectrum antimicrobial activity ~~against bacterial growth~~ at ecologically relevant concentrations ~~with ecological relevance~~ (Nylund *et al.,* 2005, 2008, 2013) confirming the potential of this species as a novel source of marine-derived antibiofilm compounds active against human pathogens. The compound also acts as a chemical grazing deterrent (Enge *et al*., 2013), which is metabolically expensive to produce but protects the seaweed against bacteria as well as grazers (Nylund *et al*., 2013).

It is interesting to note that several of these members of the Bonnemaisoniales found in Europe and containing halogenated compounds such as bromophenols (Paul *et al*., 2006)~~.~~ are aliens. These compounds undoubtedly contribute to their invasive potential by deterring grazing and allowing the establishment of ~~large~~ high biomass (Enge *et al*., 2013). This is a clear indication that alien species are worth targeting in the search for new bioactives. QSI compounds have also been described from a few non-invasive red algae, such as *Ahnfeltiopsis flabelliformis* (Gigartinales) from Korea which has been shown to produce three AHL inhibitory compounds, floridoside (Fig. 3), betonicine (Fig. 4) and isethionic acid (Fig. 5) (Kim *et al*., 2007).

Brown seaweeds (Phaeophyceae)

Brown algae have also yielded a rich chemical diversity with more than 1,140 reported secondary metabolites. The most studied and representative bioactives ~~compounds~~ of the brown seaweeds ~~include~~ comprise diterpenes, phlorotannins, and small C11 acetogenins, all with very little halogenation (Blunt *et al.,* 2007). Phlorotannins are distinguishing compounds of brown algae, with a wide range of activities of pharmacological interest including antimicrobial (Eom *et al*., 2012), antiviral (Ahn *et al.,* 2004), antidiabetic (Lee & Jeon, 2013; Kang *et al.,* 2013), anti-inflammatory (Sugiura *et al*., 2013), anti-allergic (Sugiura *et al.,*

2009), anti-cancer (Lee *et al.,* 2012), and anti-neurodegenerative diseases (Myung *et al.,* 2005, Sathya *et al.,* 2013; Jung *et al.,* 2009; Heo *et al.,* 2012) especially against Alzheimer's disease (Yoon *et al.,* 2008; Yoon *et al.,* 2009i Ahn *et al.,* 2012). The ecological role of phlorotannins in brown seaweeds appears to include defence against epiphytes (Nakajima *et al*., 2016), as well as grazing deterrence (McClintock & Baker, 2001).

Although many studies examining brown algal chemistry have focused on *Dictyota* (Dictyotaceae) and its wealth of terpenes (>250) (Munro & Blunt, 2005), several other genera display activities of pharmacological relevance. For example carotenoids from several brown algae have a wide range of bioactivities (Peng *et al.,* 2011). The meroditerpenoid methoxybifurcarenone isolated from *Cystoseira tamariscifolia* displays antifungal activity against three plant pathogenic fungi and antibacterial activity against *Agrobacterium tumefaciens* and *E. coli* (Bennamara *et al.,* 1999).

Halidrys siliquosa (family Sargassaceae) is a large temperate macroalga growing up to 120 cm long in rock pools and sometimes as forests in the shallow subtidal zone. The bioactive potential of *H. siliquosa* was identified over four decades ago. Hornsey & Hide (1974, 1976) screened crude extracts of *H. siliquosa* against a series of opportunistic human pathogens and discovered antimicrobial activity against *Staphylococcus aureus, E. coli, Bacillus subtilis, Streptococcus pyogenes* and *Proteus.* Culioli *et al.* (2008) reported the antifouling activity of meroditerpenoids isolated from this species and identified nine tetraprenyltoluquinol-related metabolites exhibiting antifouling properties and inhibiting the growth of the marine bacteria *Cobetia marina, Marinobacterium stanieri, Vibrio fischeri, Pseudoalteromonas haloplanktis,* ~~(minimum inhibitory concentrations (MICs) < 2.5 $\mu g\ ml^{-1}$)~~ Non-cytotoxic concentrations of these meroditerpenoids were found to prevent~~and preventing~~ the settlement of cyprids of *Balanus amphitrite* ~~(EC50 < 5 $\mu g\ ml^{-1}$) at nontoxic concentrations (LC50 > 5 $\mu g\ ml^{-1}$). A study of the antimycobacterial, antiprotozoal and cytotoxic potential of 21 brown algae (Phaeophyceae) from British and Irish waters found that~~ *H. siliquosa* crude extract was ~~found to be~~ active ~~active~~ against the parasites *Trypanosoma brucei rhodesiense, T. cruzi* and *Leishmania donovani* and the bacterium *Mycobacterium tubercolosis* (Spavieri *et al.,* 2010) highlighting the potential of this alga for the treatment of mycobacterial and protozoal infections.

Busetti *et al.* (2015) reported~~found~~ antimicrobial and antibiofilm activity of methanolic extracts of *H. siliquosa* against clinically relevant human pathogens of the genera *Staphylococcus, Streptococcus, Enterococcus, Pseudomonas, Proteus, Stenotrophomonas,* and *Chromobacterium*. ~~They reported MIC and minimum bactericidal concentration values (MBC) values of *H. siliquosa* methanolic extract ranging from 0.04-5 mg ml^{-1}. Moreover, b~~Biofilms of *S. aureus* MRSA ATCC 33593 and *S. aureus* MRSA NCTC 10442 were found to be susceptible to *H. siliquosa* ~~methanolic~~ extract which ac~~w~~hieved ~~ith~~ minimum biofilm

eradication concentration (MBEC) values of 1.25 mg ml^{-1} and 5 mg ml^{-1} respectively. AThe active extracts showed no toxicity against wax moth (*G. mellonella*) larvae across a wide range of concentrations up to the highest concentration tested (Busetti *et al.*, 2015). The final antimicrobial activity exhibited by the crude extract using the disc diffusion assay or the MIC assay against MRSA ATCC 33593 is the result of the additive or synergistic activity of two distinct groups of compounds whereas the activity observed against *C. violaceum* ATCC 12472 results from three distinct groups of compounds. The activity of *H. siliquosa* methanolic extracts against the emerging pathogen *Stenotrophomonas maltophilia* is study suggests the production of bioactives with the potential to be used in a treatment strategy for presence of compounds that could be used against the emerging cystic fibrosis pathogen *Stenotrophomonas maltophilia* as well as or in a treatment therapies for strategy for *Staphylococcus* biofilm-related infections. The vast arsenal of bioactive compounds produced by *H. siliquosa* renders this organism another ideal subject for the isolation and characterization of bioactive compounds displaying antimicrobial or antibiofilm activity against clinically relevant human pathogens. Moreover, the promising range of activities displayed by *H. siliquosa* organic extracts against clinically relevant, antibiotic-resistant, human pathogens re-electhighlight this alga as a candidate for further studies focused on the isolation of antibiofilm compounds and antimicrobials for the treatment of infections involving resistant multi-resistant pathogenic strains.

Macroalgal microbiomes as a source of novel bioactives of pharmaceutical relevance

In recent years, several studies characterizing algal epiphytic bacterial communities (Figs 12-13) have highlighted the presence of "core microbial species" in mutualistic or obligate association with their host (Singh *et al.*, 2015). In particular, several bacterial epiphytes have been reported to produce bioactive compounds that can protect macroalgal surfaces from biofouling (Dobretsov and& Qian, 2002). However, whereas several concerted studies have focused on characteriszing the composition of the human microbiomes as well as deciphering the physiological significance of the host-microbe interactions underlying the mutualistic relationships therein, in seaweeds the characterisation of microbiomes and the significance of their functional relationship with their hosts remain largely unexplored. The advent of culture-independent, DNA-based, metagenomic and transcriptomic methods has provided powerful new tools for the characteriszation of host-associated microbiomes as well as for the elucidation of the many, complex, yet often fundamental processes involved in host–microbe interactions, providing future studies the tools to investigate the functional microbiome involved in the often complex life cycles of macroalgae (Singh and& Reddy, 2016). The

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discoveries deriving from such studies could assist in promoting fitness and productivity in macroalgal species of commercial interest through the modulation of a functionally active microbiome as well as providing enormous potential for the discovery of novel antibiofilm or QSI compounds of clinical relevance. Epiphytic bacterial communities have been reported to play an important role in protecting macroalgal surfaces from biofouling microorganisms through production of biologically active metabolites. However, in contrast to the microbial studies associated with human skin and gut and plants that have significantly advanced our knowledge on microbiomes and their functional interactions with the host, in seaweeds the precise composition of microbiomes and their functional partnership with their hosts remain relatively unknown. Therefore, it is imperative to investigate the functional microbiome that is closely involved in the life cycles of macroalgae using high throughput techniques (metagenomics and metatranscriptomics). The findings from such investigations would help in promoting health and productivity in macroalgal species through regulation of a functionally active microbiome as well as providing enormous potential for the isolation of novel antibiofilm or QSI compounds of clinical relevance.

For example, For example, the green alga the epiphytic bacterium *Pseudoalteromonas tunicata* isolated from the surface of *Ulva lactuca* can hinderrelics on the epiphytic bacterium *Pseudoalteromonas tunicata* to block biofilm formation of competing Gram negative microbes through the synthesis of pigmented substances that inhibit LuxR-dependent AHL-dependant transcriptional control in through a comparable fashionsimilar mode of action to the furanones (McLean *et al.,* 2004). *Halobacillus salinus*, a marine Gram positive bacterium isolated from a seagrass, secretes synthesizes and releases QSI secondary metabolitesbioactives capable of quenching QS-controlled behaviours inactive against Gram negative strains (Teasdale *et al.,* 2009) through competitive binding. It is believed that these nontoxic metabolites may act as antagonists of bacterial QS by competing with AHLs for receptor binding (Teasdale *et al.,* 2009). These examples indicate that QS inhibition represents a natural, widespread, antifoulingantimicrobial strategy utilized evolved by marine organisms with significant impact on biofilm formation, making marine ecosystems an ideal source for the discovery of QS inhibitors with potentially clinically relevant antibiofilm activity.

In a recent study, an isolate belonging to the *Pseudoalteromonas* sp. genus isolatedobtained from the surface algal fronds of the red alga seaweed *Plocamium maggsiae* displayed strong potent quorum sensing inhibitory (QSI) activity against acyl homoserine lactone (AHL)-based *Chromobacterium violaceum* reporter strains ATCC 12472 and CV026 (Busetti *et al.*, 2014). The isolate's filter-steriliszed supernatant significantly reduced diminished biofilm biomass both during biofilm formation (by 63%) as well as pre- in pre-established, mature *P. aeruginosa* PAO1 biofilms (by 33%) causing a 0.97-log reduction

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(~89%) and a 2-log reduction (~99%) in PAO1 biofilm viable counts in the biofilm formation and the biofilm eradication assays. The crude organic extract obtained from this isolate extract displayed had a minimum inhibitory concentration (MIC) of 2 mg ml^{-1} against PAO1 but failed to no produce a minimum bactericidal concentration (MBC) confirming the lack of antimicrobial activity in the extract at the concentrations tested. Sub-MIC concentrations (1 mg ml^{-1}) of KSS the crude organic extract were found to significantly reduced the quorum sensing (QS)-dependent production of both the two virulence factors pyoverdin and pyocyanin in *P. aeruginosa* PAO1 without affecting growth. A combinatorial approach using tobramycin and the crude organic extract at 1 mg ml^{-1} against planktonic *P. aeruginosa* PAO1 increased the efficacy effectiveness of tobramycin ten fold by ten times, decreasing lowering the its MIC against this pathogen from 0.75 to 0.075 mg ml^{-1} (Busetti *et al*., 2014). These results of this study confirm data support the validity efficacy of combinatorial approaches strategies combining conventional current antibiotic treatment therapy with (non-antibiotic) QSI compounds derived from algal microbial epiphytes to improve the efficacy of current antibiotic treatments.

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Conclusions Future perspectives

The imminent global health threat of antimicrobial resistance with the realistic prospect of mankind entering a 'post-antibiotic era' has spurred driven research into novel innovative therapeutic strategies based relying on new different targets and approaches for the treatment of microbial infections. The discovery gradual elucidation of widespread bacterial communication (QS) systems regulated by small diffusible signal molecules as a means to coordinate group behaviours has revolutionized our classical conception of bacteria as unicellular and thus independent in nature. Targeting complex social behaviours, which include virulence and pathogenicity, regulated by chemical intra- and inter-species signal molecules which allow them to coordinate their behaviour at a community level, represents a novel target for non-antibiotic anti-infective chemotherapy.

Marine organisms are known to produce a variety of QSIs that can interfere thwart with the biofilm formation development of competing species (McClean & Winson *et al.,* 1997; Bauer & Robinson 2002; Saurav *et al*., 2017), representing an important resource for the isolation of novel "antipathogenic" antibiofilm compounds. Bacteria from algal microbiomes remain a relatively untapped source of novel candidate compounds displaying QSI activity with the potential to attenuate biofilm formation, virulence factor production or increase the antimicrobial susceptibility of clinically important pathogenic bacteria in the constant fight against emergence of multi-resistant microorganisms (Saurav *et al*., 2017).

As in many other discovery and development programs in marine bioactives, there are a multitude of challenges associated with the biodiscovery and commercialization of

macroalgal compounds as pharmaceutical agents. These include accessibility to the biodiversity, efficient screening, sustainable supply, variability in the spectrum and quantities of bioactives produced (due to factors such as seasonality and geographic distribution), elucidation of the mechanism of action, suitable pharmacokinetics/ pharmacodynamic parameters and ultimately costs associated with sustainable aquaculture and processing. Despite this, a significant body of early-stage biodiscovery research highlights marine macroalgae as promising sources of novel antimicrobials, antibiofilm compounds, antivirals, anticancer, antimicrobial, anti-inflammatory and neuroprotective agents.

Several studies have validated approaches that combine ~~conventional~~ regular antibiotic agents with non-antibiotic compounds, such as QSIs, to enhance the effectiveness~~icacy~~ of present ~~current~~ treatments, but have not yet moved to clinical trials. Drawing inspiration from nature, future studies could focus on evaluating the combinatorial effects of algal secondary metabolites with those produced by the core members of their bacterial microbiomes in an attempt to mimic the complex natural chemical mechanisms underlying the mutualistic symbiotic relationships in their environments.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

All authors contributed to the manuscript. A. Busetti prepared the first draft; A. Busetti, C.A. Maggs and B.~~-~~F. Gilmore reviewed, revised and updated the manuscript.

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Table 1. Approximate number of known natural products, bioactives and antibiotics. Adapted from Bérdy (2005, table 1).

Source	Known compounds	Bioactives	Antibiotics
Total natural products	> 1 million	200000 to 250000	25000 to 30000
Microorganisms (bacteria, Actinomycetales and fungi)	> 50000	22000 to 23000	ca. 17000
Algae, seaweeds, lichens	3000 to 5000	1500 to 2000	ca. 1000
Higher plants	500000 to 600000	ca. 100000	10000 to 12000
Protozoa	Several hundreds	100 to 200	ca. 50
Invertebrates	ca. 100000	No data	ca. 500
Marine animals	30000 to 25000	7000 to 8000	3000 to 4000
Insects/worms/etc.	8000 to 10000	800 to 1000	150 to 200
Vertebrates	300000 to 250000	50000 to 70000	ca. 1000

Data were assembled by Bérdy (2005) from his database including nearly one million references and drawing on information from other databases. Non-marine taxa (higher plants, vertebrates) are much better known than marine organisms (algae, marine animals, worms).

Figure legends

Figs 1-6. Molecular structures of acyl homoserine lactones and quorum sensing inhibitors isolated from marine algae. **Fig. 1.** General structure of acyl homoserine lactones. **Fig. 2.** Halogenated furanones. **Fig. 3.** Floridoside. **Fig. 4.** Betonicine. **Fig. 5.** Isethionic acid. **Fig. 6.** 2-dodecanoyloxyethanesulfonate. Figure adapted from Saurav *et al*. (2017).

Figs 7-10. The red algae *Bonnemaisonia hamifera* and *Bonnemaisonia asparagoides* display strong antimicrobial activity against AHL quorum sensing bioreporter strain *Chromobacterium violaceum*. Algal samples were overlaid with *C. violaceum* in 0.5% agar prior to incubation. **Fig. 47**. *B. hamifera* washed in ddH_2O. **Fig. 28**. *B. hamifera* pre-washed in 70% ethanol; QSI activity not altered by ethanol wash. **Fig. 29**. *B. asparagoides* washed in ddH_2O. **Fig. 410**. *B. asparagoides* washed in 70% ethanol, exhibiting significant loss of QSI activity which has been extracted by the ethanol wash.

Fig. 11. Structure of 1,1,3,3-tetrabromo-2-heptanone, a poly-brominated 2-heptanone produced by *Bonnemaisonia hamifera* displaying antifouling properties.

Figs 12.-13. SEM of the epiphytic microbial colonisation of *Halidrys siliquosa* algal fronds. **Fig. 12**. Diatom embedded amongst diverse prokaryotes. **Fig. 13.** Three-dimensional structure of microbial biofilm.

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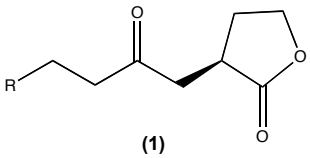

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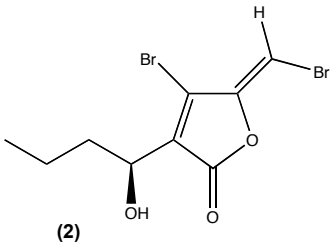

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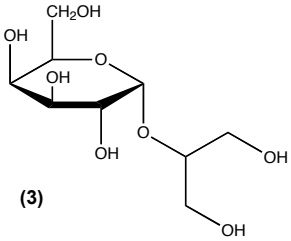

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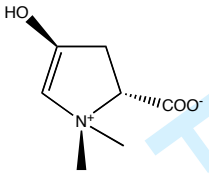

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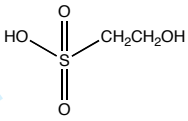

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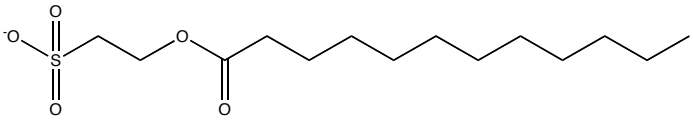

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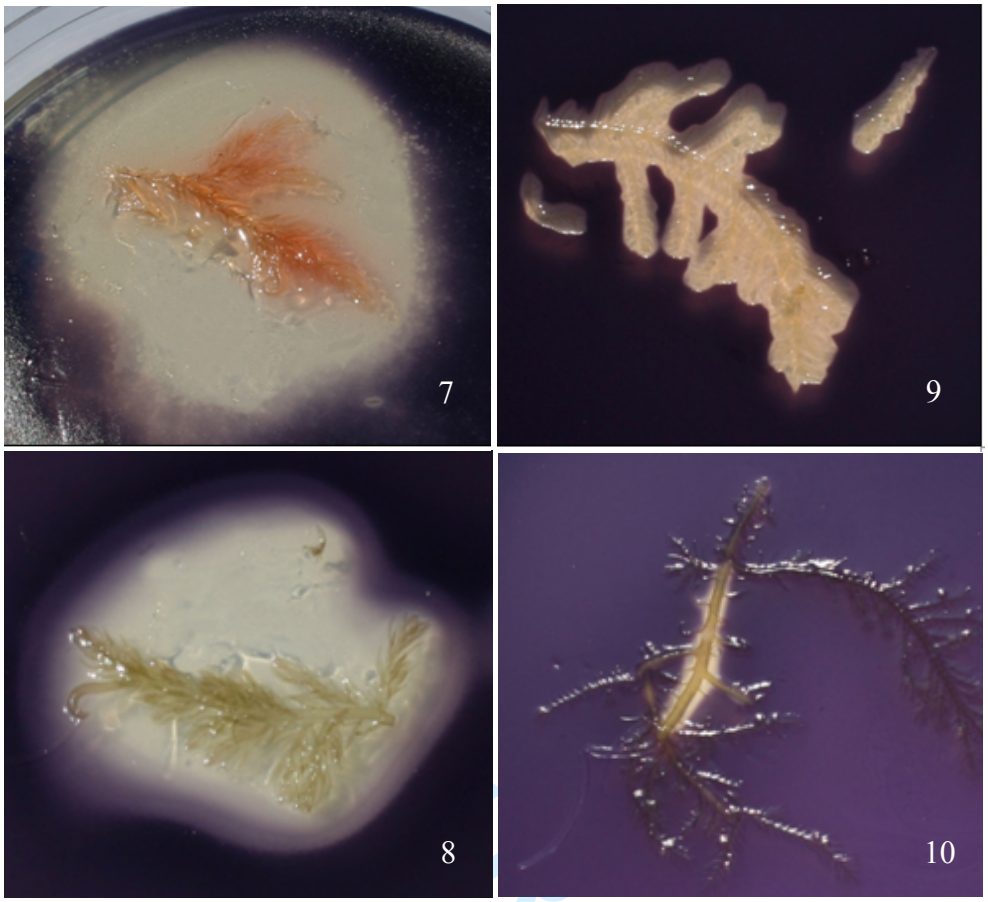

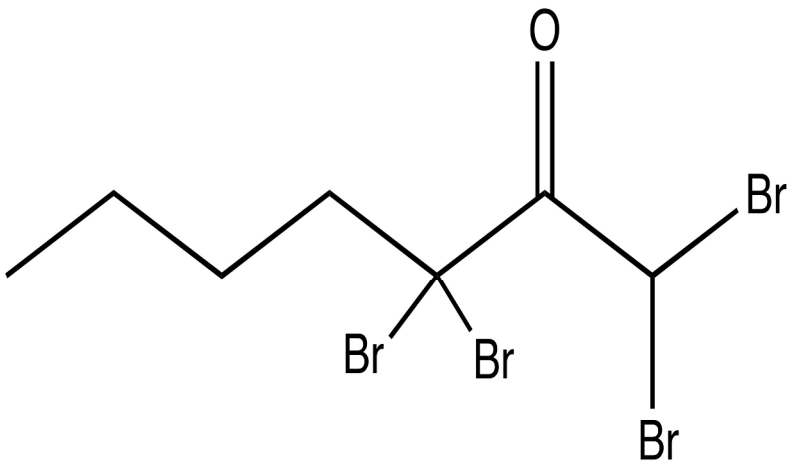

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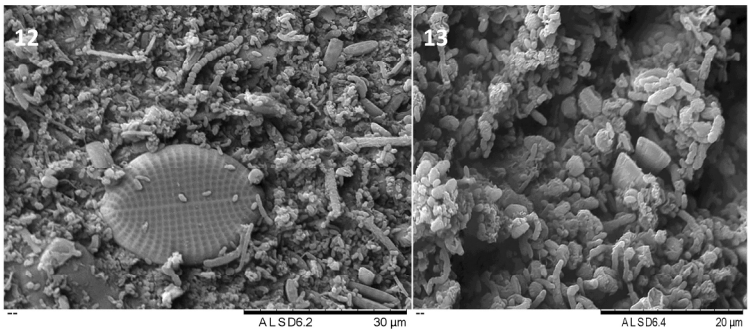

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