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 interspecific 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 RhI/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 adaptive 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, antimarial, 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 ophthalmic 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 antifilm 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 \textit{in vivo} during infection, when QS is involved in colonization, systemic spread and immune evasion (Defoirdt \textit{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 \textit{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 \textit{et al.}, 2009, 2011; Saurav \textit{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 \textit{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. \textit{In vitro}, a few substrate analogues have been found to actively block the production of AHL. For example, analogues of \textit{S}-adenosyl-\textit{L}-methionine (SAM) have proven to be potent inhibitors of AHL synthase in \textit{P. aeruginosa} (Rasmussen \& Givskov, 2006). This has yet to be tested \textit{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
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 catalyze 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

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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. Isoxobtusol, 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 isoxobtusol 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 antifilm 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), anticancer (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 tuberculosis (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 QSIIs 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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REFERENCES


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 ddH₂O. Fig. 8. B. hamifera pre-washed in 70% ethanol; QSI activity not altered by ethanol wash. Fig. 9. B. asparagoides washed in ddH₂O. 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 as we move to a global epidemic of antimicrobial-resistant infections. The discovery of new antimicrobials and antibiotics, better antimicrobial 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 how 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 an important role in regulating many aspects of host fitness involving zoology 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 combat the rise and dissemination of AMR.

Key words: Algae, antimicrobials, antimicrobial resistance, bacteria, biofilms, epiphytes, marine, microbiomes, 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 sublethal concentrations of biocides (Scenihr, 2009) and horizontal gene transfer through transformation, transduction, conjugation and conjugation events (Patrick & Lacey, 2004). 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 the manufacture of antibiotics at an industrial global scale for use in the clinical setting and for agriculture and animal care (Busetti et al., 2014) has accentuated the selective pressure on bacterial human pathogens (Hawkey et al., 2008). As a result, the emergence of drug-resistant organisms in the clinical setting has been linked to antibiotic use (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). In 2014, the World Health Organization (WHO) estimated that 1.2 million people died from infections that were resistant to at least one antibiotic (WHO, 2014). The total number of deaths is likely to be higher due to the rise of drug-resistant infections (WHO, 2014). The aim of this review is to examine the role of microbial biofilms in infection and resistance, and to discuss antimicrobial bioactives obtained from marine organisms, and to review the current state of knowledge of marine macroalgae and their associated microbiomes as a source of antimicrobial chemical diversity.

Biofilms

Biofilms are communities of microorganisms that are attached to surfaces, embedded in a self-synthesized extracellular polymeric matrix (Costerton et al., 1979). Biofilms play a crucial role in the survival and persistence of microorganisms in a wide range of environments, including the human body, where they are associated with various diseases such as dental caries, periodontal disease, and urinary tract infections. The self-synthesized extracellular polymeric matrix provides a protective niche for the microorganisms, making them resistant to antibiotics and other antimicrobial agents. The role of microbial biofilms in infection and resistance is complex and multifaceted, and understanding the factors that contribute to their formation and persistence is crucial for developing effective strategies to prevent and treat infections caused by biofilm-forming organisms. A recent study published in the European Journal of Phycology 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, and 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.
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; Hobley et al., 2011). Microbial biofilms provide a favourable propitious environment both for spontaneous mutation and vertical evolution of resistance genes (Savage et al., 2013) as well as for intraspecies and interspecies transmission and exchange of genetic elements, processes which enable bacteria harboring 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 clinical isolates of 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 healthcare 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 development and characterization of novel antibiofilm compounds and the identification of novel therapeutic strategies is crucial.

Within biofilms, both Gram-positive and Gram-negative bacteria use quorum sensing (QS), a form of cell-to-cell communication based on the release and detection of small molecules signaling molecules, to coordinate multiscale behavior and the expression of a vast array of physiological activities (Papenfort & Bassler, 2016). Some bacterial species use QS to coordinate the expression and production of virulence factors, including antibiotic resistance and biofilm formation. For example, in the opportunistic human pathogen Pseudomonas aeruginosa, 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 RhlI/RhlR, are used to regulate 170 genes via a complex network (Hentzer et al., 2002; Schuster et al., 2003; Wagner et al., 2003; Parsek & Greenberg, 2005). The coordinated, synchronized production and release of multiple proteins and enzymes is an adaptive response evolved to cope with conditions of high population density, such as those encountered in association 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.
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 multispecies communities (Mueller et al., 2006). In the photic zone they are usually dominated by phototrophic microalgae 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 larval invertebrates. Marine biofilms play a crucial role in 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 a range of stimulatory chemical cues signalling molecules that mostly remain so far mostly to be isolated and uncharacterized (Bowman, 2007).

Bioactive compounds

A “bioactive compound” can be defined as a secondary metabolite which at low concentrations can induce either beneficial or harmful effects on living organisms and is therefore of interest for potential industrial or medical applications (Rangel-Huerta et al., 2015). Some bioactive compounds have even shown activity against species of greater medical importance like tumour cells, antibiotic-resistant bacteria, anti-influenza, anti-inflammatory, anti-cancer, anti-ulcer, antimalarial, anti-bacterial, anti-fungal, and anti-parasitic activity. The diversity of natural compounds can be attributed to the process of natural selection that has driven the evolution of molecular biosources to perform their biological activities (Kaiser & Cano, 2005).

Bioactive compounds are produced by a wide range of microorganisms, plants, and animals. They play a crucial role in the survival and interaction of these organisms with their environment. For example, many antibiotics are produced by microorganisms that inhibit the growth of other microorganisms, allowing the producing organism to compete for resources. Similarly, many plants produce secondary metabolites that have antibacterial or antifungal properties, protecting them from pathogens. In addition, some animals produce bioactive compounds that influence the behavior of other organisms, such as pheromones that attract potential mates or deter predators.

Natural products are a vast and diverse group of compounds that are produced by living organisms. They include compounds such as alkaloids, terpenoids, polyketides, and steroids, among others. Many of these compounds have potential uses in medicine, agriculture, and industry. For example, alkaloids are a class of compounds found in plants that can have effects on the nervous system, such as decreasing pain or stimulating the brain. Terpenoids are a group of compounds found in plants that can have effects on the immune system, such as increasing the body’s ability to fight off infections. Polyketides are a group of compounds found in plants that can have effects on the cardiovascular system, such as lowering blood pressure.
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 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. Spontaneous and aminonucleoside 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 AraxA, 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 ophthalmic 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 unexplored resource for pharmaceutical and biotechnological biotechnology. In the marine environment, submerged non-living surfaces rapidly become macrofouled, but living surfaces of marine organisms remain relatively free from macrofouling and covered with a thin film of epibiotic bacteria (Armstrong et al., 2001). This is in part attributable to molecules effective as antifouling compounds and to the surface characteristics of marine organisms. Marine macroalgae (seaweeds) are known to exude 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 general antifouling strategy. For example, epibiotic bacteria found on the surface of aquatic larvae produce simple antibacterial antimicrobial compounds that can protect the larvae from fungal infections (Gil-Turnes et al., 1989). Bacteria isolated from the surface of tunicate larvae and grown as biofilms hindered settlement 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 and their epiphytic bacteria microbiome interact as a unified functional entity or holobiont (reviewed by Egan et al., 2012).

Quorum sensing inhibition as a novel strategy to attenuate bacterial virulence

A promising approach designed to attenuate bacterial virulence is the ability to switch off bacterial gene expression, thereby interfering with cell-to-cell communication, processes now commonly termed “quorum quenching” and “quorum-sensing inhibition” (QSI). In fact, the inability to coordinate communal behaviors can prevent bacterial pathogens from escaping or overcoming host immune responses, enabling an infection (Rasmussen & Givskov, 2006; Hentzer et al., 2003). Moreover, the ability to switch off virulence gene expression exogenously offers a novel strategy for the treatment or prevention of infection (Carani et al., 2002). Overall, the use of QSI can be used as a method to disrupt QS, thereby interfering with the expression of virulence genes and the establishment of an infection (e.g. van der Donk et al., 2008).

In certain species of bacteria, disruption of QS has been shown to affect biofilm formation (Horl et al., 2008) and differentiation (Horl et al., 2008), often rendering the biofilm more susceptible to treatment with biocides and antibiotics (Brudenell & Cunliffe, 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 (Huifor 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 percentage of infected wax moth 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, QSI-based selective pressure imposed by the use of antibiotics is particularly evident in the nosocomial clinical environment setting makes this environment a fertile ground for the emergence 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 or cell division, one can reason that its inhibition will not generate a harsh selective pressure and thus be amenable to the development of resistance (Koonin & Givskov, 2006; Spormann, 2007, Kendall & Sperandio, 2007). In fact, the persistence of QSI-based selective pressure during infection is likely a disruption of the signaling systems controlling the production and secretion of a number of virulence factors. Although it is reasonable to conclude that resistance to QS, which has been hypothesized to emerge from a combination of QSI-based selective pressure, would be selected in vivo during infection, when QSI is used to combat infections, systemic spread of the resistant strains (De Grood et al., 2010). Although a combinatorial approach relying on the use of conventional antibiotics in combination with QSI as an antivirulence approach would diminish the chance of this event considerably, joint antimicrobial/antivirulence 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, Mellybye and Schuster (Mellybye & 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 wildtype QSI-resistant mimics when grown in BSA (public nutrient acquisition) indicating QSI resistance is unlikely to spread especially during infection.
QSI targets
Marine organisms have proven to be a rich source of compounds possessing quorum sensing inhibitory activity (Dobretsov et al., 2009, 2011; Saurav et al., 2017). In a study examining the inhibition of marine biofouling by natural QSI products, more than half of the compounds derived from 78 marine organisms including sponges, algae, seaweed, fungi, bacteria, tunicates and cyanobacteria, exhibited QSI activity at micromolar concentrations. Bioactives from marine organisms such as hymenialdisin, demethoxy encecalin, microcolins A and B, and kojic acid inhibited typical LuxR-based reporter strains in a concentration-dependent manner. In particular, the compounds hymenialdisin, demethoxy encecalin, microcolins A and B, and kojic acid were found to block the production of AHL 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 antiinfective drug approach (Rasmussen & Givskov, 2006). Thus far, several substrate analogues have been found to inhibit 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 with the aid of pH control. The three principal strategies to do so include (a) neutral pH, (b) 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 control the expression 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 found 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 genus Bacillus and 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 the human pathogen P. aeruginosa PAO1 resulted in large decreases in virulence gene expression and swarming motility was achieved (Reimmann et al., 2002). Similarly, when cloned and expressed in the 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 found reported in several different types 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. 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).

In AHL-based QS, the third target for QSI is the LuxR transcription factor responsible for the regulation of downstream QS-dependent pathways in another target for QSI. The use of small AHL analogues to prevent LuxR activation has proven successful in targeting LuxR-type transcription factors. Small AHL analogues can be used to block the activation of 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 are promising leads for the treatment of biofilm-related infections. Macrocyclic bioactives such as sulphated polysaccharides and kahalalides have long been recognised for medical applications (Smit, 2004) and interest in them remains high. It...
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, few seaweed products have been tested in animal trials (Hentzer et al., 2003). Though 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). These production compounds are expressed as secondary metabolites through a complex network of regulation involving LuxR transcription factor. These furanones produced by Delisea accelerate the turnover of the LuxR transcription factor inhibiting AHL-dependent gene expression (Manefield et al., 2002). These synthetic compounds are likely to have evolved as an antifouling strategy to preserve the surface of algal fronds from the colonization by Gram negative marine bacteria.

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, with the exception of phlorotannins, which are unique to brown algae. A few red seaweed species synthesize all major classes of algal natural products (Bharti et al., 2016). Red algae primarily synthesize isoprenoid and acetogenin derivatives, as well as amino acid, diolide and methanol 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 the majority of red algal research, producing a plethora of brominated sesquiterpenes and C15 acetogenins characterized by the presence of halogen atoms in their chemical structures. This genus has been the subject of nearly 50% of all reports on red algal chemistry, producing a plethora of halogenated sesquiterpenes and C15 acetogenins characterized by the presence of halogen atoms in their chemical structures (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., 2011).
Several unidentified species of Laurencia from Malaysia and Australia exhibit 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 isoxobtusol, were isolated from this alga and structurally elucidated based on spectroscopic data, confirming the potential of these compounds as a source of pharmaceutically relevant bioactive (Vairappan et al., 2001). In extracts from Laurencia majusculemajuscula, elatol inhibited six bacterial species, with significant antibacterial activities against Staphylococcus epidermis, Klebsiella pneumoniae and Salmonella sp. Interestingly, the antibacterial activity of elatol and isoxobtusol was found to be equal or better than that of conventional antibiotics (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 were found to exhibit antimicrobial activity against clinically relevant bacteria, including Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, and Vibrio cholerae. 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, based on Ion Cyclotron Resonance Fourier Transform Mass Spectrometry (ICR-MS) analysis of the QSI-active fraction, that the compound responsible for the QSI activity was 2-decanoyloxyethanesulfonate (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 polyx halogenated compounds including 2-heptanones, 2-heptanols, acetates and acids, some of which display antifouling activity (Siuda et al., 1975; Jacobsen & Madsen, 1978; McConnell & Fenical, 1979, Nylund et al., 2008, 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 (Nylund et al., 2005, 2008, 2013), confirming the potential of this species as a novel source of marine-derived antibacterial 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 Bonnemaisoniaceae found in Europe and containing halogenated compounds such as bromophenols (Paul et al., 2006), are alien. These compounds undoubtedly contribute to their invasive potential by deterring grazing and allowing the establishment of invasive species (Engø et al., 2010). This is a clear indication that alien species are worth targeting in the search for new bioactives.

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 bioactive compounds of the brown seaweeds include tetraterpenoids, phlorotannins, and small C11 sesquiterpenoids, all with very little halogenation (Blunt et al., 2007). Phlorotannins are distinguishing compounds of brown algae, with a wide range of activities of pharmaceutical interest including antimicrobial (Kim et al., 2012), antiviral (Ahn et al., 2004), antiallergic (Lee & Han, 2013; Kang et al., 2013), anti-inflammatory (Seo et al., 2011), and anti-inflammatory (Seo et al., 2011), and anti-inflammatory (Seo et al., 2011).
2009), anti-tumour (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., 2009; 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 2.5 m 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) and 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. Caldic et al. (2008) reported the antifouling activity of meroditerpenoids isolated from this species and identified nine tetrakrenyltoluquinolxrelated metabolites exhibiting antifouling properties and inhibiting the growth of the marine bacteria Cobetia marina, Marinobacterium stanierii, Vibrio fischeri, Pseudoalteromonas haloplanktis (minimum inhibitory concentrations (MICs) < 2.5 µg ml⁻¹).

Non-cytotoxic concentrations of these meroditerpenoids were found to prevent and prevent the settlement of cyprids of Balanus amphitrite (EC50 < 5 µg ml⁻¹) at nontoxic concentrations (LC50 > 5 µg 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 against the parasites Trypanosoma brucei rhodesiense, T. cruzi and Leishmania donovani and the bacterium Mycobacterium tuberculosis (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, Enterococcus, Pseudomonas, Proteus, Stenotrophomonas, and Chromobacterium. They isolated an active compound that inhibited growth and biofilm formation of Staphylococcus aureus ATCC 29213 and S. aureus ATCC 19420, and showed the susceptibility to H. siliquosa metabolites of S. aureus MRSA ATCC 33593 and S. aureus MRSA ATCC 19420.
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eradication concentration (MBEC) values of 1.25 mg ml$^{-1}$ and 5 mg ml$^{-1}$ respectively. The active extracts showed no toxicity against wax moth (G. mellonella) larvae across a wide range of concentrations (Busetti et al., 2015).

Active extracts showed no toxicity against wax moth (G. mellonella) larvae across a wide range of concentrations (Busetti et al., 2015). The activity against the emerging pathogen Stenotrophomonas maltophilia is study suggests the production of bioactives with the potential to be used in a treatment strategy for infections involving this pathogen. The promising range of activities displayed by H. siliquosa organic extracts against clinically relevant, antibiotic-resistant, human pathogens illustrates the potential of this alga as a candidate for further studies focused on the isolation of antibiofilm compounds and antimicrobials for the treatment of infections involving 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 activity against clinically relevant 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 "true microbial species in mutualistic or obligate association with their host" (Bhagwat et al., 2013). In particular, several bacterial populations 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 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 characterization of the microbial communities with their hosts remains largely unexplored. The advent of culture-independent, DNA-based, metagenomic and transcriptomic methods has provided powerful new tools for the characterization of the microbial communities associated with macroalgae, opening new avenues for studying the functional microbiome involved in the often complex life cycles of macroalgae (Singh and Reddy, 2016). The study suggests the production of bioactives with the potential to be used in a treatment strategy for infections involving Staphylococcus biofilm-related infections.
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 antimicrobial 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 discovery of novel antibiofilm or QSI compounds of clinical relevance.

For example, the green alga *Ulva lactuca* can rely on the epiphytic bacterium *Pseudoalteromonas tunicata* isolated from the surface of *Ulva lactuca* to block biofilm formation of competing Gram negative microbes through the synthesis of pigmented substances that inhibit *LuxRx* dependent AHL transcriptional control in a comparable fashion to the furanones (McLean et al., 2004). *Halobacillus salinus*, a marine Gram positive bacterium isolated from a seagrass, secretes QSI secondary metabolites capable of quenching QS controlled behaviours in Gram negative strains (Teasdale et al., 2009) through competitive binding of QS signal molecules. These examples indicate that QS inhibition represents a natural, widespread, antifouling antimicrobial 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* genus obtained from the surface algal fronds of the red alga seaweed *Plocamium magusiae* displayed strong potent quorum sensing inhibitory (QSI) activity against *Chromobacterium violaceum* reporter strains ATCC 12472 and CV026 (Busetti et al., 2014). The isolate's filter sterilized supernatant significantly reduced biofilm biomass both during biofilm formation (by 63%) as well as in pre-established, mature *P. aeruginosa* PAO1 biofilms (by 33%) causing a 0.97 log reduction.
and a 2-log reduction in PAO1 biofilm viable counts in the biofilm formation and biofilm 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 reduce or eradicate PAO1 biofilm formation at 1 mg ml$^{-1}$. 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 antimicrobial effect of tobramycin with its minimum bactericidal concentration (MBC) reduced from 0.75 to 0.075 mg ml$^{-1}$ (Busetti et al., 2014). These results of this study confirm the antimicrobial efficacy of combining non-antibiotic compounds derived from algal microbial epiphytes to improve the efficacy of current antibiotic treatments.

**Conclusions**

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 novel therapeutic strategies targeting unexplored targets and approaches for the treatment of microbial infections. The discovery and characterisation of widespread bacterial communication (QS) systems regulated by small diffusible signal molecules as a means to coordinate group behaviors has revolutionised 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 behavior 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 with biofilm development of competing species (McChesney & Watson, 1997; Lesser & Robinson, 2002; Saurav et al., 2017), representing an important resource for the isolation of novel "antipathogenic" antimicrobial compounds. Bacteria from algal microbiomes remain a relatively untapped source of novel candidamycin compounds displaying QS activity with the potential to interrupt 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, antitumor, antimalarial, anti-inflammatory and neuroprotective agents.

Several studies have validated approaches that combine conventional antibiotic agents with non-antibiotic compounds, such as QSIs, to enhance the effectiveness of 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).

<table>
<thead>
<tr>
<th>Source</th>
<th>Known compounds</th>
<th>Bioactives</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total natural products</td>
<td>&gt; 1 million</td>
<td>200,000-250,000</td>
<td>25,000-30,000</td>
</tr>
<tr>
<td>Microorganisms (bacteria, Actinomycetales and fungi)</td>
<td>&gt; 50,000</td>
<td>22,000-23,000</td>
<td>ca. 17,000</td>
</tr>
<tr>
<td>Algae, seaweeds, lichens</td>
<td>3,000-5,000</td>
<td>1,500-2,000</td>
<td>ca. 1,000</td>
</tr>
<tr>
<td>Higher plants</td>
<td>500,000-600,000</td>
<td>ca. 100,000</td>
<td>10,000-12,000</td>
</tr>
<tr>
<td>Protozoa</td>
<td>Several hundreds</td>
<td>100-200</td>
<td>ca. 50</td>
</tr>
<tr>
<td>Invertebrates</td>
<td>ca. 100,000</td>
<td>No data</td>
<td>ca. 500</td>
</tr>
<tr>
<td>Marine animals</td>
<td>20,000-25,000</td>
<td>7,000-8,000</td>
<td>3,000-4,000</td>
</tr>
<tr>
<td>Insects/worms/etc.</td>
<td>8,000-10,000</td>
<td>800-1,000</td>
<td>150-200</td>
</tr>
<tr>
<td>Vertebrates</td>
<td>200,000-250,000</td>
<td>50,000-70,000</td>
<td>ca. 1,000</td>
</tr>
</tbody>
</table>

Data were assembled by Bérdy (2005) from his database including nearly one million references and drawing on information from other databases. Nonmarine 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. Figs 2-5. Halogenated furanones: Fig. 2. Halogened furanone. 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 ddH2O. Fig. 8. B. hamifera pre-washed in 70% ethanol, washed in ddH2O. Fig. 9. B. asparagoides washed in 70% ethanol, washed in ddH2O. Fig. 10. B. asparagoides washed in 70% ethanol, exhibiting significant loss of AHL inhibition activity compared to the control wash.

Fig. 11. Structure of 1,1,3,3-tetrabromooctadecanone, a polybrominated 2-heptanone produced by Bonnemaisonia hamifera displaying antifouling properties.

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