Innovative Approaches for the Development of New Non-Toxic Antifouling Solutions.

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Abstract

Marine biofouling can be defined as the undesirable adhesion to surfaces and further growth of organisms, mainly biofilm-forming microbes, macroalgae and invertebrates. The aim of this project was the prevention of adhesion and growth of organisms by using anti-biofilm molecules of natural origin. The antifouling properties of molecules isolated from an Artic marine sponge *Stryphnus fortis*, a sub-Artic ascidian *Synoicum pulmonaria* and micropeptides derived from the innate defence protein lactoferrin were assessed.

The bromotyrosine derivative ianthelline isolated from *S.fortis* is shown to inhibit both marine micro- and macrofoulers with a pronounced effect on marine bacteria (MIC values: 0.1–10 μg/mL) and barnacle larval settlement (IC50=3.0 μg/mL). Four molecules belonging to the recently discovered synoxazolidinone and pulmonarin families-both isolated from *S.pulmonaria* and four simplified synthetic analogs displayed minimum inhibition concentration (MIC) values in the micro- t o nanomolar range against 16 relevant marine species involved in both the micro- and macrofouling processes. Synoxazolidinone C displayed selected antifouling properties comparable to the commercial antifouling product SEA-NINE. Among the 13 micropeptides derived from the innate defence protein lactoferrin, two peptides were particularly active against the microfoulers with MIC-values ranging from 1-0.01 μg/mL and comparable to the antifouling activities of the commercial biocide SEA-NINE.The contamination of tropical marine environments (water column and sediment) by leachate from antifouling paints has led to concern regarding the effects on corals and their symbionts. Therefore, it is of high importance to evaluate the impact of antifouling compounds on key coral reef organisms. The potential toxicity of two commercial biocides

(SEA-NINE and Irgarol), one synthetic biocide (thiram) and two biocides of natural origin (myristic acid and Totarol) were assessed at environmental concentrations, toward the survival of two species of *Symbiodinum* sp.: *Symbiodinium microadriaticum* and *Symbiodinium voratum*. SEA-NINE-and Irgarol affect the growth and survival rate of the two species of symbiont but myristic acid, thiram and Totarol have shown no long term effect on *Symbiodinium spp*. Application of thermal stress (+2°C) increased the toxicity of SEA-NINE-and Irgarol to these species. The effect of increased mortality of these symbionts on coral bleaching as sea temperatures rise is unclear.

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Author's declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions presented in this thesis are the work of the named candidate and have not been submitted for any other research award.

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Dissemination

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Trepos R., Cervin G., Hellio C., Pavia H., Stensen W., Stensvåg K., Svendsen J., Haug T., Svenson J. (2014) Antifouling Compounds from the Sub-Arctic Ascidian *Synoicum pulmonaria*: Synoxazolidinones A and C, Pulmonarins A and B, and Synthetic Analogs, Journal of Natural Products, 77(9): 2105-13.

Trepos R., Pinori E., Jonsson P.R., Berglin M., Svenson J., Coutinho R., Lausmaa J. and Hellio C. (2014) Innovative approaches for the development of new copper-free marine antifouling paints. Journal of Ocean Technology, December 2014, vol 9 (4): 7-18.

Hansenn K., Cervin G., Trepos R., Petitbois J., Haug T., Hansen E., Andersen J., Pavia H., Hellio C. and Svenson J. (2014). The Bromotyrosine derivative Ianthelline isolated from the artic marine sponge *Stryphnus fortis* inhibits marine micro and macro biofouling. Marine Biotechnology, July 2014, 16(6):684-94.

Zhou A, Debbab A, Wray V, Lin W, Schulz B, Trepos R, Pile C, Hellio C, Proksch P, Aly A. (2014) Marine Bacterial Inhibitors from the Sponge Derived Fungus *Aspergillus sp*. Tetrahedron Letters Volume 55, Issue 17, 23 April 2014, Pages 2789– 2792.

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Abbreviations

AF: antifouling

AI: auto-inducer

CFU: colony forming units

EC: effective concentration

EPS: extracellular polymeric substance

FSW: fresh sea water

HPLC: high performance liquid chromatography

IR: infrared

IMO : International marine organisation

LD : lethal dose

LDOPA : L-3,4-dihydroxyphénylalanine

MIC: Minimum inhibitory concentration

MNP: marine natural product

MRSA: methicillin resistant *S.aureus*

OD: optical density

PAM: pulse amplitude modulated fluorometry

PS II: photosystem II

QS: quorum sensing

RPM: revolution per minute

RT: room temperature

NMR: nuclear magnetic resonance

SEA-NINE: SEA-NINE™ 211 = DCOIT: 4,5-dichloro-2-octyl-2H-isothiazol-3-one

SRB: sulfo-reducing bacteria

SW: sea water

TBT: Tributyltin

TBT-SPC: tributyltin self-polishing coating

Trademarks

SEA-NINE™ is a trademark of the Dow Chemical Company

Irgarol® is a trademark of BASF

Totarol™ is a trademark of Mende Biotech

Chapter 1

Chapter 2 : Introduction

1.1 Biofouling

1.1.1 Definition

Marine biofouling can be defined as the undesirable adhesion to surfaces and further growth of organisms, mainly biofilm-forming microbes, macroalgae and invertebrates that are defined as foulers or biofoulers (Fig. 1.1).

The process of colonization of an unprotected surface can be described as follows: Following adsorption of a conditioning film of macromolecules, primary colonizers, consisting of microorganisms such as bacteria, microalgae and other pioneer microorganisms, are found on surfaces after only a few minutes to hours of immersion. Secondary colonizers, consisting mostly of protozoa and spores of macroalgae, settle and start to grow on untreated surfaces within a week. Finally, larvae of hard macrofoulers (or tertiary colonizers, e.g. barnacles, mussels, tubeworms, bryozoans) will settle on unprotected surfaces 2-3 weeks after immersion (Wahl, 1989; Raikin, 2004; Fig. 1.2).

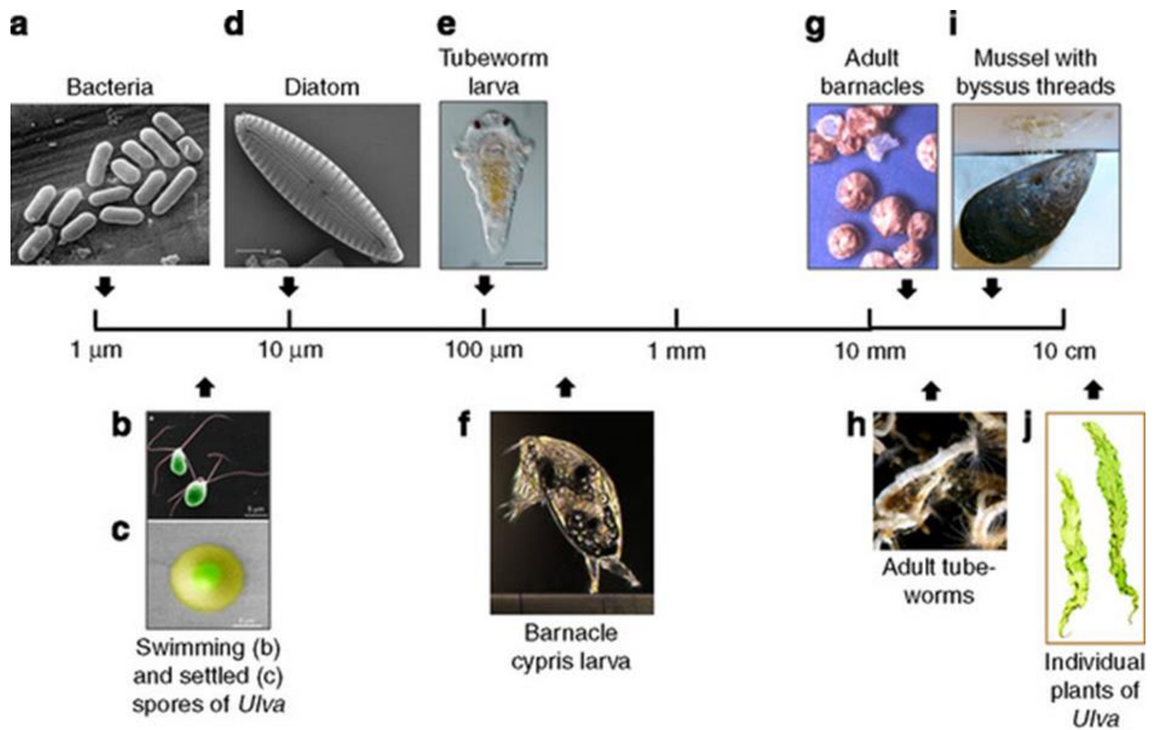

Figure 2.1: Organisms involved in the fouling process (from Callow *et al.*, 2011).

(**a**) Bacteria (scanning electron micrograph (SEM)), (**b**) false-colour SEM of motile, quadriflagellate spores of the green alga (macroalgae) *Ulva*, (**c**) false-colour environmental SEM image of settled spore of *Ulva* showing secreted annulus of swollen adhesive, (**d**) SEM of diatom (*Navicula*), (**e**) larva of tube worm, *Hydroides elegans* (image courtesy of B. Nedved), (**f**) barnacle cypris larva (*Amphibalanus amphitrite*) exploring a surface by its paired antennules (**g**) adult barnacles (**h**) adult tubeworms (*H. elegans)*, (**i**) adult mussels showing byssus threads attached to a surface, (**j**) individual thallus *Ulva*.

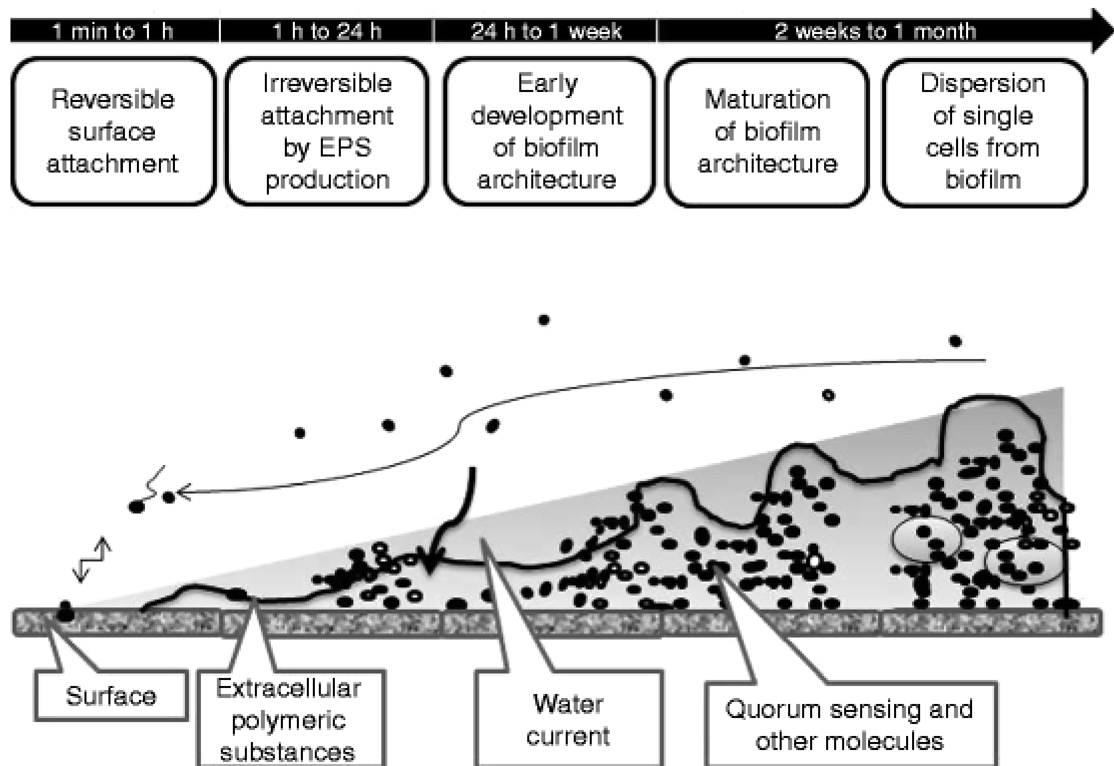

Figure 2.2: Fouling process (figure adapted from Qian *et al.* 2012)

More than 4000 marine species have been reported on surfaces globally, most of which live primarily in the shallower water along the coast and in harbours that provide abundant nutrients (Yebra *et al.*, 2004). The severity of biofouling depends on a large number of parameters, including temperature, salinity, light, location, season, depth, and in case of ship travel, speed (Admiraal *et al.*, 1997; Yebra *et al.*, 2004; Bressy *et al*., 2014). For example, biofouling is generally more serious in areas with high water temperature and long period of or intensity of light because temperature and light are the principal conditions determining microalgal blooms and thus breeding periods and rates of growth of biofouling organisms (Rascio *et al.*, 2003; Fig 1.3).

Figure 2.3: World map showing those areas with higher fouling risk from (Hellio and Yebra, 2009)

Any new structures immersed in seawater can be attractive for foulers and can thus lead to extra costs due to increased maintenance, and/or even mechanical wear, and/or biodeterioration and biocorrosion that require costly repairs (Maréchal and Hellio, 2009; Langhamer *et al.,* 2010; Trepos *et al*., 2014).

1.1.2 Biofouling organisms and their adhesion mechanisms

Biofoulers can be divided into two major categories: microfoulers (e.g. bacteria and diatoms) and macrofoulers organisms (e.g. macroalgae, barnacles, mussels, polychaete worms, bryozoans).

1.1.2.1 Microfouling

The adhesion of bacteria to surfaces relates to such factors as surface charge, surface energy, and the characteristics of polymers on bacteria. Bacterial adhesion is a consequence of the balance of attractive and repulsive physicochemical interactions between bacteria and surfaces.

First, through a simple physical reaction, a layer of conditioning film composed of organic materials (e.g. protein, polysaccharide, and proteoglycan) is formed on the substrate surface (Langmuir *et al.*, 2008; Bressy *et al.*, 2014). Microfoulers colonization involves two distinct steps: reversible adsorption, and irreversible adhesion. The reversible adsorption is governed mainly by physical effects such as Brownian motion, electrostatic interaction, gravity, water flow and van der Waals forces (Fletcher *et al*., 1984; Cao *et al*., 2011). The irreversible adhesion occurs mainly through biochemical effects such as secretion of extracellular polymeric substances (EPS) and leads to the formation of a biofilm. The biofilm has a three-dimensional structure, it is a heterogeneous community of microbial cells, bacteria, fungi and diatoms, enclosed in an exopolysaccharide matrix that is irreversibly attached to an inert or living surface. EPS is mainly composed of polysaccharides produced by microorganisms (such as colanic acid, chitosan, alginate), other components such as enzymes, DNA, RNA, nutrients, proteins, surfactants (Flemming *et al.*, 2007, Sohm *et al*., 2011). The exact role of the matrix is not yet completely elucidated but it has been demonstrated that the matrix acts as a protective layer (Fux *et al.,* 2005) and is microenvironment-conservative (Beech, 2004). The biofilm is a highly organized community usually formed by a number of similar or homologous and mixed species, which has beneficial effects for the microorganisms (Costerton *et al*., 1995; Flemming *et al*., 2001; Hellio and Yebra, 2009). After maturation of the biofilms, the latter disperse cells into the water to expand the spread of the species (Fig. 1.4). The dispersion can be due to various factors including, fluid dynamics and shear effects of the bulk fluid (Brugnoni *et al.*, 2007; Besemer, 2015).

Bacterial biofilms are organised communities, which form intricate architectures with microcolonies of homogenous and mixed species, and water channels inside the matrix that can transport nutrients or metabolites through convective flow (Costerton, 1995; Kristensen *et al*., 2008).

It has been reported that microfouling alone can increase fuel consumption by up to 18%, and reduce the sailing speed by at least 20% (Lewis *et al.,* 1984).

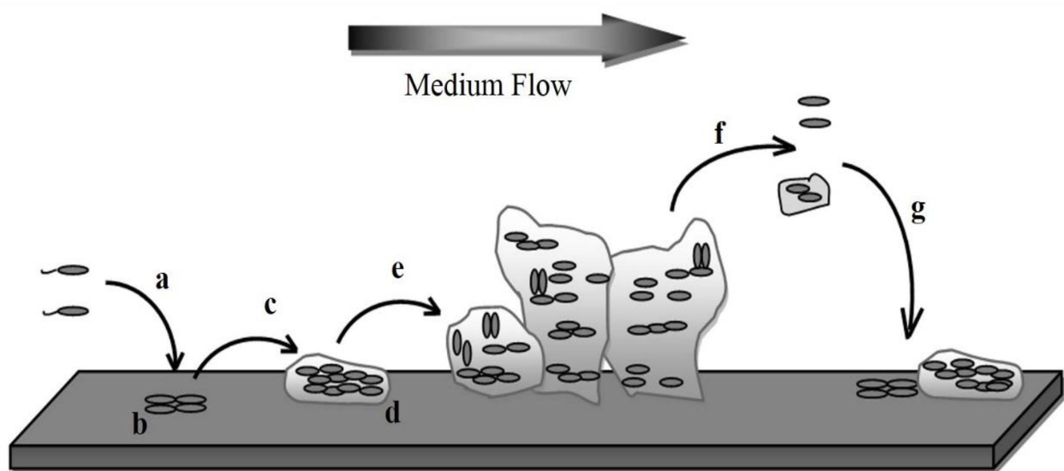

Figure 2.4: Process of biofilm formation and propagation (Meireles *et al*., 2015).

Biofilm formation steps: (**a**) transport of planktonic cells (**b**) adsorption of cells at the surface; (**c**) starting of EPS formation and production of cell-cell signalling molecules; (**d**) irreversible adsorption of cells; (**e**) biofilm maturation; (**f**) biofilm removal by detachment or sloughing; and (**g**) biofilm recolonization.

1.1.2.1.1 Bacterial adhesion

Bacterial adhesion occurs as a result of the interaction of planktonic cells with the surface by physical reactions, such as electrostatic interactions, gravity and water flow (Fletcher *et al.*, 1979; Walt *et al.*, 1985; Manivasagan *et al*., 2014). After the initial reversible absorption, bacteria use extracellular polymeric substances (EPS) to

temporarily adhere to the surface. These polymers are mainly glucose – and fructose – based polysaccharide fibrils (Abartua *et al.*, 1984, Wingender *et al.*, 1999; Manivasagan *et al*., 2014). The premise of the phenotypical change between reversible and irreversible states is a cell density-dependent system called quorum sensing (Miller *et al.,* 2001; Ben Jacob *et al.,* 2008; Delle *et al.,* 2016). The bacteria cells are able to sense that it is part of a concentration of cells of a certain size (the quorum), by recognition of specific low-molecular-weight signal compounds secreted and accumulated by the cells in the quorum. In quorum sensing low molecular weight compounds called auto-inducers regulate synchronized group behaviours which increase their ecological impact. An example of quorum sensing is illustrated in the Figure 1.5.

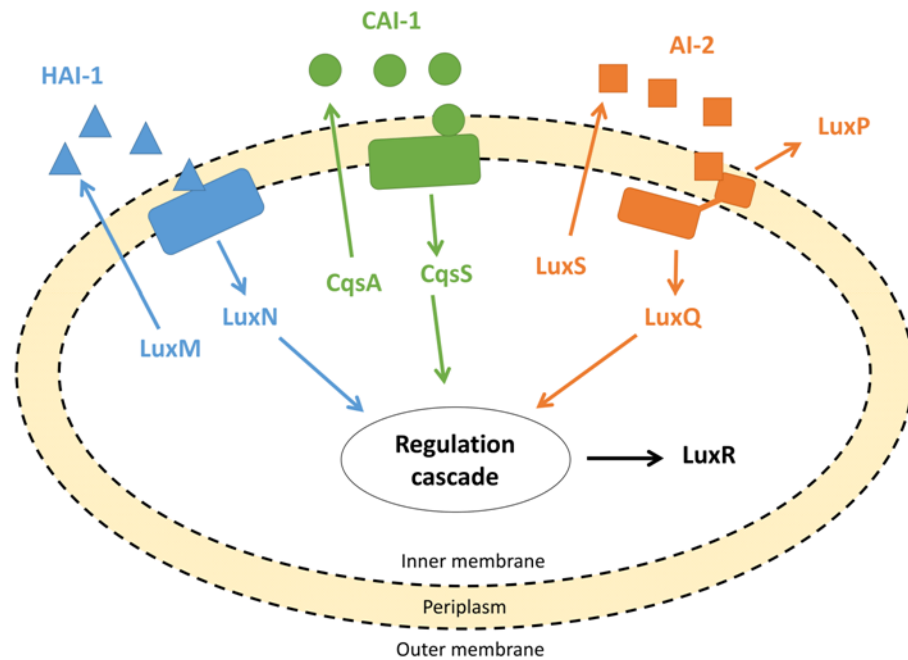

Figure 2.5: Illustrative scheme of the quorum sensing circuit in *V. harveyi* (from Delle *et al*. 2016). Bacteria realize QS through the synthesis, secretion and detection of some chemical signaling molecules known as autoinducers (AIs)Three different AIs (HAI-1, CAI-1 and AI-2) are produced by the corresponding synthase (LuxM, CqsA and LuxS). The three signals are then detected by their cognate receptors, i.e. LuxN, CqsS and the couple LuxP+LuxQ. The information relative to the three AIs are then channeled on the same regulation cascade that converges on the protein LuxO. When the threshold is reached, a positive feedback loop stimulates a further release of AIs. Below the QS threshold, LuxO negatively regulates the production of the genes encoding for bioluminescence (I.e luxL and luxM genes). Specifically, this inhibits the production of a key protein, LuxR. Above the threshold, LuxO is inactivated and LuxR is produced. This starts the QS controlled behaviors. In particular, LuxR is responsible for the transcription of the *luxCDABE* operon, which controls the production of both the luciferase and the long chain fatty aldehyde (RCOH).

Quorum sensing has been demonstrated to regulate many bacterial behaviours, including biofilm formation, antibiotic production, bioluminescence, and bacterial-eukaryotic interactions (Miller et al., 2001; Waters et al., 2005; Reuter et al., 2016). The mass of cells in biofilms accounts for only 2%–5% of the total weight with the remainder contributed by the EPS matrix, which includes a variety of extracellular carbohydrates, proteins, nucleic acid, glycoprotein, phospholipids and other surfactants. The ratio of these various extracellular compounds excreted by various species is quite different (Jayaraman *et al*., 2003; Krug *et al.,* 2006; Manivasagan *et al*., 2014), and even the same species secret different EPS compounds under different circumstances (Lasa *et al.*, 2006). Among those compounds, the polysaccharides are highly heterogeneous, containing different sorts of monosaccharide units and inorganic materials (Kristensen *et al*., 2008; Fig.1.6). The secreted proteins, many of which are polymer-degrading enzymes, also have heterogeneous compositions, although there is evidence that different proteins share some common substances or features. For instance, the surface protein Bap, amyloid fibril and β-1,6-N-acetyl glucosamine (Latasa *et al*., 2006; Larsen *et al*., 2007; Wetzel *et al*., 2007), and the sequence GGDEF/EAL (Lasa *et al*., 2008) are found in different proteins.

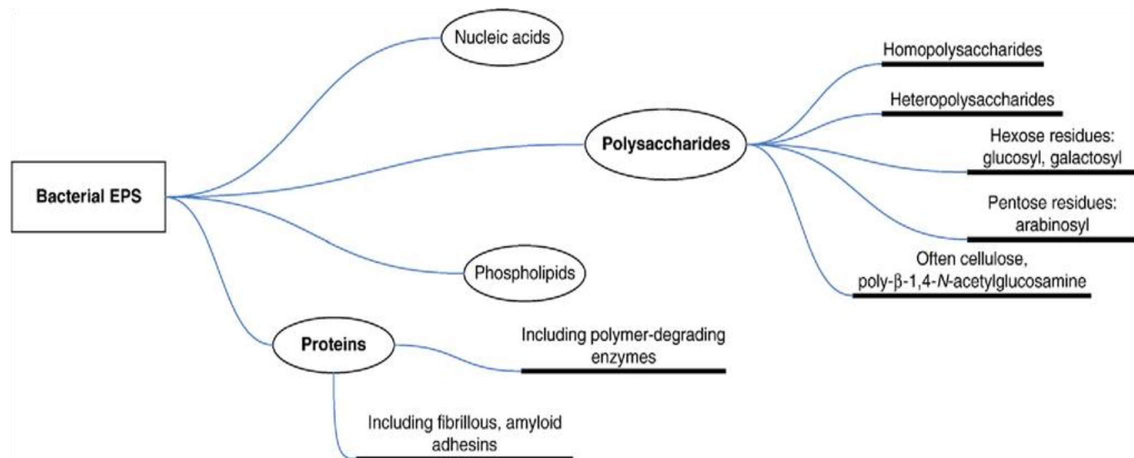

Figure 2.6: Schematic overview of the structural component chemistry of extracellular polymeric substances (EPS) involved in bacterial biofilms (Kristensen *et al*., 2008).

Once bacteria have irreversibly attached to a surface, the process of biofilm maturation begins. The overall density and complexity of the biofilm increase as surface-bound organisms begin to actively replicate and extracellular components generated by attached bacteria interact with organic and inorganic molecules in the immediate environment to create the glycocalyx (Carpentier and Cerf, 1993; Morente *et al*., 2013). The maturation of biofilm generates many processes already having taken place, such as quorum sensing (Nadell *et al*., 2008; Zhang *et al*., 2012), gene transfer (Molin *et al*., 2003; Harder *et al.*, 2014) and persister cell development (Lewis *et al*., 2005). All of these processes contribute to the community life of the biofilm and play an important role in both biofilm survival and spreading, since they allow also detachment of biofilm parts and release of free cells, which is the most common way for biofilm to spread (Kaplan *et al*., 2003; Dang *et al*., 2014).

Gram negative bacteria form biofilms more readily because EPS are found in greater abundance. This fact is of importance as Gram negative bacteria constitute the majority of the bacterial populations found in aquatic environments (An *et al*., 1998).

1.1.2.1.2 Microalgae adhesion

The major eukaryotic marine fouling microorganisms are diatoms, fungi, and protozoan, and the dominant organisms are diatoms (Abazua *et al.*, 1995; Luciana *et al*., 2009). Diatom adhesion is a complex process, as most of the diatoms lack flagella, they cannot actively approach a given surface but rather passively land on the substratum. Benthic diatoms approach surfaces through the effects of either gravity (Korbio *et al.,* 1993) or water currents (Finlay *et al*., 2002; Jeffery *et al*., 2004). Planktonic diatoms, which have almost the same specific gravity as seawater, land on surfaces mainly via turbulence (Wetherbee *et al*., 1998; Chiovitti *et al*., 2006). Moreover, electrostatic interactions such as Coulomb attraction and electrostatic contact potential are also involved (Gebeshuber *et al.,* 2005). During contact between diatoms and a surface, van der Waals forces may also operate (Autumn *et al*., 2000, Murguia *et al.*, 2015). After the diatoms land on the surface, they actively form the reversible primary adhesion through secretion of EPS. The diatoms then reorient themselves and move along the surface (Fig. 1.7). This process is called diatom gliding and is an important process resulting of an actin-myosin motility system meditated by extracellular proteoglycans (Poulsen *et al*., 1999; Yamaoka *et al*., 2016).

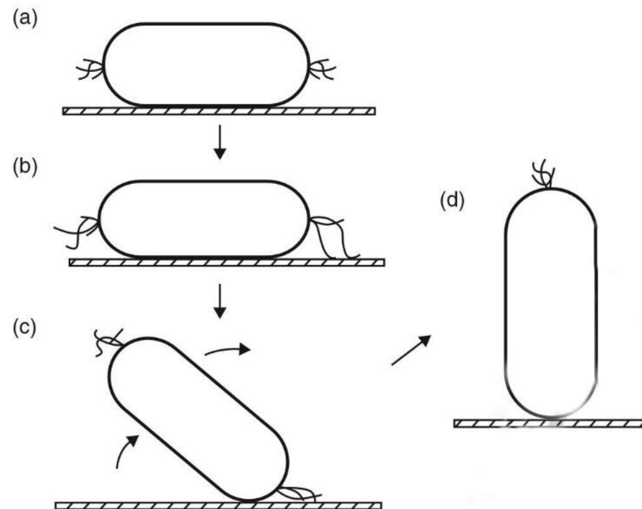

Figure 2.7: Diagram showing the initial adhesion process of the diatom *Stauroneis decipiens* (from Wetherbee *et al.*, 1998).

(**a**): landing on the girdle or the side, (**b**): contact and interactions between the motility and the adhesion complex, (**c**) and (**d**): pull of the cell onto the raphe.

EPS of diatoms is composed of carboxylated or sulphated acidic polysaccharides, which are involved in the primary adhesion, and proteoglycans, which are involved in diatom gliding and cross-linking stabilization of the biofilm matrix (Lind *et al*., 1997; Zhang *et al*., 2015). The composition of EPS produced by different types of diatoms are diverse (Wustman *et al*., 1997; Railkin., 2004), and include various protein fractions and complex anionic polysaccharides with heterogeneous combinations of monosaccharide (Chiovitti *et al*., 2006; Zhang *et al*., 2015; Fig. 1.8). In addition, at least two types of mucilage can be detected for the same species of diatom (De Brouwer et *al*., 2006; Kristensen *et al.*, 2008). After the adhesion, the microorganisms begin to multiply while sending out chemical signals that intercommunicate among the bacterial cells.

The cells multiply within the embedded exopolysaccharide matrix, thus giving rise to formation of micro-colonies (Sekar *et al*., 2004; Zhang *et al*., 2015).

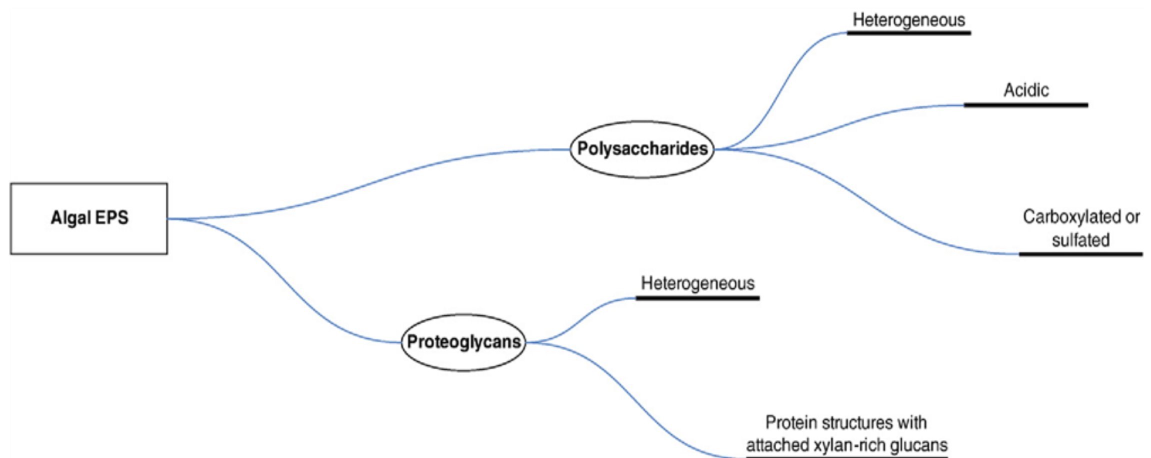

Figure 2.8: Schematic overview of the structural component chemistry of extracellular polymeric substances (EPS) involved in microalgal biofilms (from Kristensen *et al.,* 2008).

1.1.2.2 Macrofouling

After the formation and development of the biofilm, larvae or spores of macrofoulers will attach to the surface. The adhesion of macroorganisms usually occurs after biofilm formation (Costerton *et al*., 1978; Yebra *et al*., 2006). However, the adhesion of larvae of some species (e.g. bryozoans, polychaetes) could occasionally happen before biofilm formation (Maki *et al.*, 1989; Hung *et al*., 2005). Therefore, the process of biofouling occurs by both physical and biochemical-based reactions. The physical reactions are governed by factors such as electrostatic interactions and water flow and lead to the formation of the conditioning biofilm and adsorption of microorganisms. The physical reactions are usually reversible. The biochemical reactions include EPS secretion, movement and secondary adhesion of microorganisms, formation of the biofilm and adhesion of macrofoulers, these reactions are irreversible.

Adhesion mechanism of macroorganims are quite different in specific organisms. *Balanus* sp. and *Ulva* sp. have been investigated respectively as representative's species of invertebrate (Rittschtoff *et al.,* 1992; Marechal *et al*., 2004; Aldred *et al*., 2008) and macroalgae (Hellio *et al*., 2002; Maggs and Callow, 2003; Da Gama Perez *et al*., 2014, Martinelli *et al*., 2015).

1.1.2.2.1 Barnacle settlement

The settlement mechanism of barnacles has been studied extensively (Yule and Walker, 1984; Aldred and Clare, 2008; Yorisue *et al*., 2016). The cyprid antennule consists of four segments that are responsible for crawling, attachment and sensory functions (Gohad *et al*., 2012; Fig 1.9). When an appropriate surface is found, the cyprid will adhere by secretion of granulated cement containing high concentrations of proteins with small side chain amino acids (Kamino *et al.,* 1996). This cement embeds the antennular attachment organs and hardens because of protein polymerization (Ödling *et al*., 2007). After stable settlement, cyprids metamorphose into juvenile barnacles, and finally become adults.

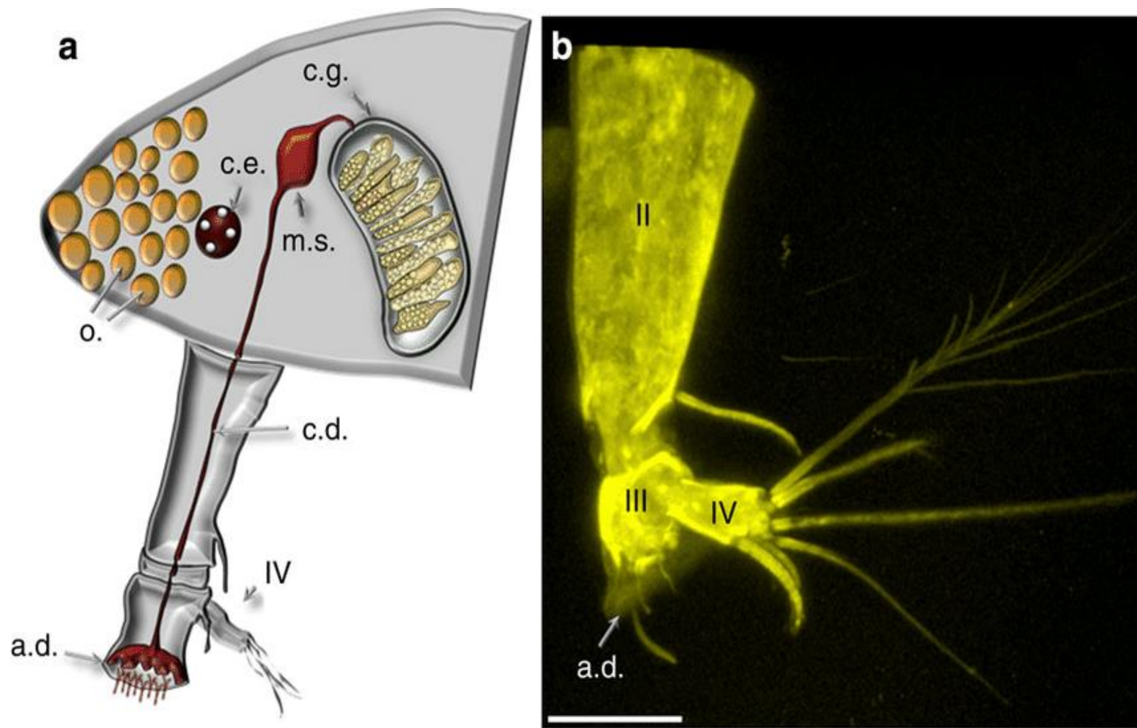

Figure 2.9: Morphology of the cyprid adhesive apparatus and the expressed adhesive plaque.

(**a**) A schematic of the anterior section of a barnacle cypris larva, with particular focus on the cementation apparatus: c.g., cement gland; m.s., muscular sac; c.d., cement duct; a.d., adhesive disc; o., oil bodies; c.e., compound eye; IV, fourth antennular segment, (**b**) A confocal volume projection of a cyprid antennule labelled with a membrane dye showing the 2nd, 3rd and 4th segments of the antennule, the adhesive disc (a.d.) and setae of the 4th segment (IV) (modified from Gohad *et al.*, 2013).

1.1.2.2.2 Macroalgae settlement

Ulva spores are extremely important in biofouling because of their abundance in seawater and adaptability to different environments (Callow and Callow, 2000). The motile spores have four flagella and no polysaccharide-rich cell wall. *Ulva* spores adhere to the surfaces by secreting glycoprotein, and then retract the flagella to form a cell wall (Fletcher and Callow, 1992; Hellio and Yebra, 2009; Fig. 1.10).

Freshly released glycoprotein from *Ulva* spores has strong adhesion strength, and the spores cannot be removed under the speed of most vessels and calculations have shown that the attachment is so tenacious that the operating speed of most vessels would be insufficient to shear off the zoospore, which can then evolve into adult macroalgae (Callow *et al*., 2000; Finlay *et al*., 2002).

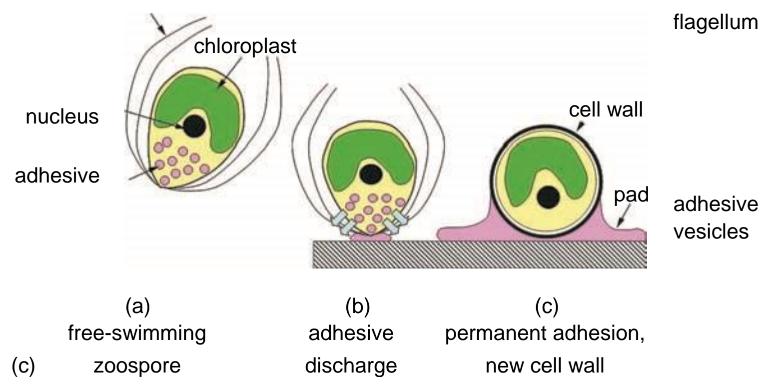

Figure 2.10: Course of events involved in the settlement and adhesion of *Ulva* spores (from Callow *et al*., 2006).

1.2 Impacts linked to biofouling

1.2.1 Environmental impacts

The expansion of goods transportation by maritime routes has led to significant energy and environmental impacts. Thus, according to the International Maritime Organisation (IMO), the world trading fleet is responsible for about 90% of the global trade of goods and a large contributor to the so-called 'welfare society': the total fuel consumption for ship transportation was estimated to be approximatively 370 million tons per years in 2007 (with corresponding 1120 million tons of CO_2 emitted) and will be burning about

half a billion tons of fuel per year by 2020 (1475 million tons of CO_2 emitted). According to some estimates (Schultz, 2007) the potential absence of fouling protection on ship hulls may roughly require an increase of propulsive power by 70% compared to a largely fouling-free hull (Fig. 1.11).

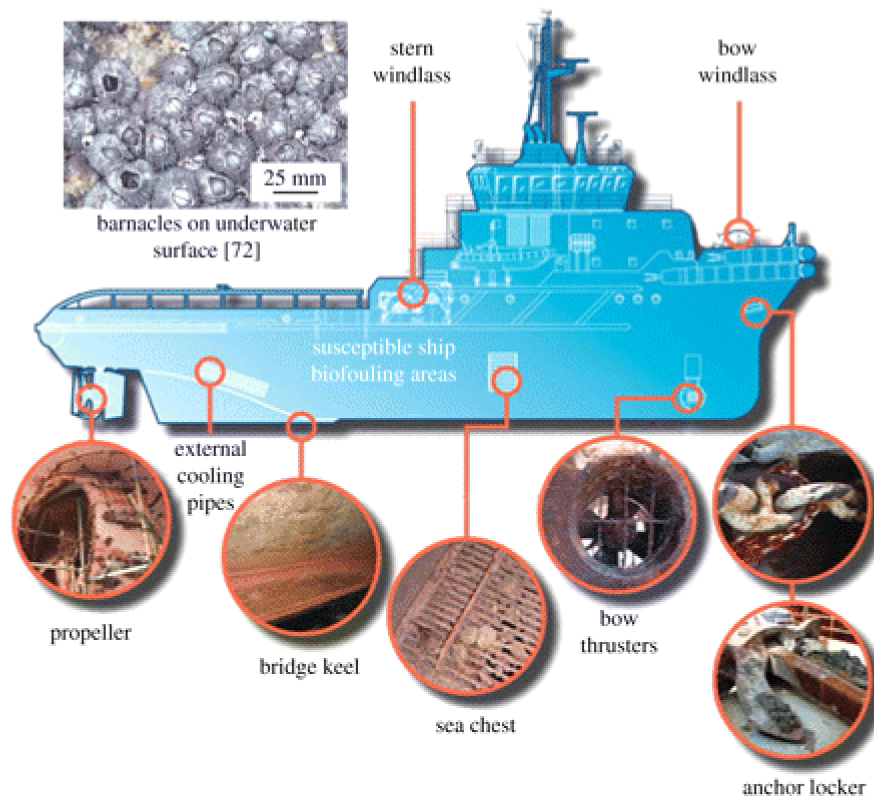

Figure 2.11: Area susceptible to biofouling on a ship (adapted from Bixler and Bhushan, 2012).

Based on these data, it has been proposed that a highly efficient antifouling (AF) protection will be saving over 150 billion dollars per year globally and about 450 million tons of CO_2 from being emitted in the atmosphere every year (excluding indirect costs resulting from transport delays, hull repairs, sunk vessels due to biocorroded hulls, etc.). Besides shipping industry and maritime transportation, other key sectors are negatively impacted by the development of biofouling communities.

For example, biofouling lead to significant problems for the energy production industry by obstructing the cooling systems of some nuclear power stations (Flemming, 2002) and by affecting the operation of immersed new renewable energy infrastructure. For example, Orme *et al*. (2001) demonstrated that the colonization of barnacles on marine current turbines has a detrimental effect on turbine efficiency reduced by up to 50%. A study by Wilhelmsson and Malm (2008) further showed that offshore wind power plants offer atypical substrates for fouling assemblage in terms of orientation, depth range, structure and surface texture, and thus efficiency can be affected. Biofouling can also severely affect wave energy plants by having a negative impact on material, weight, shape and efficiency (Langhamer *et al*., 2010). Biofouling is further responsible for a significant gain in weight of static structures in aquaculture and sometimes cause mechanical failure (Braithwaite and McEvoy, 2004; Wood *et al*., 2010). Fouling organisms growing on immersed man-made surfaces can also trigger biocorrosion (Eashwar *et al*., 1995; de Messano *et al.*, 2009; Sangeetha *et al*., 2010) (see section I.2.2), and transport of biofoulers may promote the spread of non-indigenous species (see section I.2.3).

1.2.2 Biocorrosion

Induced microbial corrosion is one of the major aspect of biofouling consequence. Although metal corrosion is a natural problem and can occur without microorganisms, microbial activities accelerate the reaction rate (Horn *et al*., 2002). Metabolic products such as organic acids, sulphide, protein and polysaccharides affect the kinetics of cathodic and/or anodic reactions and also modify the properties of the metal surface as well as passive layers, which are formed on the metal surface (Beech *et al*, 2004) (Fig. 1.12).

Microbiological influenced corrosion or biocorrosion occurs in all aqueous environments and affects an extensive range of structures from bridges to buildings, pipeline marine ships and offshore systems. Biocorrosion reportedly contributes up to 20% of the total corrosion damage of all metals and building materials (Geesey *et al*., 2000). Annually the direct cost of microbiological influenced corrosion is estimated at 30 -50 billion US Dollar (Flemming, 1996). In oil and gas industry, biocorrosion accounts for up to 30% of the corrosion cases. Underground corrosion of steel gas or water pipes which cost 0.5 to 2 billion US dollars per year is the best known economic disaster engendered by sulfate reducing bacteria (SRB) and acid producing bacteria (APB), groups of bacteria involved in microbiological induced corrosion (Fig. 1.12).

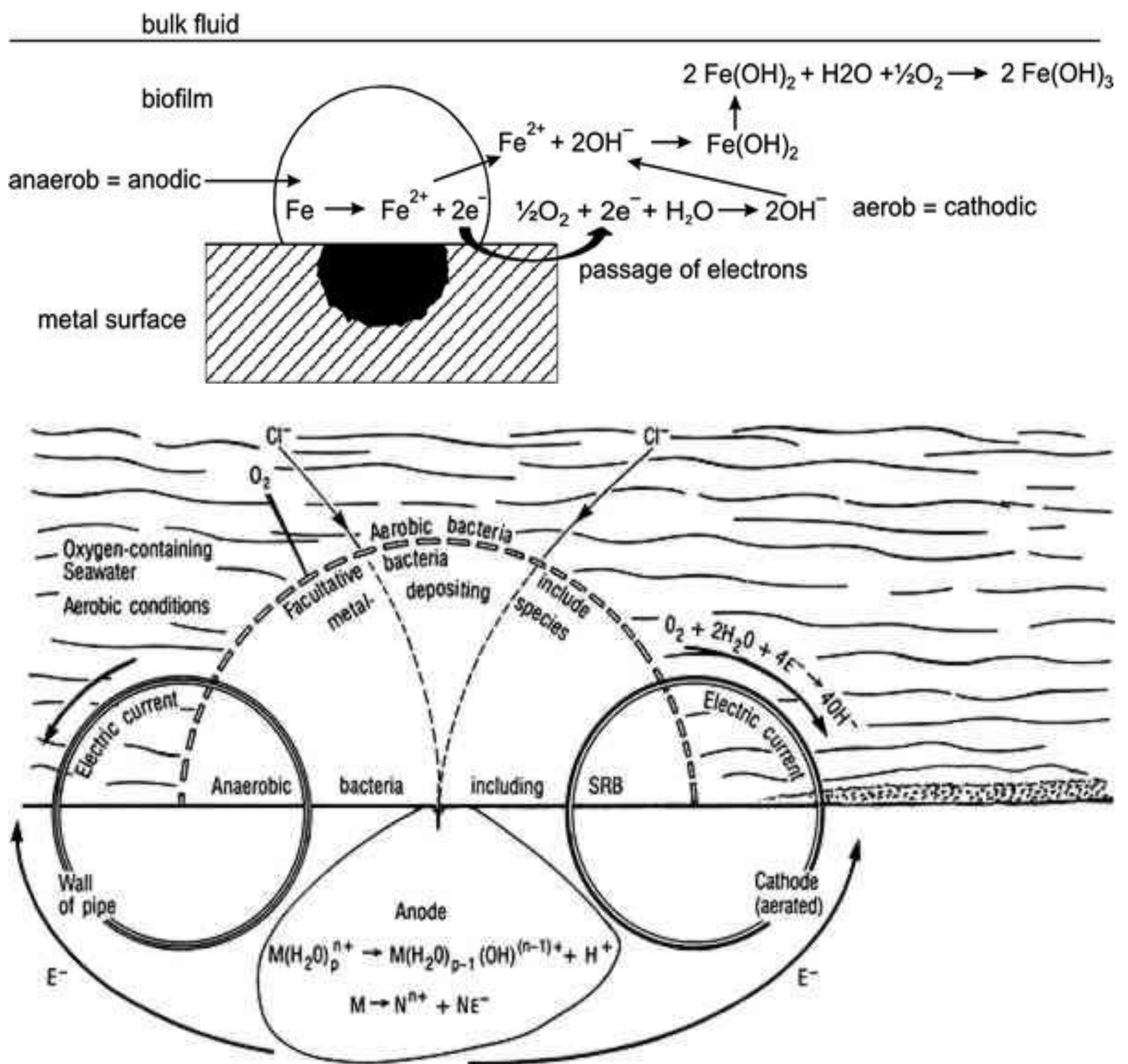

Figure 1.2.12: Microbial induced corrosion (from Koch *et al.*, 2014 and Mc Neil *et al.*, 1992). Heterogeneous biofilm including sulphate reducing bacteria (SRB) form regions which are depleted of oxygen. The different oxygen (O_2) concentrations create a difference in electric potential and form corrosion cells. The anode is the corroded metal. The cathode is protected by readily accepting electrons.

1.2.3 Ecological impacts: Invasive species

The introduction of invasive aquatic species to new environments by ships has been identified as a major threat to the world's oceans and to the conservation of biodiversity (Roberts and Tsameniy, 2008; Frey *et al.*, 2014).

Numerous marine species, carried either in ship's ballast water or on ship's hulls, may survive to establish a reproductive population in the host environment, becoming invasive, out-competing native species and multiplying into pest proportions (Piola *et al*., 2009; Fernandes *et al*., 2016). The problem of invasive species carried by ships has intensified over the last few decades due to the expanded trade and traffic volume and, since the volumes of seaborne trade continue to increase, the problem may not yet have reached its peak (Piola *et al*., 2009). The effects in many areas of the world have been devastating. Quantitative data show that the rate of bio-invasions is continuing to increase at an alarming rate and new areas are being invaded all the time (Frey *et al*., 2014).

The spread of invasive species is now recognized as one of the greatest threats to the ecological and the economic well-being of the planet (Darrigran *et al*., 2015). These species are causing enormous damage to biodiversity and the valuable natural riches of the earth upon which we depend. Direct and indirect health effects are becoming increasingly serious and the damage to the environment is often irreversible. Moreover, significant economic impact occurs to industries that depend on the coastal and marine environment, such as tourism, aquaculture and fisheries, as well as costly damage to infrastructure. Invasive aquatic species are introduced to new environments by ships mainly through ballast water or hull fouling. While ballast water is essential for safe and efficient modern shipping operations, the multitude of marine species carried in it may pose serious ecological, economic and health problems (Barry *et al*., 2008). These include bacteria, microbes, small invertebrates, algae, eggs, cysts and larvae of various species (Table 1.1). Biofouling is also considered one of the main vectors for bioinvasions, the undesirable accumulation of microorganisms, plants, algae and animals on submerged structures.

Studies have shown that biofouling can be a significant vector for the transfer of invasive aquatic species (Hellio and Yebra, 2009; Fernandes *et al*., 2016). Biofouling on ships entering the waters of states may result in the establishment of invasive aquatic species, which may pose threats to human, animal and plant life, economic and cultural activities and the aquatic environment. The potential for invasive aquatic species transferred through biofouling to cause harm has been recognized by the IMO, the Convention on Biological Diversity (CBD), several UNEP Regional Seas Conventions (e.g. Barcelona Convention for the Protection of the Mediterranean Sea Against Pollution), the Asia Pacific Economic Cooperation forum (APEC) and the Secretariat of the Pacific Region Environment Programme (SPREP) (MEPC.1/Circ.811; 2013).

Table 2.1: Examples of the major invasive species introduced via ballast water and ships hulls

Name	Native to	Introduced to	Impact
Asian paddle crab *Charybdis japonica*	Ranges from the North-west Pacific (China, Japan, Korea) to the east Asian Seas (Thailand, Malaysia)	New Zealand; detected but not established in Australia	May carry the White Spot Syndrome virus. Can affect biodiversity (predation or alteration of trophic levels)
Colonial tunicate *Didemnum vexillum*	North-west Pacific	North-east and north-west Atlantic, north-east Pacific, New Zealand	Fouler of hydrotechnical constructions, ships, aquaculture infrastructures and cultured molluscs. Affects the biodiversity of existing communities
North Pacific seastar *Asterias amurensis*	North-west Pacific	North-east Pacific, Southern Australia	Pest to native species, such as the endangered spotted handfish (*Brachionichthys hirsutus*) Impacts on mollusc aquaculture and wild fisheries (predation on mussels, scallops and clams)
Asian green mussel *Perna viridis*	Occurs from the Persian Gulf through to the Philippines, throughout the East Asian Seas and up to eastern China	The Caribbean, South Atlantic, South Pacific; detected in far North Queensland, Australia but not established	Foulers of hydrotechnical constructions, ships and aquaculture infrastructure. Affects the biodiversity of existing communities and can alter trophic levels.
Black striped mussel *Mytilopsis sallei*	North-west Atlantic, the Caribbean and South Atlantic	India, East Asian Seas (Malaysia, Singapore), South Pacific, North-west Pacific (Japan, Taiwan, Hong Kong); detected in Darwin, Australia but eradicated	Quick impact on the biodiversity by forming dense mat. Foulers of hydrotechnical constructions, ships and aquaculture infrastructures
European fan worm *Sabella spallanzanii*	North-east Atlantic, Mediterranean	South-west Atlantic, Southern Australia, New Zealand, North-west Pacific	Foulers of hydrotechnical constructions, ships and aquaculture infrastructures Competes with native filter-feeding organisms for habitat and food.
Bay barnacle *Amphibalanus improvises*	Thought to be the east coast of North-east and North-west Atlantic	South-west Atlantic, Caribbean Sea, Atlantic, Baltic Sea, Black Sea, Caspian Sea, North-west Pacific, East Asia Seas; detected but not established in Australia and New Zealand	Foulers of hydrotechnical constructions, ships and aquaculture infrastructures; corrosion, technical problems and loss of efficiency. Able to affect biodiversity, changes community structures and alters trophic levels.
Wakame seaweed *Undaria pinnatifida*	North-west Pacific	Mediterranean, North-east Atlantic, South-west Atlantic, North-east Pacific, South-east Australia, New Zealand	Foulers of hydrotechnical constructions, ships and aquaculture infrastructure Able to affect biodiversity, change community structures and alter trophic levels.
European shore crab *Carcinus maenas*	North-east Atlantic, The Baltic Sea	West Africa (Mauritania to South Africa), Mediterranean, North-west Atlantic (Delaware to Nova Scotia), South-west Atlantic (Panama to Argentina), East Africa (Red Sea to South Africa; Madagascar), North-west Pacific (Japan), North-east Pacific (South-east Alaska to California), East Asian Seas (Burma), Central Indian Ocean (Sri Lanka), South Pacific, South-eastern Australia	Voracious predator, preying on molluscs and other crustaceans, including commercially important species. Disrupts existing community structures through competition (habitat and food) and behavioural activities (burrowing).

1.3. Antifouling

The battle to control biofouling goes back to the dawn of civilization when man constructed boats and began his ocean travels. The earliest documented accounts date from the Greek and Roman civilizations when copper or lead sheathing was used to protect wooden boats (Anon, 1952) (Fig. 1.13). In modern time Tributyltin (TBT) was widely used from the 1960 to its recent ban in 2008. Its efficiency was the best ever recorded for antifouling (AF) formulations. However, the use of TBT-based paints was phased out after being shown to be highly toxic to many aquatic organisms and accumulated in biological tissues leading to contamination through the marine food chain (Ellis, 1991).

Oldest	Copper sheathing on wooden ships
2000 BC	Ships were fastened with copper bolts and the entire bottom was sheathed with lead
300 BC	Arsenic compounds, sulfur, etc. were formulated in wax, tar-type coatings
19th century	The first industrial use of antifouling paints: a soap based on copper sulfide in rosin
Pre-1960s	Conventional antifouling paints were largely based on copper(I) oxide as the toxicant. Other antifoulants: organomercury, organolead, organoarsenic, organohalogen (e.g. DDT), organosulfur compounds
1960–	Organotin antifoulants
1974–	Ablative self-polishing organotin polymers: tributyltin methacrylate/methylmeth-acrylate copolymer
1980–	Polysiloxane polymers
1990–	Tin-free antifoulants

Figure 2.13: Chronogram of the development of antifouling technologies (Evans and Clarkson, 1993).

Nowadays, most AF paints rely on the use of seawater-soluble copper oxide combined with various organic booster biocides (e.g. Irgarol 1051, diuron, SEA-NINE, zinc pyrithione, zineb) to prevent biofouling (Fig. 1.14).

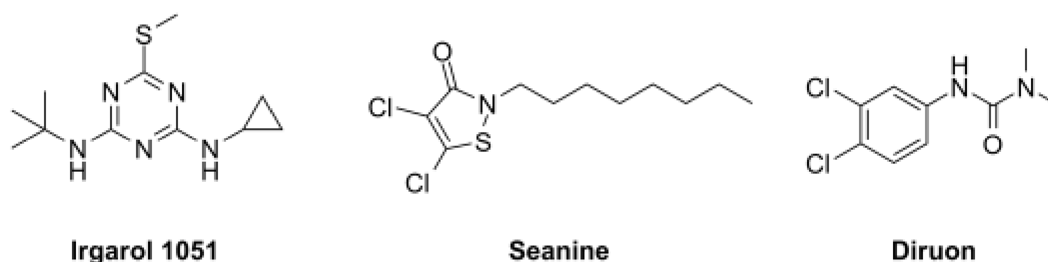

Figure 2.14: Examples of commercial organic booster biocides in current use together with copper salt in AF paints.

1.3.1 Antifouling methods

Antifouling (AF) methods can generally be divided into three categories: chemical, physical, and biological methods.

1.3.1.1 Traditional chemical methods

Biofouling has been recognized as problematic for more than 2000 years (Callow, 1990), and many types of AF methods have been investigated over this time (Yebra *et al.*, 2009). Since the late 20th century, organic tin and its derivatives have been widely used as AF coatings because of their activity against a wide range of fouling species. Organotin compounds that have been used as antifoulants include tributyltin oxide and tributyltin fluoride.

Those AF organotin compounds are powerful fungicides and will completely inhibit the growth of most fouling organisms at a very low concentration (Terlizzi *et al.*, 1998). The paints containing these compounds can be classified as those with insoluble and soluble

matrices, according to the chemical characteristics of the binder and their water solubility (Fig. 1.15). Insoluble matrix AF paints have a polymer matrix (such as vinyl and epoxy) that will not erode in water (Yebra *et al*., 2004; Bressy *et al*., 2010). When the coating is immersed in seawater, the soluble toxic materials dissolve, which leaves a multiporous structure known as the leached layer. Seawater then penetrates deeper into the film and more poisonous materials dissolves in the water. The advantage of this kind of paint is that the structures are mechanically strong and stable to oxidation and photodegradation. Thus the coatings can be made very thick to increase the content of toxic materials. However, at some stage the leached layer will be so thick that water cannot penetrate any deeper, and the rate of release will fall under the minimum value required for AF. Therefore, the lifespan of insoluble matrix AF paints is as short as 12-18 months (Chambers *et al*., 2006).

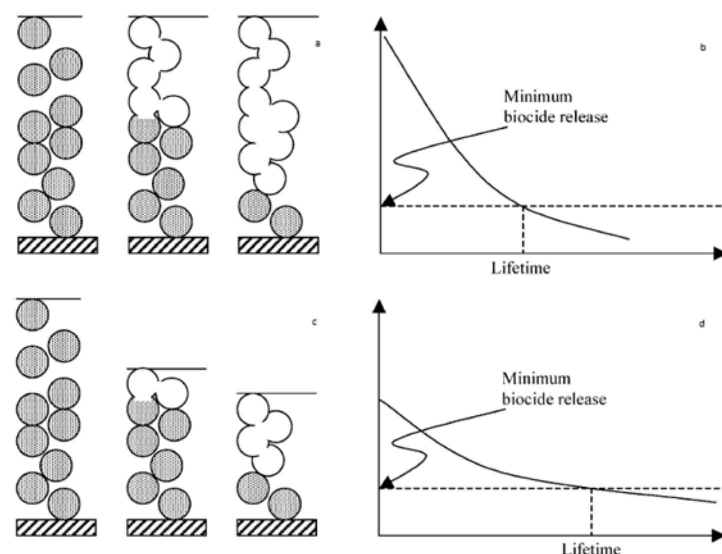

Figure 2.15: Working schemes and biocide release rates of traditional insoluble (**a** and **b**) and soluble (**c** and **d**) matrix AF paints.

“Minimum biocide release” indicates the limit for efficient protection against fouling (dependent on the fouling conditions) (Yebra *et al.*, 2004).

In insoluble matrix paints (also termed contact leaching or continuous contact), the polymer matrix is insoluble and does not polish or erode after immersion in water. Soluble matrix paints were developed in order to avoid the loss of AF efficiency with time by incorporating a binder which could be dissolved in sea water. To lengthen the lifespan of AF coatings, soluble matrix AF coatings were developed. As implied by the name, both the toxic materials and matrix, which contains a great amount of resin, can dissolve in seawater. In this case, the leached layer can be much thinner and toxic materials deeper in the film can be easily exposed to water, which lengthens the lifespan of the AF coating (Yebra *et al*., 2004). The release rate will exponentially increase as the sailing speed increases. However, during the static conditions that favour settlement of fouling organisms the pores of this coating can become blocked by insoluble salts, which greatly reduces the release of biocides (Lejars *et al*., 2012). In addition, because of the resin's brittleness and instability to oxidation, its mechanical properties are inferior to those of insoluble matrix coatings.

1.3.2 Modern chemical antifouling methods

1.3.2.1 Tributyltin self-polishing copolymer coatings.

Both insoluble and soluble matrix AF coatings have their weakness in term of efficiency. Consequently, alternative coatings have been investigated. In 1974, Milne and Hails patented the first tributyltin (TBT) self-polishing copolymer (TBT-SPC) technology, which provided an excellent AF effect that revolutionized the entire shipping industry (Yebra *et al*., 2004). TBT-SPC paints based on acrylic polymer (usually methyl methacrylate) with TBT groups bound to the polymer backbone by an ester. When immersed in water, the soluble pigment particles (such as zinc oxide) begin to dissolve (Fig 1.16).

The polymer of TBT methacrylate and methyl methacrylate is hydrophobic, which prevents water from infiltrating the paint film. Therefore, water can only fill the pores generated by the dissolution of soluble pigment particles. Moreover, the carboxyl-TBT linkage is easily hydrolysed in slightly alkaline environments such as in seawater (pH 7.5–8.5). This results in cleavage of the TBT portion from the copolymer, and releases the biocides into the water (Yebra *et al.*, 2004). Once many TBT portions have been cleaved, the partially reacted brittle polymer backbone can be easily washed off by the moving seawater, which exposes a fresh coating surface. The hydrolysis process provides a low hull roughness (about 100 µm), so as not to increase the resistance of the ship's hull. One of the major advantages of an AF coating such as this is that manipulating the polymer chemistry can control the polishing rate.

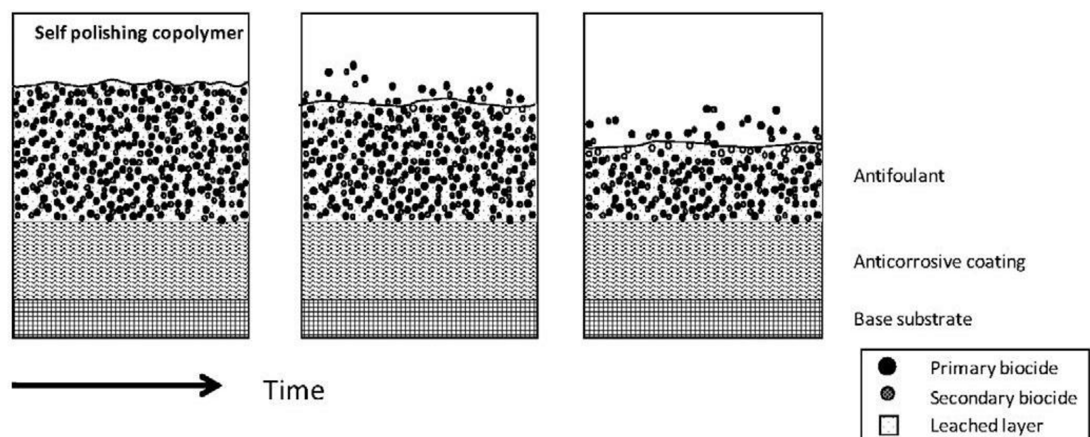

Figure 2.16: Diagrammatic representation of a self-polishing copolymer (SPC) coating (adapted from Dafforn *et al.,* 2011).

Therefore, it is possible to balance high effectiveness and a long lifespan; the coatings can be customized for ships operating under different conditions, such as duration of their idle periods and sailing speed (Hellio and Yebra, 2009).

It has been shown that release rate of TBT in seawater is almost constant with sailing speed, and thus high AF performance can be obtained even if the ship is not moving. In addition, the maintenance is convenient and low cost. Moreover, TBT-SPC paints have high mechanical strength, high stability to oxidation, and short drying times. The unavoidable formation of biofilm does not largely affect the net biocide leaching and binder reaction rates (Yebra *et al*., 2006). Therefore, in general, the TBT-SPC AF coating was widely applied in the shipping industry before it was banned (Davies *et al*., 1998).

TBT was initially anticipated to be environmentally safe (Evans and Hill, 1983; Lewis *et al*., 2010) but appeared to belong to the most toxic pollutants known so far for aquatic life (Hellio and Yebra; 2009). TBT contamination was found to occur worldwide in regions with shipping and boating activity. The main ecotoxicological effects linked to TBT are: dysfunction of calcium homeostasis, inhibition of mitochondrial oxidative phosphorylation and ATP synthesis, inhibition of photophosphorylation in chloroplast and of ATPases and cytochrome P450 monooxygenases (Lee *et al*., 1989; Fent, 2006; Hallers-Tjabbes *et al.*,2009). Malformations of oyster shells were documented in the late 1970s (Alzieu *et al*. 1989) in Arcachon Bay (France) (Fig. 1.17).

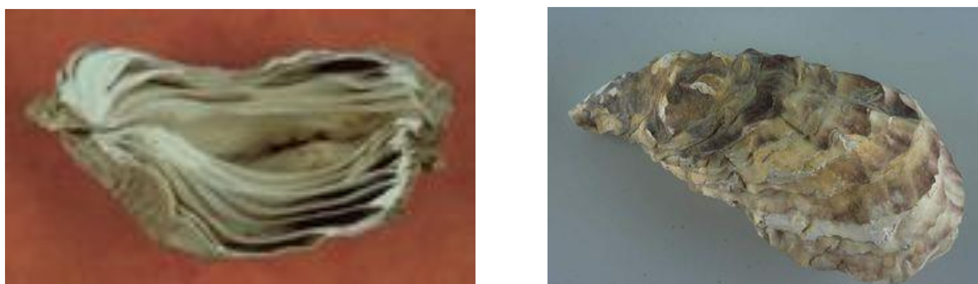

Figure 2.17: Pictures of an oyster with a shell malformation induced by TBT (on the left) and an oyster without a shell malformation (on the right) (courtesy of Olivier Barbaroux and Ifremer).

Some mollusc like *Nucella lapillus* have developed a reproductive system abnormality, named "imposex" when exposed to even extremely low levels of TBT (Gibbs and Bryan, 1986; Fig. 1.18; Ellis and Pattisina, 1990;). Imposex is the imposition of male sexual characteristics on female gastropods and has been found to be one of the most sensitive indicators of environmental TBT exposure (Nicolau and Barry, 2015).

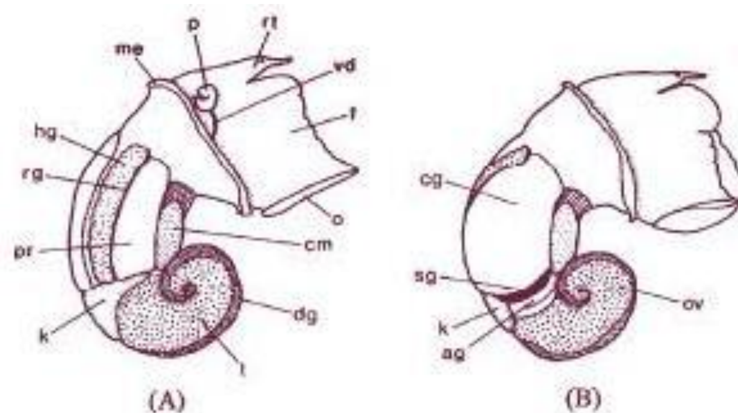

Figure 2.18: *Nucella Lapillus*. External features of mature male (**A**) and mature female presenting an imposex (**B**) after removal of the shell (from Gibbs *et al.,* 1987).

Abbreviations: ag, albumen gland; cg, capsule gland; cm, columella muscle; dg, digestive gland; f, foot; hg, hypobranchial gland; k, kidney; me, mantle edge; o, operculum; ov, ovary; p, penis; pr, prostate; rg, rectal gland; rt, right tentacle; sg, sperm-ingesting gland; t, testis.

Significant levels of TBT (up to 190 $ng.g^{-1}$) were measured in sediments and tissue of marine animals high in the food chain, including seals and dolphins (Tanabe *et al*., 1999; Takahashi *et al*., 2000, Sudaryanto *et al.,* 2004).

1.3.2.2 Tin-free self-polishing copolymer technology

TBT-SPC coatings have many advantages as AF coatings, as discussed previously. However, the damaging effect of TBT on non-target organisms cannot be ignored (see previous paragraph I.3.1.2.1). Consequently, TBT has been restricted as of the International Maritime Organization (IMO) conference in 1998, and these coatings have been banned from January 2008 (Yebra *et al.*, 2004). Therefore, TBT-free systems have been commercially introduced.

The tin-free AF coatings can be divided into two categories: controlled depletion systems (CDPs), and tin-free self-polishing copolymers (tin-free SPCs). Their AF efficacy lasts between 12 months (contact leaching coatings) and up to 90 months for the most efficient SPCs. These chemically active AF paints typically contain 35-50 wt.% of dicopper oxide as the main biocide, and less than 10 wt.% of co-biocides or booster biocides such as copper pyrithione, zineb, and DCOIT (4,5-dichloro-2-octyl-2H-isothiazol-3-one). The former coatings upgrade the traditional soluble matrix technology by incorporating modern reinforcing resins with the same AF mechanism as the conventional resin matrix paints. The latter coatings function in a similar manner to TBT-SPC but do not contain tin. Beside Zinc, the majority of tin-free AF paints currently available contain copper, and some contain silver (Lejars *et al*., 2012). Currently, the major copper compounds used for AF include metallic copper, cuprous thiocyanate, and cuprous oxide (Omae, 2003). Copper ions as Cu^{2+} have a major role in AF (Brooks and Waldock, 2008). Therefore, some booster biocides that are highly toxic to macroalgae, barnacles, and bryozoans are added to improve the AF proprieties. These biocides include Irgarol 1051 and diuron (Karlson and Elkund, 2004), copper pyrithione and Isothiazolone (Thomas, 2001) (see also Fig. 1.14).

There is concern about the influence of coatings containing copper and booster biocides on the marine environment (Marechal and Hellio, 2009, Trepos *et al*., 2014). The heavy metals do bioaccumulate in the internal organs of marine life. Numerous scientific publications have highlighted that copper is toxic also to a broad range of non-target marine species. The three most sensitive animal species are the embryo/larvae of the oyster, mussel and sea urchin; among microbes, the cyanobacteria are the most sensitive group to copper toxicity (Brooks and Waldock, 2009). Recent data have shown toxicity in salmon due to copper-induced olfactory impairment leading to neurobehavioral response changes critical to survival, which depends on successful migrations (Baldwin *et al*., 2003; Lee *et al*., 2010). Copper toxicity from AF sources becomes a problem in the marine environment, especially in isolated water bodies such as enclosed marinas and harbours that experience little water exchange combined with high levels of boating activity (Parks et al., 2010). For example, it has been estimated that in San Diego's Shelter Island Yacht Basin, an estimated 2.5 tons of copper leaches from the hulls of 2,000 boats each year. It is thus urgent to develop new environmentally friendly AF solutions. In the US, western states are leading the campaign against copper AF paints. Beginning in 2020 owners of recreational boats (under 65 feet) in Washington will be prohibited to buy and apply bottom paint that contains more than 0.5% copper. Starting in 2018, recreational boats on the market will need to be stripped of copper paint or sealed. In California, a similar bill to ban copper paints for recreational boats has been passed in the State Senate enforcing that from 2015, there will be a ban on the sale of new recreational boats with copper bottom paint, followed by total ban of usage for recreational boats in 2019.

Protection against biofouling and biodeterioration of renewable energy devices in the marine environment - both important areas in which the global society will invest more in the future - will be crucial for service life and efficiency. The solutions proposed in the past years have revealed numerous drawbacks and several approaches have been banned. The currently most used solution, paints containing copper, is not sustainable. It is thus urgent to develop new environmentally friendly AF solutions using new innovative concepts such as biomimetics or low-emission coatings.

1.3.3 Physical solutions

Fouling also occurs on the surfaces of living marine organisms and, e.g. leads to problems in aquaculture of seaweeds and shellfish (Gomez-Gil *et al*., 2004). Many sessile marine organisms do not possess any physical or mechanical means of defense against possible colonizers, or predators, but nevertheless resist colonization and overgrowth by epibionts. Studying the natural defenses against fouling of organisms has led to a biomimetic approach that can be used to better understand the structure and function of biological systems as models or inspiration for the sustainable design and engineering of materials and machines (Salta *et al.,* 2010). Ralston and Swain (2011) have reviewed the natural AF strategies developed by marine organisms and could classify them into four categories: chemical, physical, mechanical and behavioral. So far, with special focus on biofouling, two main topics have been studied for biotechnological applications: chemical defense and effects of surface topography. A strategy is to exploit physical defense mechanisms shown by some marine organisms, e.g. molluscan shells, sloughing of epidermis, and shark skin teeth. Some studies have attempted to replicate such natural hostile surfaces and assess possible AF activity. Most focus has been on microtextured surfaces (e.g. Berntsson *et al*., 2000a; Chambers *et al*., 2006; Scardino *et*

al., 2009). The effectiveness of topography as an AF strategy appears to be a function of the scale of surface texture and the settling organism (Ralston and Swain, 2011; Myan *et al*., 2013). One limitation of using microtextured surfaces in AF is that a specific scale of the microtexture may be effective only against some foulers, as e.g. micro- and macrofoulers differ significantly in their perception of hostile surfaces. Myan *et al.* (2013) concluded that macrotopographies (1-100 mm) are not suitable for AF applications as they are attractive to many marine fouling taxa such as hydroids, bryozoans and ascidians (Bixler *et al.*, 2012). Microtopographies (1 to ≤ 1000 µm), usually consisting of one uniform arrangement of geometry and scale, provide better AF efficiency but again depending on the fouling community. An interesting and promising approach to improve efficiency against several foulers is the development of multi-scale topographies (Bers and Wahl, 2004) consisting of periodic sine wave-like riblets (5 nm – 500 µm in wavelength) with smaller micro peaks (1 – 5 µm in width and height). Such multi-scale textures reduced both barnacle and mussel settlement (Bers and Wahl, 2004). The approach of surface modifications in AF has shown success against some fouling species or groups but has failed to achieve broad-spectrum fouling resistance and inhibition of biofilm formation. Concerns can also be raised about the long-term efficacy, which may be impaired due the build-up of biofilms, which will ultimately hide the original surface topographical features. Another constraint of micro-textured surfaces is that they need to be hydrodynamically smooth not to increase hull friction (Berntsson *et al*., 2000b). Similar to natural products there may be technological constraints in the upscale production of microtextured surfaces for AF coatings, despite continuing advances in large scale nano- and microfabrication.

Nevertheless, non-biocidal based approaches, relying on the physical properties of the surface either to deter the organisms from settling by topographical features or to

reduce their adhesion strength (fouling release) have shown promising results (Marabotti *et al.*, 2009; Martinelli *et al.*, 2011; Mielczarski *et al.,* 2010). The environmental cost/benefit analyses are here very good while economic cost/benefit analyses are still not comparable with the biocide-based coatings.

1.3.4 Biological methods

Some organisms can secrete enzymes or metabolites to inhibit the growth of their competitors. As early as 1991, it was reported that actives substances secreted by microalgae such as cyanobactericin (Abarzua *et al*., 1999) and fisherellin (Dahms et al., 2006) could inhibit the growth of diatoms (Cao *et al.*, 2011). Functional AF components have also been discovered in other organisms such as fungi (Dobretsov *et al*., 2006), sponges (Hansen *et al*., 2014), macroalgae (Hellio *et al*., 2002), microalgae (Dahms *et al*., 2004) and bacteria (Dobretsov and Qian, 2002).

1.3.4.1 Enzymatic methods

The application of enzymes as AF agents has been successfully investigated recently. Many types of enzymes, such as oxidoreductases, transferases, hydrolase, lyase, isomerase, and ligase, have been reported to have AF capabilities (Kristensen *et al*., 2008). From the perspective of enzymatic AF technology, biofouling problems are caused by the formation and reproduction of biofilms, and the adhesion of spores and larvae of macroorganisms. The functions of enzymes for AF applications can be divided into the following four categories: degradation of adhesives used for settlement, disruption of the biofilm matrix, generation of deterrents/biocides, and interference with intercellular communication (Kristensen *et al*., 2008). In the case of macrofouling, proteins and proteoglycans have a dominant role in the adhesion process.

Proteases can hydrolyse peptide bonds at different sites and it can be used to degrade mucilage based on peptide and hence prevent biofouling. For example, the attachment of *Ulva* sp. spores, barnacle cyprids and bryozoans can be effectively inhibited by serine protease (Dobretsov *et al*., 2007) without any toxic or deterrent effect. Quorum sensing has an important role in the formation of biofilms (see section I 1.2.1). Using AHL-acylase to degrade AHL may thus prevent the development of bacterial fouling (Leroy *et al.*, 2008) and then disrupt the macrofouling process.

To date the research for an active enzymatic AF method is facing several challenges. The seawater temperature ranges from –2°C to 30°C, which can largely affect enzyme catalytic activity and stability. Each enzyme will itself decompose if the temperature is too high, and then the lifespan of the enzymatic AF coating will also decrease. In addition, balancing the effectiveness and lifespan will be a major challenge. The design of an appropriate coating matrix to contain the enzymes will be another crucial step for successful application (Olsen *et al*., 2007).

1.3.4.2 Natural Products with AF activities

The ability of some organisms to chemically combat fouling by epibionts is associated primarily with production of so called secondary metabolites (de Nys *et al*., 1998; Hellio *et al*., 2002; Da Gama *et al*., 2014).

Secondary metabolites are compounds not involved in core life-supporting systems (the primary metabolism) and are commonly involved in chemical defense against predators, pathogens and also against fouling (e.g. McClintock and Baker, 2001). The discovery of potent AF compounds such as brominated furanones (Fusetani, 2004; De Nys, 1995), meriditerpenoid (Culioli *et al*., 2008), floridoside (Hellio *et al*., 2004), ianthelline (Hanssen *et al*., 2014), synoxazolidinones (Trepos *et al*., 2014) and pulmonarins (Trepos

et al., 2014) has highlighted the potential of natural products as a source of potent new AF compounds (Plouguerné *et al.*, 2010). A selection of marine AF compounds is shown in Figure 1.19.

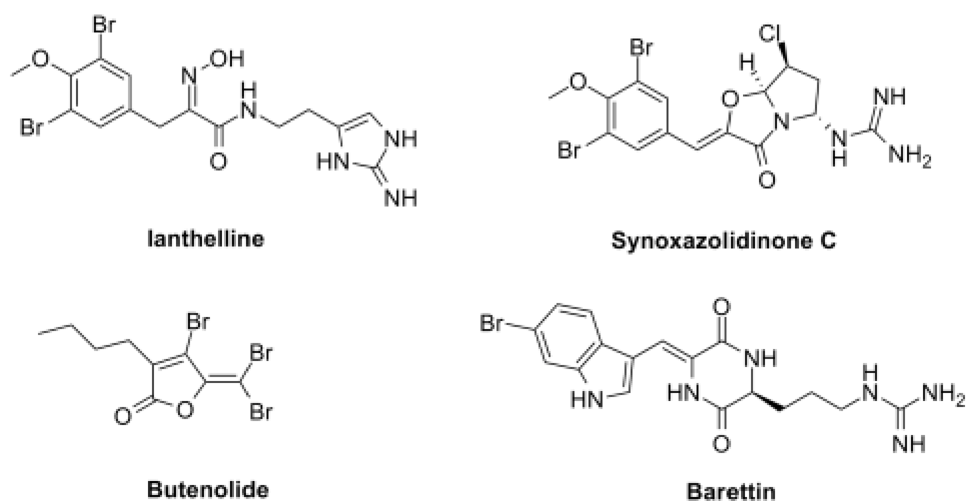

Figure 2.19: Examples of small halogenated natural AF compounds produced by sessile marine organisms that have been evaluated as new "green" AF compounds in biomimetic AF solutions.

The discovery and further development of a new lead compound with AF activity requires large quantities of product for laboratory screening, field assays, and tests of paint formulations (Hellio, 2010). However, the real challenge using natural products is the up-scaled production for the coating market. There are four main strategies to produce larger quantities of natural products. Firstly, natural products can be extracted from organisms collected in the field. This is the first step during development but is usually prohibitive during up-scaled production because of unsustainable use of natural resources. Controlled harvesting is an ideal solution when bioactive compounds are produced from unwanted biomass such as marine invasive species (Hellio *et al.*, 2004) but a major constraint consists of the potential natural variation of bioactivity (Hellio *et*

al., 2004; Maréchal *et al.*, 2004) and ecological impact of harvesting should be carefully monitored.

A second option is to extract natural products from cultured organisms and plants (e.g. capsaicin from pepper plants). Aquaculture and mariculture can be used as a possible strategy for production of marine natural products for initial studies. Economic projections suggest that in-sea culture can be a cost-effective option for the supply of natural compounds. Culturing may be a particularly successful strategy if the natural product has a microbial origin (e.g. avermectins). However, most organisms are very difficult to culture.

A third solution is to culture recombinant microorganisms that are transfected with genes involved in the synthesis of the natural product. This is usually only practical if the desired natural product is a direct gene product, i.e. a peptide. However, the recent development in *in vitro* synthesis using isolated enzymes may be used to design new synthetic pathways that produce novel bio-inspired compounds (e.g. Houssen *et al.*, 2014).

Finally, the fourth option is to produce the natural product through chemical synthesis. The success here depends on the cost of initial reactants and the number of synthetic reactions, which largely determines the yield of the final product. In practice, many natural products are too complex and expensive to synthesize and the synthesis of natural products is still considered highly inefficient (Li and Trost, 2008) as the exact build-up of functional groups with a complex molecule still represents a challenge. An attractive alternative is to synthesize analogues of the natural product that are less expensive but with similar bioactivity.

1.3.5 Combined AF methods

Based on experiences from research on natural products and surface topographies, there is still much to learn from the strategies used by marine organisms to inhibit epibiosis. AF solutions based on natural products or natural topographies face a key challenge, which is to achieve a good efficiency and to maintain surface performance for long periods. When looking closer at how most of the marine organisms repel fouling, it appears unlikely that a single natural AF mechanism (based on behavior, chemistry or physics) will be sufficient to prevent fouling. As demonstrated by Ralston and Swain (2011) most of the marine organisms that are successful at inhibiting epibiosis do indeed use a combination of defenses. So far, most of the research investigating biomimetic solutions have mainly focused on single mechanisms. Natural products often have specific bioactivity, e.g. AF effects against one or a few taxonomic groups of biofoulers. The advantage of specificity is that natural products may have a reduced broad-spectrum toxicity compared to traditional biocides such as copper, thus avoiding unwanted effects on non-target marine organisms. A potential problem with specificity is that one natural product may not offer sufficient AF protection. Many marine organisms counteract this by producing a cocktail of different AF compounds. Future, environmentally friendly AF technologies may thus depend on a combination of several strategies.

Several natural products may together cover a broader range of AF species (e.g. Sjögren *et al*., 2011). Natural products may also be combined with biocide-free technologies, e.g. silicone coatings that reduce adhesion and promote fouling release are boosted with natural products with AF effects (Barrios *et al*., 2005), or combined with hostile, textured surfaces (Maréchal and Hellio, 2009) (Fig. 1.20 and 1.21). Recently, a promising

study by Chapman (Chapman *et al.*, 2013) based on biomimicry showed synergistic AF effects between surface topography and chemistry.

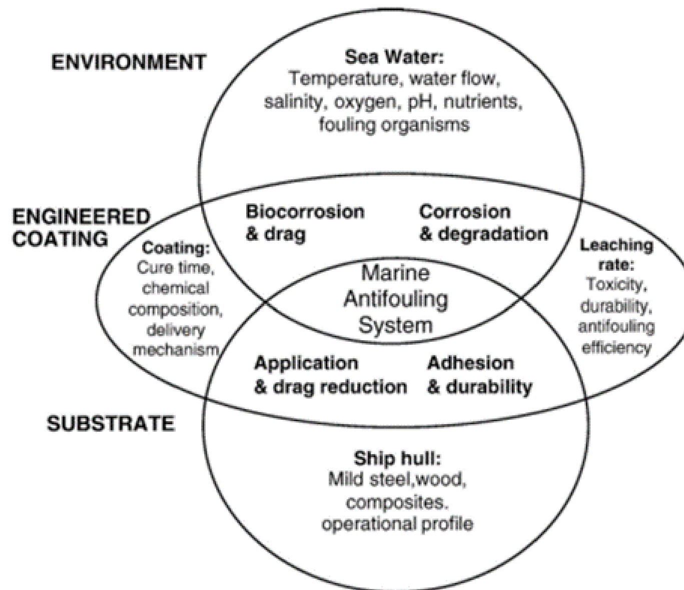

Figure 2.20: Key interactive parameters affecting AF coating system (Chambers *et al.*, 2006).

Must be:	Must not be:
Anticorrosive	Toxic to the environment
Antifouling	Persistent in the environment
Environmentally acceptable	Expensive
Economically viable	Chemically unstable
Long life	A target for non-specific species
Compatible with underlying system	
Resistant to abrasion/biodegradation/erosion	
Capable of protecting regardless of operational profile	
Smooth	

Figure 2.21: Requirement for an optimal AF coating (Chambers *et al.*, 2006).

The aim of their work was to create an AF material with a combined bio-inspired defence mechanism taken directly from two macroalgae: *Saccharina latissima* and *Fucus guiryi*.

For this purpose, the macroalgal surfaces were characterized and further replicated using polymeric reproduction methods. Concomitantly, the AF efficiency of a pre-extracted algal natural product (a brominated furanone) was evaluated. From this study, it appears that the highest AF potential was obtained when using a combination of chemistry and topography. These results demonstrate that future work should focus on combining several types of AF solutions and this opens the door to a new range of innovative solutions. There are still many challenges to develop AF solutions from natural products, such as difficulties and drawbacks in up-scaled production, but despite these, numerous fields studies provided proof of efficiency of AF coatings enriched with marine natural products (Chambers *et al.*, 2011; Acevedo *et al.*, 2013; Chambers *et al.*, 2014; Perez *et al.,* 2014).

1.3.6 Assessment of toxicity of new antifouling compounds

Bioassays have been developed to determine whether novel natural products inhibit organisms known to be implicated in biofouling. Bioassays may be based on growth, adhesion, toxicity and/or behavior (Briand, 2009). A reliable bioassay must be reproducible and use appropriate controls (negative and positive). The results can be expressed as effective concentration (EC), lethal dose (LD) or minimum inhibitory concentration (MIC). MIC is defined as the lowest concentration of a chemical that will inhibit the growth of a microorganism (Andrews, 2001). MICs are considered the "gold standard" for determining the susceptibility of organisms to antimicrobials (Andrews, 2001). Such parameters allow comparisons between the relative efficacy of a novel compounds and standard biocides tested under the same conditions. The biotechnological potential of bioassays lies in the screening of bioactive substance effectiveness as well as in the monitoring of their side-effects (Dahms and Hellio, 2009).

Owing to the new legislation, AF compounds had to be active at non-toxic concentrations against non-target species, as a result, ecotoxicity evaluation is now on the top list priority in the screening process. Toxicity tests have been developed primarily to screen bioactive compounds as potential antifoulants. Molecules or extracts are tested at the same concentrations against the same organisms (Briand, 2009). Toxicity assays are also frequently conducted in parallel to settlement and/or assays to assess the potential of active molecules to leach from what are intended to be environmentally benign coatings (Majumdar et al., 2008a).

Washington recently became the first US state to ban copper-based paints beginning in 2020 when owners of recreational boats (under 65 feet) will be prohibited to buy and apply bottom paint that contains more than 0.5% copper. Starting in 2018, recreational boats on the market will need to be stripped of copper paint or sealed. In California, a similar bill to ban copper paints for recreational boats has been passed in the State Senate enforcing that from 2015, there will be a ban on the sale of new recreational boats with copper bottom paint, followed by total ban of usage for recreational boats in 2019. Protection against biofouling and biodeterioration of renewable energy devices in the marine environment both important areas in which the global society will invest more in the future, will be crucial for service life and efficiency. The solutions proposed in the past years have revealed numerous drawbacks and several approaches have been banned. The currently most used solution, copper containing paints, is not sustainable. It is thus urgent to develop new environmentally friendly AF solutions.

The Biocidal Products Directive 98/8/EC require testing of new possible active substance to make AF paint, before marketing authorisation (Hellio and Yebra, 2009). In European biocidal legislation, the ecotoxicity of a biocide is specified as its acute effects on fish, invertebrate and algae combined with its environmental fate. Nevertheless, despite

numerous studies (Watanabe *et al*., 2007; Van dam *et al*., 2015; Booij *et al*., 2015) describing the negative impact of herbicides commonly used as AF (e.g. Diuron, Irgarol) on coral reefs, no specific assays are currently design to assess the toxicity of new AF compounds on corals and their symbionts. Most reefs building corals rely on symbiotic microalgae (genus *Symbiodinium*) to supply a substantial proportion of their energy requirements (Fig. 1.22). The *Symbiodinium* cells are localized within the endodermal cells (Jones *et al*. 2003, Yellowlees *et al*. 2008) in vacuoles surrounded by a host-derived membrane (Rands *et al*. 1993).

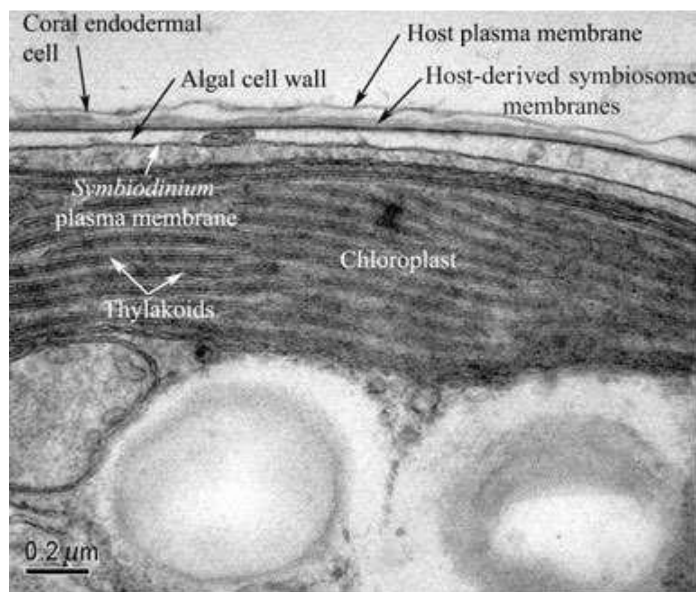

Figure 2.22: The arrangement of algal cellular structures, algal plasma membrane and cell wall in the cnidarian-*Symbiodinium* symbiosis (Yellowlees *et al.*, 2008).

Loss of *Symbiodinium sp*. have been linked to the coral bleaching event (Fig 1.23). Coral bleaching is defined as the loss of *Symbiodinium sp.* from their hosts. Coral bleaching is one of the major threats to corals and thus is an evidence that the reef coral symbiosis is obligatory for the coral hosts.

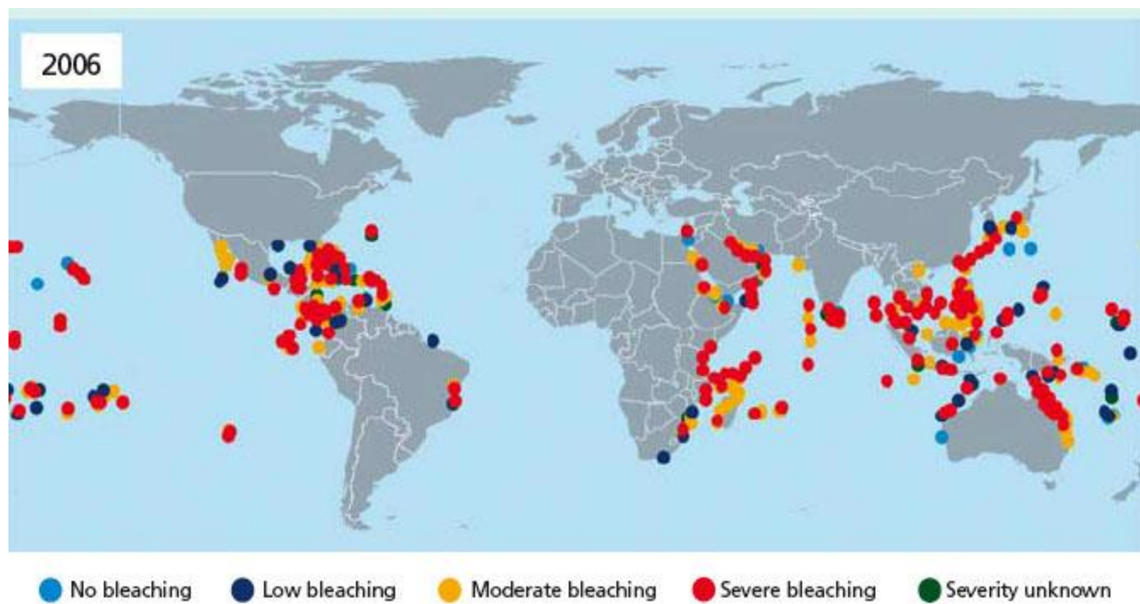

Figure 2.23: Global trends in coral bleaching (from Marshall and Schuttenberg, 2006).

In order to develop new eco-friendly AF solutions, it is of high importance to evaluate the impact of new AF compounds on key coral reef organisms.

1.4 Aims of the thesis

Within this project, we were aiming at the prevention of adhesion and growth of micro-organism by using anti-biofilm molecules of natural origin. For the research of compounds active towards marine strains of microorganisms, the compounds and extracts were tested against 10 strains of marine bacteria and 4 strains of microphytobenthic microalgae (Baltic sea, Atlantic Ocean): *Cylindrothecca closterium* (AC 170), *Amphora coffeaformis* (AC 231), *Porphyridium purpureum* (AC 121) *and Pleurochrysis roscoffensis* (AC 32). These organisms are known to be involved in the fouling process (Chambers *et al*., 2011; Marechal *et al*., 2004). Among the bacteria, five of the strains: *Halomonas aquamarina (ATCC 14400), Polaribacter irgensii (ATCC 700398), Shewanella putrefaciens (ATCC 8071), Roseobacter Littoralis (ATCC 495666) and Pseudomonas Elyakovii* are commonly involved in biofilm formation and the other five strains*: Vibrio aestuarianus (ATCC 35048), Vibrio harveyi* (ATCC 14126), *Vibrio natriegens (ATCC 14058), Vibrio proteolyticus (ATCC 53559) and Vibrio carchariae (ATCC 35084)* are known pathogens. The inhibition of adhesion and growth of bacteria and microalgae and the toxicity of the products on microorganisms (bacteria and microalgae) were assessed.

Chapter 1 provides an introduction to the biofouling process and the research of new AF compounds. The inhibition of marine biofouling by the bromotyrosine derivative ianthelline, isolated from the Arctic marine sponge *Stryphnus fortis*, was tested in several bioassays (**Chapter 2)**. The antifouling properties of four members belonging to the recently discovered synoxazolidinone and pulmonarin families – both isolated from the sub-Arctic sessile ascidian *Synoicum pulmonaria,* collected off the Norwegian coast – and four simplified synthetic analogs were assessed (**Chapter 3**). Thirteen short synthetic amphiphilic cationic micropeptides derived from antimicrobial iron-binding

innate defense protein lactoferrin found in breast milk and tear fluid have been evaluated for their capacity to inhibit the marine fouling process (**Chapter 4**). The potential toxicity of two commercial biocides (SEA-NINE and Irgarol), one synthetic biocide (thiram) and two biocides of natural origin (myristic acid and Totarol) were assessed at environmental concentrations, toward the survival of two species of *Symbiodinum* sp.: *Symbiodinium microadriaticum* CCMP 2467 and *Symbiodinium* sp. AC 561 (**Chapter 5**). Finally, **Chapter 6** of this thesis presents the conclusion of the research and recommendations for future research investigations.

Among these chapters, **Chapter 2** has been published in *Marine Biotechnology:* The bromotyrosine derivative ianthelline isolated from the arctic marine sponge *Stryphnus fortis* inhibits marine micro- and macrobiofouling, 2014, 16(6), 684-94. **Chapter 3** has been published *in Journal of Natural Product*: Antifouling Compounds from the Sub-Arctic Ascidian *Synoicum pulmonaria*: synoxazolidinones A and C, pulmonarins A and B, and synthetic Analogs, 2014, 77(9). **Chapter 4** has been accepted in publication in *Biofouling*: Evaluation of cationic micropeptides derived from the innate immune system as inhibitors of marine biofouling, 2015, 31(4), 393-403. 2105-13.

Chapter 2

Chapter 3 : The Bromotyrosine Derivative Ianthelline Isolated from the Arctic Marine Sponge *Stryphnus fortis* Inhibits Marine Micro- and Macrobiofouling

2.1 Abstract

The inhibition of marine biofouling by the bromotyrosine derivative ianthelline, isolated from the Arctic marine sponge *Stryphnus fortis*, is described. All major stages of the fouling process were investigated. The effect of ianthelline on adhesion and growth of marine bacteria and microalgae was tested to investigate its influence on the initial microfouling process comparing with the known marine antifoulant barettin as a reference. Macrofouling was studied via barnacle (*Balanus improvisus*) settlement assays and blue mussel (*Mytilus edulis*) phenoloxidase inhibition. Ianthelline was shown to inhibit both marine micro- and macrofoulers with a pronounced effect on marine bacteria (minimum inhibitory concentration (MIC) values 0.1–10 µg/mL) and barnacle larval settlement (IC_{50}=3.0 µg/mL). Moderate effects were recorded on *M. edulis* (IC_{50}=45.2 µg/mL) and microalgae, where growth was more affected than surface adhesion. The effect of ianthelline was also investigated against human pathogenic bacteria. Ianthelline displayed low micromolar MIC values against several bacterial strains, both Gram positive and Gram negative, down to 2.5 µg/mL. In summary, the effect of ianthelline on 20 differents representatives marine antifouling organisms and seven human pathogenic bacterial strains is presented.

This work has been published in a refereed journal, Marine Biotechnology, and is presented below in identical form.

The citation for the original publication is:

Kine Hanssen, Gunnar Cervin, Rozenn Trepos, Julie Petitbois, Torn Haug, E Hansen, JH Andersen, Claire Hellio, Johan Svenson. (2014). The bromotyrosine derivative Ianthelline isolated from the arctic marine sponge *Stryphnus fortis* inhibits marine micro- and macrobiofouling. Marine Biotechnology, 16(6):684-94.

The author designed the experiments and performed the antibacterial and antimicroalgal assays. The co-authors have collected the organisms and purified the compounds and performed the *Balanus* cyprids settlement assays and the phenoloxydase assay. The manuscript was written in collaboration with the co-authors.

2.2 Introduction

Rapid colonisation and successive overgrowth of epibiotic organisms on a surface immersed in water is a process known as biofouling. It is a fast and complex sequence of events which is initiated by surface adhesion of organic molecules, followed by the rapid settlement of microfoulers (marine bacteria, protozoans and microalgae) and, subsequently, macrofoulers (macroalgae and invertebrates) (Yebra *et al*., 2004; Qian *et al*., 2007). Biofouling is a natural process, yet it is highly undesired for a wide range of marine economic sectors such as shipping, aquaculture and the offshore petroleum industry. The growth on associated man-made structures leads to substantial economic losses in these sectors mainly due to increased fuel usage, maintenance costs and process parameter interference (Yebra *et al*. 2004; Fitridge *et al.* 2012). Employment of antifouling (AF) paints has been the most successful strategy to prevent biofouling on such structures (Almeida *et al*. 2007).

The most efficient AF compounds such as organotin and copper oxide have been shown to come with several serious environmental drawbacks (Nakanishi, 2008; Okoro *et al*. 2011; Kotake, 2012) due to the collateral damage inflicted on the marine environment and non-target species. The high toxicity and induction of imposex, i.e. the development of male characteristics by females, amongst marine molluscs (Ellis and Pattisina 1990; Nakanishi, 2008) resulted in a total ban on the use of organotin-based compounds in the marine environment, which was implemented in 2008.

Not only man-made structures submerged in water but also sessile marine organisms such as sponges and tunicates serve as surfaces for biofouling and are under a constant threat of being overgrown by epibionts. Despite this, many sessile marine organisms appear to have developed strategies to combat epibiosis and maintain a clean exterior surface, free of settling species (Muller *et al*. 2013). This observation has motivated the

search for marine compounds with AF activities from their arsenal of secondary metabolites (Tsukamoto *et al*., 1996; Fusetani, 2004; Hellio *et al.* 2005, Fusetani, 2011). Marine sponges in particular are a rich source of bioactive secondary metabolites, some with documented AF activity (Sipkema *et al*., 2005; Stowe *et al*., 2011; Gerwick and Moore, 2012). Sponges are primitive sessile filter-feeders that lack a specialised immune system and the ability to remove themselves from predators and settling organisms. To prevail, they have developed physical and chemical defence strategies to protect themselves against over-growth by epibionts (Muller *et al.*, 2013). Sponges commonly host a range of microbial symbionts that may produce compounds protecting their host. Sponges therefore represent a particularly rich marine source for discovering novel compounds (Ortlepp *et al*., 2007; Gerwick and Moore, 2012; Gerwick and Fenner, 2013).

One group of marine compounds described in the literature that display particular potential as antifoulants are bromotyrosine-derived sponge metabolites (Ortlepp *et al.,*2007). Several such compounds have been studied, and their inhibition of balanide larval settlement has been described (Ortlepp *et al*., 2007). Similar brominated marine compounds such as Barettin and Oroidin (Fig. 2.1) have also been extensively investigated as potential leads for additives in marine paints as “green” settlement deterrents (Yamada *et al*., 1997; Sjogren *et al*., 2004). Both barretin and Oroidin and their synthetic derivatives have been incorporated in paint formulations and shown to prevent biofouling in field studies (Sjogren *et al*. 2004; Melander *et al*. 2009). Synthetic analogues of bromotyrosines isolated from sponges have further been shown to inhibit phenoloxidase from blue mussels at low micromolar concentrations (Bayer *et al*., 2011). Following on the ban on organotin compounds, marine natural products have received an increased focus and have even been heralded as the way forward towards

environmentally friendly AF solutions (de Nys and Steinberg, 2002; Bhadury and Wright, 2004; Marechal and Hellio, 2009; Qian *et al*., 2010). Searches for such compounds within the phylum Porifera are ongoing and provide sound basis that an environmentally friendly compound may be isolated from marine sponges (Tsukamoto *et al*. 1996; Santos Acevedo *et al.*,2013). Being of natural origin does not guarantee that the marine AF compounds are non-toxic, but they come with the advantage of having been tuned by evolution for their task. The ideal "green" AF compound should be deterring rather than toxic to the target species (Dobretsov *et al.*, 2006; Chambers *et al.* ,2006; Marechal and Hellio 2009).

In this study, we report the AF activities of ianthelline (displayed in Fig. 2.1 together with structurally related marine AF compounds), a derivative isolated from the Arctic sponge *Stryphnus fortis* (Hanssen *et al.;* 2012).

The structure of ianthelline was determined in 1986 (Litaudon and Guyot, 1986), and its synthesis was recently described by Shearman *et al.* (2010).

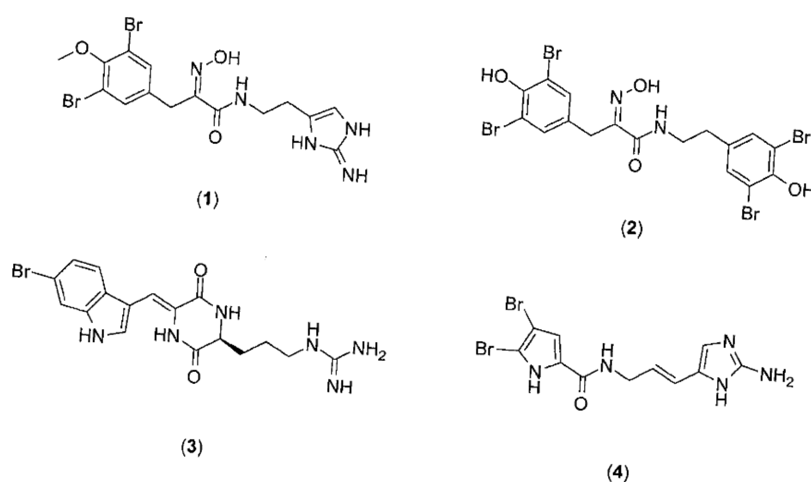

Figure 3.1: Structure of ianthelline (**1**) and structurally and marine related compounds that have been evaluated as AF compounds: 5,5'-dibromohemiBastadin-1 (**2**), Barettin (**3**) and Oroidin (**4**)

Ianthelline was originally isolated from the Caribbean sponge *Ianthella ardis* and has been shown to be a major secondary metabolite in several other warm water sponges (Litaudon and Guyot, 1986; Ciminiello *et al*., 1995). It is a bioactive compound, and the antitumoral activity of ianthelline was published in 2012 by Hanssen *et al*. The initial isolation paper briefly reported antibacterial and antifungal bioactivities against one bacterial and one fungal strain (Litaudon and Guyot, 1986). Apart from those studies, little is known about the potential bioactivities of Ianthelline, and its ecological role is not understood. A fractionated extract from the Caribbean sponge *Aiolochroia crassa* containing Ianthelline has been reported to inhibit the attachment of marine bacteria to agar blocks (Kelly *et al*., 2003 and 2005). The pooled fractionated extracts prepared in that particular study was studied at natural volumetric concentrations. Thus, the amount, purity, and bioactive concentration of ianthelline were unknown. The attachment inhibition of *Vibrio harveyi* was examined, and growth values ranging from 14 to 61 %, compared to growth control, in five different fractions rich in ianthelline was reported (Kelly *et al.,*2005).

The optimisation of synthetic bromotyrosine derivatives for phenoloxidase inhibition revealed a pharmacophore that is highly analogous to the substructures displayed by ianthelline, suggesting additional bioactivities for this compound (Bayer *et al*., 2011). Ianthelline contains an oxime moiety, postulated by Proksch and co-workers, to be essential for AF activity, and it represents an interesting compound for further studies towards AF solutions (Ortlepp *et al*., 2007).

The present study is thus an important continuation of the previously limited studies of the bioactivity and potential ecological role of ianthelline and also represents an assessment of the exploitability of ianthelline for biotechnological and medical purposes. Herein, we describe how ianthelline is isolated from an Arctic source and

tested in a wide array of assays (Svenson, 2013). In the marine environment, biofouling is undertaken by a great diversity of species, and marine AF compounds should therefore ideally possess a broad-spectrum activity to contend with the vast amount of epibionts. The effect of ianthelline on the growth and settlement inhibition of 10 marine bacterial and 8 microalgal strains relevant to the formation of the biofouling film as well as studies on *Balanus improvisus* barnacle larvae settlement inhibition is described. A potential effect on bivalves is also studied using the *Mytilus edulis* phenoloxidase assay. Finally, the effect of ianthelline on a test panel of seven human pathogenic bacterial strains is investigated to evaluate the inhibitory effect on medically important strains. Comparisons with relevant structurally related, marine secondary metabolites are included and discussed.

2.3 Materials and Methods

2.3.1 Organism and Purification of ianthelline and barettin

The specimen of *S. fortis* was collected off the coast of Spitsbergen (79° 33’ N, 8° 53’ E) in September 2007. Extraction, purification and structure determination of ianthelline was carried out as previously described (Hanssen *et al*., 2012).

For comparison, barettin was isolated from *Geodia barretti*, collected off the coast of Tromsø, Norway, in 2009. The compound was extracted according to previous methods and purified from a desalted 40 % RP-SPE extract (Tadesse *et al*., 2008) using a Waters SunFire Prep C18 HPLC column (10×250 mm, 5 µm particle size). A linear gradient from 15 % to 30 % acetonitrile in ultra-pure water containing 0.1 % TFA (*v/v*) was applied at 4 mL/min over 50 min to elute 3. The compound was identified by high-resolution mass spectrometry (HR-MS and HR-MS/MS) using a LTQ Orbitrap XL Hybrid Fourier Transform

mass spectrometer (Thermo Fischer Scientific, MA, USA) and by comparison with previously published data on barettin (Lidgren and Bohlin, 1986; Solter *et al*., 2002).

2.3.2 Marine Antibacterial Assays

10 marine bacterial strains from the bacterial collection of the University of Portsmouth, School of Biological Sciences were used in the current study (Table 2.1).

These selected strains are representative of fouling species in both estuarine and marine environments (Chambers *et al*., 2011). They were grown at 26 °C in a marine medium, composed of 0.5 % peptone (neutralised bacteriological peptone, Oxoid LTD) in filtered (Whatman 1,001-270, pore size 11 µm) natural seawater from the Solent (UK). Ianthelline and Barettin were dissolved in 100 % methanol (general purpose grade, Fisher Chemical) and transferred to clear polystyrene 96-well plates (Fisher Scientific), dried under vacuum and sterilised under UV illumination.

Wells were prepared using concentrations of 0.01, 0.1, 1 and 10 µg/mL, and each concentration was replicated six times (Bressy *et al.*, 2010). Both growth and adhesion inhibition were studied.

Table 3.1: Marine bacterial strains used in the present study

Marine bacteria	ATCC[1]
Halomonas aquamarina	14400
Polaribacter irgensii	700398
Pseudoalteromonas elyakovii	700519
Roseobacter litoralis	49566
Shewanella putrefaciens	8071
Vibrio aestuarianus	35048
Vibrio carchariae	35084
Vibrio harveyi	700106
Vibrio natriegens	14058
Vibrio proteolyticus	53559

[1]American tissue culture code

2.3.2.1 Growth inhibition experiments

100 µL of bacterial culture (2×10^{8} colony forming units (CFU) mL^{-1}) was added to each well under aseptic conditions and incubated for 48 h at 26 °C. The minimum inhibitory concentration (MIC) was defined as the minimum concentration resulting in no change in optical density at 630 nm after incubation for 48 h (Thabard *et al.*, 2011).

2.3.2.2 Adhesion inhibition experiments

Microplates were prepared and inoculated as stated above. After a 48-h incubation, wells were emptied and rinsed once with 100 µL of sterile seawater to remove the non-attached cells and air-dried at room temperature. The remaining bacterial biofilm was

stained with 100 µL of 0.3 % (*v/v*) aqueous crystal violet, and the optical density (OD) was measured at 595 nm (Sonak and Bhosle, 1995).

Media was used as blank, and MIC was defined as the lowest concentration that produced a reduction in adhesion.

2.3.3 Microalgal Assay

8 pure, but non-axenic, marine microalgae (obtained from Algobank, Caen, France) were used in this study (Table 2.2).

All the included strains are involved in surface colonisation and can lead to increased rates of biocorrosion (Jellali *et al*., 2013). Each algal strain was grown for 5 days prior to use at 20 °C in F/2 medium.

After 5 days, microalgal concentration was assessed via analysis of the chlorophyll *a* content (Chambers *et al*., 2011), and dilutions of the stock culture were made accordingly to generate stock solutions of each microalgae containing 0.1 mg/L chlorophyll *a*. 100 µL of the stock solutions were transferred to the wells of black 96-well plates prepared with ranging concentrations of ianthelline and barettin as described above. Both adhesion and growth inhibition were studied.

Table 3.2: Microalgae used in the present study

Microalgae	**Algobank code**
Cylindrotheca closterium	AC 170
Exanthemachrysis gayraliae	AC 15
Halamphora coffeaeformis	AC 713
Pleurochrysis roscoffensis	AC 32
Porphyridium purpureum	AC 122
Hymenomonas coronata	AC 115
Rhodosorus marinus	AC 119
Pleurochrysis carterae	AC 1

2.3.3.1 Growth inhibition experiments

The inoculated plates were grown for 5 days under constant light exposure (140 µmol m^{-2} s^{-1}) at 20 °C. After the incubation, the microplates were centrifuged at 4,100 rpm for 10 min at 4 °C using a Beckman Coulter Allegra 25R centrifuge and subsequently emptied. 100 µL of 100 % methanol was added to each well to liberate chlorophyll *a*. The pigment concentration was quantified employing the fluorimetric method (Chambers *et al*., 2011). MIC values were calculated as explained for bacteria (based on OD measurements).

2.3.3.2 Adhesion inhibition experiments

Black microplates were prepared and inoculated as stated above. Media were used as blank. After 5 days of incubation, the medium was gently removed using a multichannel pipette to eliminate all the non-attached cells. 100 µL of 100 % methanol was then

added to each well to liberate chlorophyll *a*. The pigment concentration was quantified employing the fluorimetric method explained above.

2.3.4 Balanus Cyprid Settlement

Cyprid larvae of *B. improvisus* were reared in a laboratory cultivating system at Tjärnö Marine Biological Laboratory, Sweden, as described by Berntsson *et al.* (2000). The settlement assays with *B. improvisus* cyprids were conducted in non-treated polystyrene Petri dishes (Ø 48 mm, Nunc #150340) containing 10 mL filtered (0.2 µm) seawater.

Iantheline was dissolved in DMSO and then serially diluted with DMSO to give the desired concentration series of which 10 µL was added to each test dish.

A total of 18–22 newly moulted cyprids were added to each test dish which were incubated at room temperature (20–25 °C) for 5 days. At the end of the experiment, the number of metamorphosed juvenile barnacles, as well as live and dead cyprids, was assessed under a dissection microscope. Each concentration of ianthelline was replicated four times (n=4), and dishes with 10 µL DMSO served as controls. The concentration of ianthelline leading to 50 % inhibition of the settlement compared to the control was reported as the IC_{50} value.

2.3.5 Inhibition of Phenoloxidase from the Blue Mussel *M. edulis*

Phenoloxidase was isolated from the byssus gland of the blue mussel (*M. edulis*) as previously described (Hellio *et al*., 2000; Bayer *et al*., 2011). Solutions of 1 mg/mL *M. edulis* phenoloxidase, 0.4 mM $_{L}$-dihydroxyphenylalanine (DOPA), and ianthelline (2–2,000 µg/mL) were prepared. 1ml of the LDOPA solution, 10 µL of ianthelline (or 10 µL of water in the case of the control samples), and 10 µL *M. edulis* phenoloxidase (added last) were mixed in a test tube. Phosphate buffered saline (pH 6.8) was added to the

solution to make a total volume of 2 mL. The solution was placed in a cuvette, and the change in absorbance at 475 nm was measured after 30 min using a UV-VIS spectrophotometer. The blank control used was the sample without the enzyme solution. The assay was run in triplicate, and the concentration of ianthelline leading to 50 % inhibition of the phenoloxidase activity result was reported as the IC_{50} value.

2.3.6 Terrestrial Antibacterial Assay

The MIC of ianthelline was determined against seven human pathogenic bacterial strains presented in Table 2.3.

Enterococcus faecalis and *Streptococcus agalactiae* were grown in brain-heart infusion broth (BHI; Oxoid, Hampshire, England). *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli* and *Pseudomonas aeruginosa* were grown in Mueller Hinton Broth (MH; Merck, Darmstadt, Germany), and Staphylococcus epidermidis in tryptic soy broth (TS; Merck, Darmstadt, Germany). Both growth and biofilm inhibition studies were performed.

Table 3.3: Human pathogenic bacterial strains used in the current study

Bacterial strain	**ATCC[1]**
Gram positive	
Staphylococcus aureus	25923
MRSA	33591
Staphylococcus epidermidis	35984
Streptococcus agalactiae	12386
Enterococcus faecalis	29212
Gram negative	
Escherichia coli	25922
Pseudomonas aeruginosa	27853

[1]American tissue culture code

2.3.6.1 Growth inhibition experiments

Suspended bacteria in log phase grown at 37 °C in growth medium were added to 96-well microtiter plates resulting in 1,500–15,000 CFU/mL. A serial dilution of ianthelline was subsequently added and left to inoculate for 24 h before growth inhibition was observed with a Victor multilabel counter at 600 nm. Growth medium diluted with water (1:1) was used as negative control, and bacteria suspension diluted with water (1:1) was used as positive control. Gentamicin at ranging concentrations from 0.015 to 16 µg/mL was used as positive assay control. The MIC was defined as the minimum concentration resulting in no change in optical density after incubation for 24 h at 37 °C. Ianthelline was tested at concentrations ranging from 2.5 to 160 µg/mL.

2.3.6.2 Inhibition of biofilm formation

S. epidermidis was used to assess the effect of ianthelline on biofilm formation. An overnight culture of *S. epidermidis* grown in TS was diluted with fresh TS containing 1 % glucose (1:100). Aliquots of 50 µL were transferred to a 96-well microtiter plate, and 50 µL of ianthelline, dissolved in water at ranging concentrations, was added. After overnight incubation at 37 °C, the bacterial suspension was carefully discarded and the wells washed with water. The plate was dried and the biofilm fixed by incubation for 1 h at 55 °C before the surface attached cells were stained with 100 µL of 0.1 % crystal violet for 5 min. The crystal violet solution was removed and the plate once more washed with water and dried at 55 °C for 1 h. After adding 70 µL of 70 % ethanol, the plate was incubated at room temperature for 10 min. Biofilm formation was observed by visual inspection of the plates.

The MIC was defined as the lowest concentration where no biofilm formation was visible. A *S. epidermidis* suspension, diluted with 50 µL of water, was used as a positive control, and 50 µL *Staphylococcus haemolyticus* suspension with 50 µL of water was employed as a negative control. A mixture of 50 µL water and 50 µL TS was used as assay control.

2.4 Results and Discussion

Biofouling is a complex chemical and biological process, and no single bioassay can be used to accurately imitate it. Several different assays and organisms are hence needed to evaluate the AF potential of a compound (Briand, 2009; Dahms and Hellio, 2009). The progression from adsorption of organic molecules to a marine surface to the growth of macroepibionts such as macroalgae, crustaceans and mussels also involves

microorganisms such as bacteria and microalgae. It is therefore unrealistic to assume that a single non-toxic AF compound will display activity against all organisms associated with it. In fact, limited access to comprehensive screens against all the different stages of the AF process may lead to the discovery of promising compounds that are later shown to be inactive in complementary studies against other types of foulers (Qian *et al*., 2010). In the current study of ianthelline, we have included bioassays targeting the main groups of marine organisms involved in the different stages of the biofouling process. The well-established AF brominated diketopiperazine barettin (3), isolated from *G. barretti*, was included for comparison and as a reference in selected assays.

The initial microfouling of bacteria and microalgae was studied via both adhesion and growth inhibition assays. It is generally accepted that the microfouling can facilitate the macrofouling (Beech *et al.,* 2005; Qian *et al*., 2007).

Targeting the initial settlement and production of these marine biofilms may thus have beneficial effects for limiting the fouling of macroorganisms. The presence of such marine biofilms on surfaces also increases the rate of biocorrosion (Beech and Sunner, 2004). The growth inhibition was studied via standard serial dilution methods against ten relevant marine bacterial strains. Since biofouling is a process associated with the adhesion of organisms to a surface, the study of bacterial attachment is highly relevant and that was therefore also studied for the same bacterial strains. In addition, eight microalgal strains including the diatoms *Cylindrotheca closterium* and *Halamphora coffeaeformis* were also studied. Diatoms represent particularly relevant strains as they commonly rapidly form resilient slimy layers on marine surfaces (Molino and Wetherbee, 2008). The data from the microorganism screening is compiled in Table 2.4.

Table 3.4: The effect of a range of concentration (0.01-10µg/ml) of ianthelline (1) and barettin (3) on the growth and adhesion of fouling of marine bacteria and microalgae. Results are expressed as MIC (µg/ml) (*n*=6).

Microorganism	**MIC 1 (µg/mL)**		**MIC 3 (µg/mL)**	
	Growth	Adhesion	Growth	Adhesion
Marine bacteria				
Halomonas aquamarina	>10	>10	>10	>10
Polaribacter irgensii	1	>10	>10	>10
Pseudoalteromonas elyakovii	1	>10	>10	>10
Roseobacter litoralis	1	>10	>10	>10
Shewanella putrefaciens	0.1	>10	10	>10
Vibrio aestuarianus	0.1	0.1	0.01	>10
Vibrio carchariae	>10	>10	>10	>10
Vibrio harveyi	10	>10	>10	>10
Vibrio natriegens	>10	0.1	>10	10
Vibrio proteolyticus	10	>10	10	>10
Microalgae				
Cylindrotheca closterium	>10	>10	0.1	0.1
Exanthemachrysis gayraliae	>10	>10	>10	>10
Halamphora coffeaeformis	>10	>10	>10	>10
Pleurochrysis roscoffensis	10	>10	>10	>10
Porphyridium purpureum	>10	>10	0.1	1.0
Hymenomonas coronata	1	n.d.[1]	n.t.[2]	n.t.[2]
Rhodosorus marinus	1	n.d.[1]	n.t.[2]	n.t.[2]
Pleurochrysis carterae	0.1	n.d.[1]	n.t.[2]	n.t.[2]

[1]Not determined due to unfavourable growth conditions for the adhesion study

[2]Not tested

Only concentrations of ianthelline at or below 10 µg/mL were evaluated for the microorganism studies. This represents a threshold for compounds to be regarded as highly active and of further interest from a biotechnological viewpoint (Hellio *et al*., 2009). From Table 2.4, it is clear that ianthelline has a pronounced effect on the inhibition of marine bacterial growth. All the tested marine bacteria are Gram-negative, and most display MIC values below 10 µg/mL, some as low as 0.1 µg/mL. Only the growth of three bacteria (*Halomonas aquamarina*, *Vibrio carchariae* and *Vibrio natriegens*) of the tested ten strains tested remained unaffected by Ianthelline at 10 µg/mL, illustrating a strong and broad antibacterial activity. In the bacterial adhesion studies, the MIC values were higher. Only the two *Vibrio* species *V. natriegens* and *Vibrio aestuarianus* displayed high sensitivity against Ianthelline with MIC values of 0.1 µg/mL. The high sensitivity of *V. natriegens* in the adhesion assay is of interest as it displayed a MIC >10 µg/mL in the growth assay. *V. natriegens* is a very rapidly growing sulfate-reducing bacterium found on microfouled surfaces (Cheng *et al.,* 2009). Sulfate-reducing bacteria are especially known for their role in biocorrosion (Beech and Sunner, 2004), and finding a compound inhibiting the adhesion of *V. natriegens* would be highly interesting for reducing the biocorrosion process. The oyster industry has grown to be very important for many regions of the world, contributing substantially to social and economic activity in the coastal zones. *V. aestuarianus* is a known pathogen of the commercial pacific oyster *Crassostrea gigas* (Labreuche *et al*., 2006; De Decker *et al*., 2011) causing massive mortality outbreaks and a shortage in shellfish. ianthelline inhibited the growth and adhesion of *V. aestuarianus* at very low concentrations.

In previous studies, ianthelline has been included in unspecified natural concentrations which are most likely significantly higher than those in the current study.

This is based on the amounts of similar brominated secondary metabolites found and analysed in other tropical sponge extracts which frequently contain such compounds (e.g. Oroidin, aeroplysinin and sceptrin) in high (0.8-4.9 mg/mL) natural volumetric concentrations (Kelly *et al*., 2003). *S. fortis* is also rich in ianthelline, and approximately 0.05 % of the organism wet weight is ianthelline, indicating that it is an important secondary metabolite. The antibacterial activity of ianthelline is similar to the bromotyrosine derivative zamamistatin isolated from the Okinawan sponge *Pseudoceratina purpurea* by Takada *et al*. (2001). In comparison to barettin, which is both structurally related and a thoroughly studied marine AF compound, ianthelline displays a higher antibacterial activity. Barettin previously isolated from *G. barrette* was included in the antimicrobial assay and was active against both *Vibrio proteolyticus* and *Shewanella putrefaciens* at 10 µg/mL and highly active (MIC=0.01 µg/mL) against *V. aestuarianus* in analogy to ianthelline. In the bacterial adhesion assay, barettin was only active at 10 µg/mL against *V. natriegens*. No other bacterial strains were affected in the concentration range employed.

Microalgae forms slimy layers on marine surfaces that are generally challenging to prevent (Molino and Wetherbee, 2008), and it was shown that ianthelline was active against half of the tested microalgal strains. *Hymenomonas coronata*, *Rhodosorus marinus* and *Pleurochrysis carterae* were all sensitive to ianthelline in solution with MIC values ranging from 0.1 to 1 µg/mL while *Pleurochrysis roscoffensis* displayed a MIC value of 10 µg/mL. The other four strains, including the two diatom species, were unaffected at the tested concentrations. In analogy to the bacterial studies, microalgal adhesion was also evaluated. No effect on algal adhesion was seen for ianthelline.

Growth conditions for the three most sensitive species in the growth assay were unfavorable at the time of the adhesion studies, and no data could be obtained for those species.

No algal toxicity was seen upon extended exposure times. In contrast, barettin was active against both the growth and adhesion of the diatom *C. closterium* at 0.1 µg/mL and *Porphyridium purpureum* at 0.1 and 1 µg/mL, respectively.

The major macrofoulers, barnacles and mussels, were studied via both enzyme and settlement inhibition assays. Attachment of bivalves was studied by inhibition studies of phenoloxidase isolated from the byssus gland of the common blue mussel, *M. edulis*. Inhibition of the production of byssus threads by mussels has become an established method for evaluating their settlement (Hellio *et al*., 2000). Other marine invertebrates such as barnacle larvae also depend on phenoloxidase for generating a secure surface attachment. The function of phenoloxidase is to oxidise both phenylalanine and tyrosine in protein secretions to quinone derivatives that crosslink to generate a strong biopolymer enabling the organism to anchor to a surface (Bayer *et al*., 2011). The release of active phenoloxidase is controlled by Ca^{2+} -dependent signalling pathways, and therefore, the intracellular concentration of Ca^{2+} is thus also an interesting target for the development of AF strategies. An inhibitory effect of ianthelline on phenoloxidase was seen at 1 µg/mL, and the IC_{50} was determined to 45.2 µg/mL. Such an IC_{50} suggests only moderate affinity of ianthelline for the phenoloxidase despite the α-oxo-oxime functionality previously reported to be beneficial for blue mussel phenoloxidase inhibition. The synthetic 5,5'-dibromohemiBastadin-1 (2 in Fig. 2.1) is a strong phenoloxidase inhibitor with an IC_{50} value of 0.84 µg/mL (Bayer *et al*., 2011). Ianthelline and 5,5'-dibromohemiBastadin- share several structural features such as the dibrominated phenol ring, the overall molecular length and the placement of the α-oxo-

oxime functionality. The α-oxooxime functionality has been shown to be involved with complexation of the two copper(II) ions coordinated by six histidine residues in the catalytic centre of the phenoloxidase (Kim and Uyama, 2005), and the oxime functionality was crucial for the strong inhibition of synthetic bastadin derivatives (Bayer *et al*., 2011). The main structural differences between ianthelline and 5,5'-dibromohemibastadin lie in the methoxylated phenolic hydroxyl in ianthelline and the cationic iminoimidazole ring. The inhibitory effect of ianthelline was reduced by nearly 50-fold in comparison to 5,5'-dibromohemiBastadin , and it is clear that the α-oxo-oxime functionality is not sufficient for a high affinity interaction with the catalytic site of the enzyme given the other structural features of ianthelline. Ianthelline is also positively charged, and charge repulsion within the active site of phenoloxidase is a potential explanation for the lower inhibitory activity. The lack of a phenolic hydroxyl is also believed to additionally impair the enzyme binding as it represents the natural enzyme substrate binding motif (Kim and Uyama, 2005).

While phenoloxidase inhibition is a common representative indicator of a compound's ability to reduce the settling abilities for a range of invertebrates (Bayer *et al*., 2011), studies on *B. improvisus* larvae were also performed to assess the potential effects ianthelline may specifically have on barnacles. Based on the moderate phenoloxidase inhibition, a similar moderate deterring effect on *Balanus* settlement was anticipated. The most commonly studied barnacle in AF studies is *Balanus amphitrite* which is found in tropical waters. The sponge *S. fortis* (Fig. 2.2), from which Ianthelline was isolated, is a cold water species, which is why we in this study chose to target *B. improvisus*, which is also found in cold water.

Figure 3.2: A specimen of *Stryphnus. fortis* from which ianthelline (1) was isolated for the present study.

Scale bar represents 2 cm

From a chemical ecology perspective, it has been established that when working on marine natural products, it is more judicious to sample organisms and also test them primarily on fouling species from the same area (Marechal and Hellio, 2011). *B. improvisus* is commonly found in the shallow littoral zone attached to stones, algae or man-made constructions (Barnes and Barnes, 1962). It is a notorious fouler on smooth artificial substrates such as ship hulls, which has enabled its rapid spread amongst continents during the last century.

The species has a planktotrophic larval development consisting of six naupliar stages and one non-feeding cyprid stage, the latter being responsible for finding and attaching to a suitable substratum. The processes of attachment and metamorphosis of the cyprid larva into an adult barnacle are collectively referred to as settlement. The cyprids are capable of exploring surfaces actively, and the site of settlement is determined using physical and biochemical properties of the substratum (Berntsson *et al*., 2000). The effect of Ianthelline on *B. improvisus* cyprid larvae is presented below in Fig. 2.3.

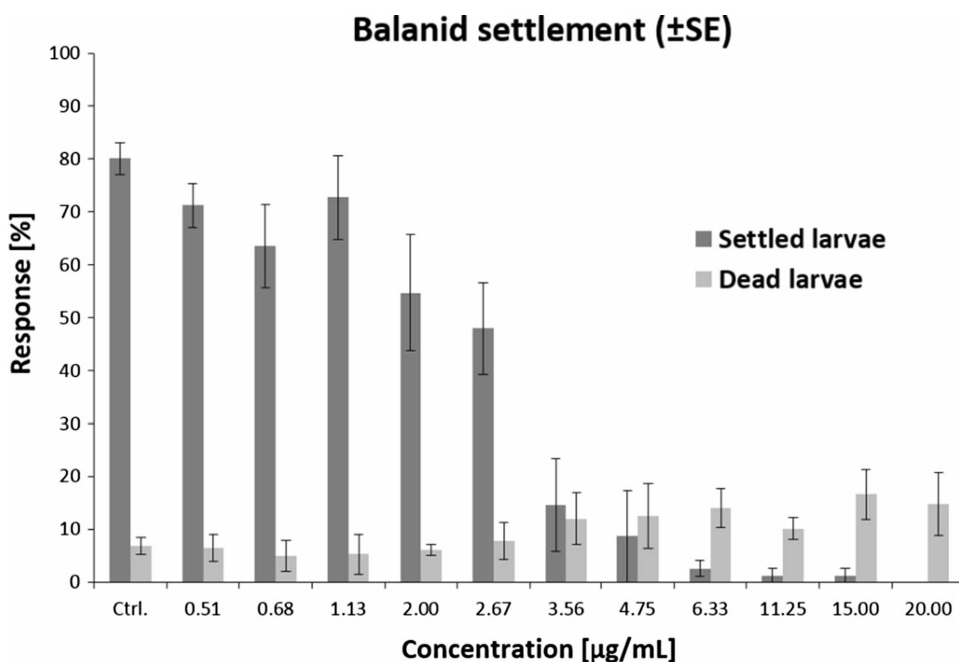

Figure 3.3: Effect of ianthelline (1) on the settlement of *Balanus improvisus* cyprid larvae as presented as percentages of settled (*dark grey columns*) and dead cyprids (*light grey columns*) and given as means ±SE (*n*=4).

IC_{50} was determined to 3.0 µg/mL. 0.1 % DMSO (*v/v*) in fresh sea water was used as negative control

As evident from the settlement and metamorphosis inhibition data, ianthelline represents a potent AF compound against *B. improvisus* cyprid larvae. An IC_{50} of 3.0 µg/mL indicates a high deterring effect and makes Ianthelline significantly more active against balanide settlement than most reported bromotyrosine derived sponge metabolites and their synthetic analogues (Tsukamoto *et al.,* 1996. Ortlepp *et al*., 2007). Ianthelline is four times as active as 5,5'-dibrolohemibastadin-1, and no apparent coupling between the moderate phenoloxidase inhibition and *Balanus* settlement is seen for ianthelline, as has been previously reported for the synthetic hemibastadins (Bayer *et al*., 2011). Barettin displays an IC_{50} around 1 µg/mL (Sjogren *et al*., 2004) while the structurally related dibromopyrrole oroidin (4 in Fig. 2.1) exhibits an IC_{50} of 19 µg/mL

against the settlement and metamorphosis of *B. amphitrite* cyprids (Tsukamoto *et al*., 1996).

Ianthelline displays activity similar to ceratinamide B but is one order of magnitude less active than ceratinamide A, a highly potent AF bromotyrosine derivate with low toxicity isolated from the marine sponge *P. purpurea* (Tsukamoto *et al*., 1996). Even at the highest concentration tested (20 μg/mL), ianthelline exhibited low toxicity. Furthermore, an increase in larval time in the water column caused by settlement inhibition will inevitably result in higher larval mortality, which may partly explain the tendency for a higher cyprid mortality at the highest test concentrations of ianthelline (Fig. 2.3).

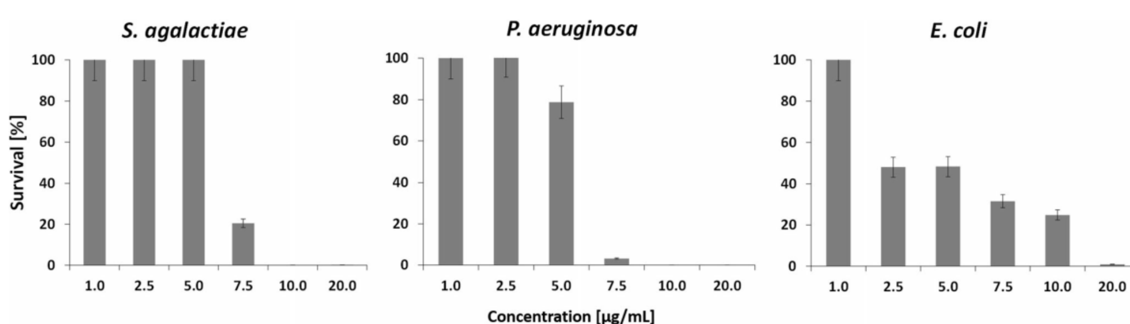

Figure 3.4: Effects of ianthelline (1) on selected bacterial strains illustrating the threshold concentrations needed for activity, particularly against Gram-positive bacteria. (*S.agalactiae* and *P.aeruginosa*). Results are presented as percentages of survival and given as means ±SE (*n*=4).

When the isolation of ianthelline was initially reported by Litaudon and Guyot in 1986, an activity against *S. aureus* using a disc diffusion assay (15 mm growth inhibition radius using a disc loaded with 50 μg of 1) was included (Litaudon and Guyot, 1986). Based on that initial data, a screening against human pathogenic bacterial strains was also performed to provide a comprehensive overview of the antibacterial effects of

ianthelline. Strains resistant to conventional antibiotics and biofilm forming bacteria were included in the test panel of seven bacterial strains. Serial dilution experiments were performed to assess the antibacterial activity of Ianthelline against human pathogenic bacterial strains, and the data is compiled in Table 2.5.

Table 3.5: Antibacterial activity of a ianthelline (concentrations 2.5µg-160µg/ml) against 7 human pathogenic bacterial strains. Results are expressed as MIC (µg/ml)

Bacterial strain	**MIC (µg/mL)**
Gram positive	
Staphylococcus aureus	2.5
MRSA	20
Staphylococcus epidermidis	50
Streptococcus agalactiae	7.5
Enterococcus faecalis	22.5
Gram negative	
Escherichia coli	7.5
Pseudomonas aeruginosa	7.5
Biofilm inhibition	
Staphylococcus epidermidis	30

The Gram-positive bacteria displayed sensitivities towards ianthelline ranging from 2.5 to 50 µg/mL while the two Gram-negative strains were both highly sensitive with MIC values of 7.5 µg/mL. *S. aureus* displayed the highest sensitivity towards ianthelline at 2.5 µg/mL, and the methicillin-resistant strain was slightly more tolerant. These activities are similar to those reported for synoxazolidinone A, which is a closely structurally

related marine compound isolated from the Arctic ascidian *Synoicum pulmonaria* (Tadesse *et al.,* 2010). In this molecule, the central oxime is replaced with an oxazolidinone core.

Previous cytotoxicity studies indicate that ianthelline does not act via cellular membrane disruption against mammalian cells (Hanssen *et al*., 2012). Instead, the kill kinetics suggests intracellular targets as no biological effect was seen until cells were incubated with Ianthelline for extended periods and at higher concentrations. Even though the antibacterial mode of action of ianthelline was not studied in detail, it appears from the data that the antibacterial effect is primarily induced at the bacterial membrane interface. The rapid killing, once a threshold concentration on ianthelline has been reached, is typical for a compound acting by a nonspecific membrane depolarisation mechanism (Shai, 1999) and is exemplified for selected strains in Fig. 2.4.

The lipid composition of the bacterial cell membrane differs significantly from the neutral mammalian cell membrane (Lohner *et al*., 2008), and a different mode of action on the anionic bacterial cell membrane is suspected. Cationic amphiphilic antimicrobial peptidic derivatives, which ianthelline can be regarded as, are generally active at the bacterial membrane interfaces via several mechanisms (Melo *et al*., 2009), and a pronounced antibacterial effect can be seen even for small di- and tri-peptidomimetics (Flaten *et al*., 2011).

The inhibition of *S. epidermidis* biofilm formation at 30 µg/mL is seen at a lower concentration than the MIC for growth inhibition and may suggest an additional mode of action for ianthelline.

Given the many promising bioactivities of ianthelline and the relative ease of preparing it, and similar analogues, synthetically (Ortlepp *et al*., 2007; Shearman *et al*., 2010) it is

realistic to assume that libraries of analogues of 1Ianthelline is within reach. Ianthelline can be dissected into smaller building blocks allowing for alternative molecular assemblies where the key functional groups such as the tyrosine moiety, the central oxime and the cationic iminoimidazole can be substituted with analogous chemistries. Several different structural motifs displayed by ianthelline can be thus be targeted to generate diverse libraries of compounds that would aid in future structure activity relationship studies (SAR) as depicted in Fig. 2.5.

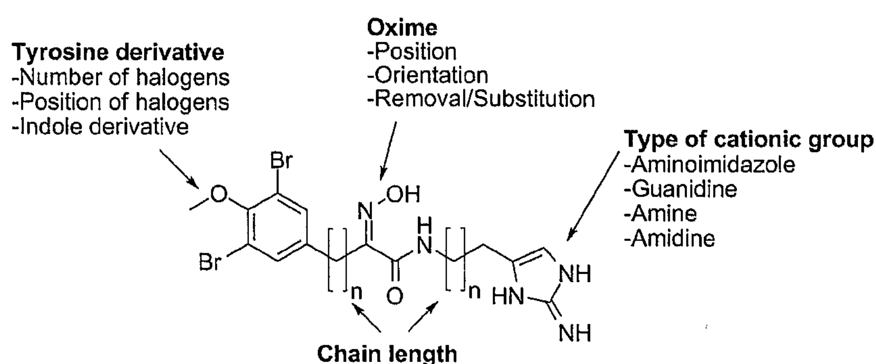

Figure 3.5: Synthetic targets for structure activity relationship studies for establishing the AF pharmacophore of ianthelline.

It is expected that such analogues will be active in several assays and also structurally simplified in comparison with one. This is certainly a prerequisite if a large-scale marine use is considered. The structurally related 5,5'-dibromohemibastadin-1 has undergone extensive SAR studies, and those libraries of synthetic analogues have been shown to display several medically relevant bioactivities (Richards *et al.*, 2008 and 2009) and are being studied further.

The studied extract of *S. fortis* contains a range of secondary metabolites (unpublished data), but ianthelline is by far the most abundant compound constituting 0.05 % of the

organism's wet weight. This strongly indicates that ianthelline serves important ecological functions such as inhibition of the recruitment of potential epibionts and competitors, maybe in synergy with other compounds. A tissue specific production or localisation of ianthelline to exposed areas of the organism may further aid to increase the local concentration to levels higher than those evaluated in the present study to effectively prevent settlement to generate a broad resistance against competing marine species.

2.5 Conclusion

Brominated secondary metabolites are common in the marine environment, and the sponge-derived tyrosine derivatives are particularly interesting due to their diverse bioactivities and their potential application areas. In the present study, ianthelline was shown to be a powerful micro- and macro-AF compound, mainly against marine bacteria and the settlement of barnacles. A lower activity is seen against microalgae, and a moderate bioactivity is reported for blue mussel phenoloxidase inhibition. In addition, ianthelline is active against all tested human pathogenic bacterial strains included in the current study, both Gram positive and Gram negative. Based on the structural similarities with barettin, oroidin and the synoxazolidinones, it is anticipated that ianthelline also exhibits other biological activities yet to be investigated.

Chapter 3

Chapter 4 : Antifouling Compounds from the Sub-Arctic Ascidian *Synoicum pulmonaria*: Synoxazolidinones A and C, Pulmonarins A and B, and Synthetic Analogs

3.1 Abstract

The current study describes the antifouling properties of four members belonging to the recently discovered synoxazolidinone and pulmonarin families, both isolated from the sub-Arctic sessile ascidian *Synoicum pulmonaria* collected off the Norwegian coast. Four simplified synthetic analogs were also prepared and included in the study. Several of the studied compounds displayed MIC values in the micro-nanomolar range against 16 relevant marine species involved in both the micro- and macrofouling process. Settlement studies on *Balanus improvisus* cyprids indicated a deterrent effect and a low toxicity for selected compounds. The two synoxazolidinones displayed a broad activity and are shown to be amongst the most active natural antifouling bromotyrosine derivatives described. Synoxazolidinone C displayed selected antifouling properties comparable to the commercial antifouling product SEA-NINE. The pulmonarins prevented the growth of several bacterial strains at nanomolar concentrations but displayed a lower activity towards microalgae and no effect on barnacles. The linear and cyclic synthetic peptidic mimics also displayed potent antifouling activities mainly directed against bacterial adhesion and growth.

This work has been published in a refereed journal, Journal of Natural Product, and is presented below in identical form.

The citation for the original publication is:

Rozenn Trepos, Gunnar Cervin, Claire Hellio, Henrik Pavia, Wenche Stensen, Klara Stensvåg, John-Sigurd Svendsen, Tor Haug, Johan Svenson (2014). Antifouling Compounds from the Sub-Arctic Ascidian *Synoicum pulmonaria*: Synoxazolidinones A and C, Pulmonarins A and B, and Synthetic Analogs, Journal of Natural product,77(9):2105-13.

The author designed the experiments and performed the antibacterial and antimicroalgal assays. The co-authors have collected the organisms and purified the compounds and performed the *Balanus* cyprids settlement assays and the phenoloxydase assay. The manuscript was written through contributions of all authors.

3.2 Introduction

Sessile benthic marine organisms are a rich source of diverse chemical scaffolds and novel compounds with promising bioactivities (Gerwick *et al*., 2012). Their stationary lifestyle forces the organisms to develop effective chemical defensive means to avoid predation or colonisation and successive overgrowth by epibionts (Procksh, 1994; Müller *et al*., 2013). Overgrowth of an organism relying on filtering of sea water for nutritional intake can have fatal consequences and maintaining an unhindered passage of water through the organism is vital. The production of a chemical arsenal capable of preventing such overgrowth is thus promoted and many sessile organisms are remarkably clean and devoid of growth (Müller *et al*., 2013).

Bromotyrosine derived secondary metabolites from marine sponges have been ascribed powerful antifouling activities (Ortlepp *et al*., 2007) and compounds such as barettin (Sjogren *et al*., 2004), ianthelline (Hanssen *et al*., 2014), the bastadins (Bayer *et al*., 2011) and the similar oroidins (Tsukamoto *et al*., 1996) have been extensively studied and evaluated in fouling assays (Fig. 4.1).

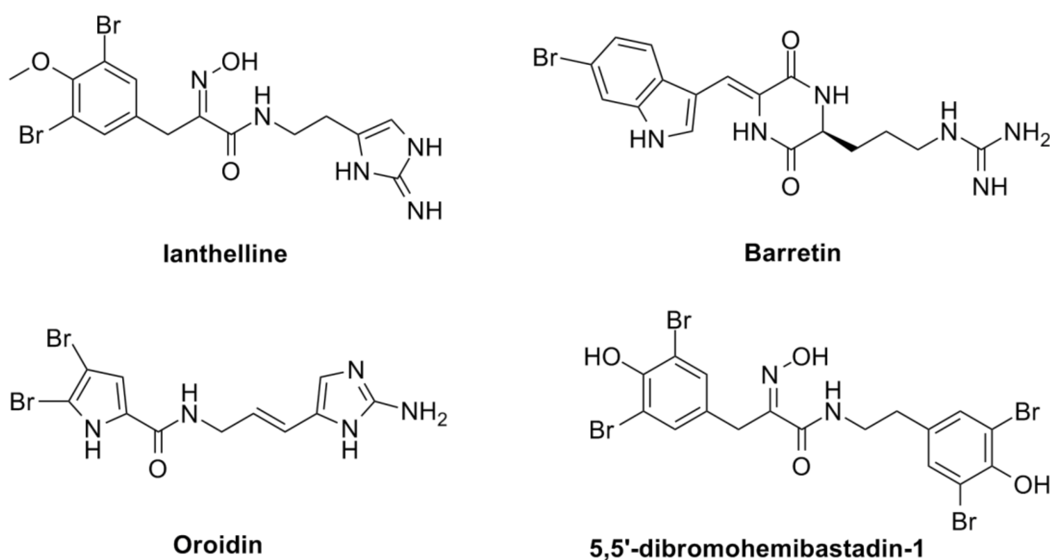

Figure 3.4.1: Structure of ianthelline and structurally and marine related compounds that have been evaluated as AF compounds: barettin, oroidin and 5,5'-dibromohemibastadin-1

These compounds share several structural features and occur in high concentrations in the organisms and often as major components in extracts thereof, suggesting a common role in the sponges and a similar mode of action (Hanssen *et al*., 2014). Several such compounds have been evaluated for commercial applications and both barettin and oroidin have been included in field studies and paint formulations (Sjogren *et al*., 2004; Melander *et al*., 2009). Most studies have been devoted to the inhibition of barnacle cyprid settlement (commonly *Balanus improvisus* or *Balanus amphrite*) which are major hard macrofoulers on many surfaces (Berntsson *et al*., 2000). However, fouling is a complex stepwise process (Qian *et al*., 2007; Yebra *et al*., 2004) involving both microorganisms such as bacteria and microalgae (microfouling) and larger organisms such as macroalgae and invertebrates (macrofouling) as illustrated in Fig. 3.2.

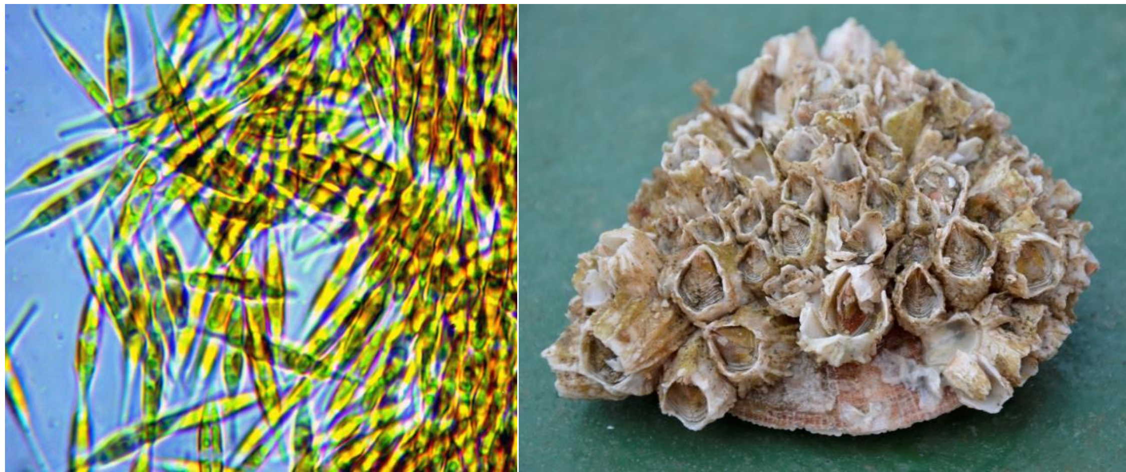

Figure 3.4.2: Examples of micro and macrofouling in cold waters.

To the left, a microscope image (×400, each organism is 60 to 70 µm) of a growing biofilm of the diatom *Cylindrotheca closterium*. To the right, extensive, hard macrofouling of the shell of *Chlamys islandica* (Iceland scallop) by *Balanus* (Acorn barnacles) (Photo by J. Svenson and R.A. Ingebrigtsen).

Microfouling enables and sometimes facilitates the macrofouling development (Beech *et al*., 2005; Qian *et al*., 2007;) and activity against several different classes of organisms may thus result in synergistic overall antifouling effects.

A complete assessment of a compounds ability to interfere with several types of settling organisms is thus necessary before drawing conclusions regarding a compound's potential as a broad spectrum antifoulant (Briand *et al*., 2009). Such an overview cannot be rapidly obtained via a single assay and furthermore it is unlikely that a compound will display activity towards the diverse set of involved organisms without being a general biocide and therefore of less interest for further development into an antifouling product (Dahlstrom *et al*., 2000; Dahms and Hellio, 2009). The use of multiple assays therefore enables the discovery of broad-spectrum antifoulants and also increases the chances of finding group-specific inhibitory effects. A narrow but highly potent

antifouling activity against a single species or group of organisms deserves attention and can lead to commercial products. This is exemplified by Medetomidine (Selektope(R)) which is under development by the company I-tech (Sweden) and only displays activity against barnacle larvae settlement via a reversible non-toxic deterring mechanism (Dahlstrom *et al*., 2000; Lind *et al*., 2010).

Ascidians are stationary marine filter feeders and they have been shown to be rich sources of novel marine natural products (Faulkner *et al*., 2002; Menna *et al*., 2011). Specimens of the colonial sub-Arctic ascidian *Synoicum pulmonaria* are generally free from epibionts and appear to be a particularly efficient producer of bromotyrosine derived secondary metabolites (Tadesse *et al*., 2008). *S. pulmonaria* have recently been shown to produce a complex cocktail of brominated secondary metabolites involving a range of novel bioactive compounds such as the synoxazolidinones and the pulmonarins (Tadesse *et al*., 2011; Tadesse *et al*., 2014). Other related cold water *Synoicum* species such as the Antarctic *S. adareanum* have also been shown to be rich producers of novel bioactive scaffolds (Miyata *et al*., 2007; Diyabalanage *et al*., 2006) and illustrates how the *Synoicum* genus produce diverse chemistries (Nunez-Pons *et al.*, 2012).

The synoxazolidinones have been shown to be both antibacterial and antitumoral (Tadesse *et al*., 2010; Tadesse *et al*., 2011) whereas the pulmonarins have recently been reported to display selective acetylcholine esterase (AChE) inhibitory activity (Tadesse *et al*., 2014). The current paper describes the evaluation of the antifouling properties of synoxazolidinone A (**1**) and C (**2**) and pulmonarin A (**3**) and B (**4**), all recently isolated from *S. pulmonaria* as novel compounds (Fig. 3.3).

The effect of the compounds on the growth and settlement inhibition of 10 marine bacterial and 5 microalgal strains relevant to the formation of the biofouling film is evaluated. Studies on settlement inhibition of barnacle larvae from the hard

macrofouler *B. improvisus*, is also included. One synthetic linear and three cyclic peptidic analogs of synoxazolidinone A and synoxazolidinone C were designed, prepared and evaluated and structural reasons underlying the observed differences in bioactivities between the natural and synthetic compounds are discussed. The commercial antifouling product SEA-NINE was further included in the assays and used as a relevant positive control. SEA-NINE is an herbicide that is commonly used in antifouling paints formulation and act as an inhibitor of PSII electron transport (Gardiola *et al.*, 2012).

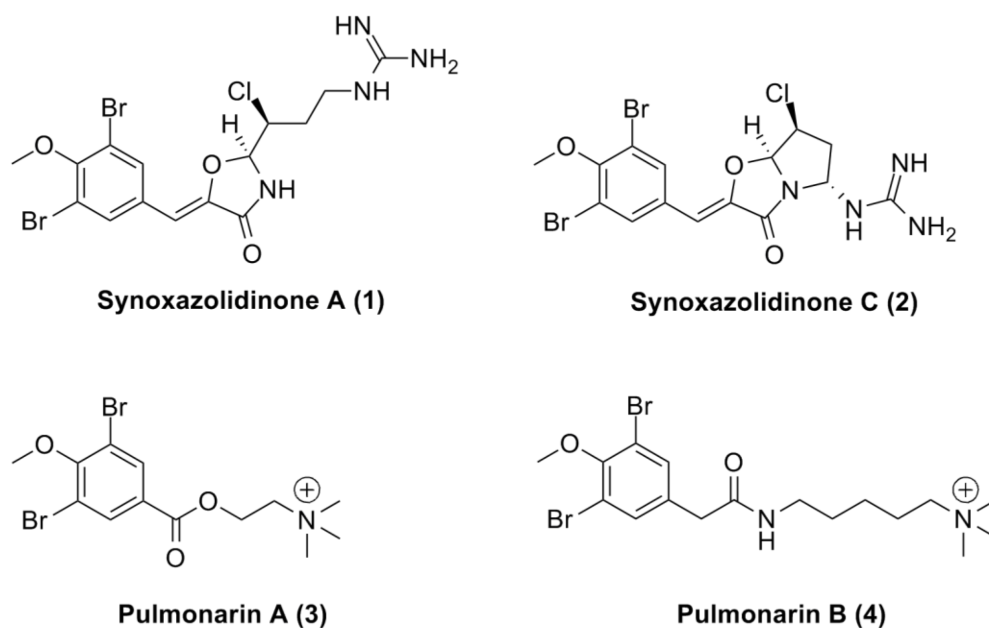

Figure 3.4.3: Chemical structures of synoxalidinone A and C and Pulmonarin A and B

3.3 Materials and Methods

3.3.1 General experimental procedures

Infrared spectra were recorded on an Avatar 320 FT-IR spectrometer from Nicolet. NMR spectra of synthetic compounds were recorded on a Varian 7000e 400 MHz spectrometer in CD_3OD. Chemical shifts were referenced to the residual solvent peaks, δ^1H 3.31 and δ^{13}C 49.0 ppm. Mass spectra were acquired on a LTQ Orbitrap XL Hybrid Fourier Transform mass spectrometer from Thermo Scientific and the Thermo Scientific Accela HPLC-LTQ Ion Trap-Orbitrap Discovery system. HPLC of synthetic compounds was performed on a Waters 600 system with a 2487 dual λ absorbance detector. Microwave assisted couplings were performed using a Biotage Initiator 2.0. All starting materials were purchased from Sigma-Aldrich. Chemicals were used without further purification.

3.3.2 Natural compounds and organisms

Synoxazolidinone A and synoxazolidinone C were isolated as described previously from the ascidian *S. pulmonaria* (Ellis and Solander, 1986) collected at Rya (69°33.16N, 18°42.53E) off the Norwegian coast in 2006 (Tadesse *et al*., 2010; Tadesse *et al*., 2011). The organisms are stored at the Norwegian College of Fishery Science, University of Tromsø, Norway. Pulmonarin A and pulmonarin B were synthesised as recently described (Tadesse *et al*., 2014). The ten marine bacterial strains, representative of fouling species in marine environments (Chambers *et al*., 2011), were obtained from the ATCC bacterial collection. Five marine microalgal strains involved in surface colonisation and biocorrosion (Jellali *et al*., 2013) (obtained from Algobank, Caen, France) were also evaluated together with reared *B. improvisus* cyprids. The included microfouling organisms are compiled in Table 3.1.

Table 3.1: Marine bacterial and microalgal strains used in the present study

Marine bacteria	**ATCC**
Halomonas aquamarina	14400
Polaribacter irgensii	700398
Pseudoalteromonas elyakovii	700519
Roseobacter litoralis	49566
Shewanella putrefaciens	8071
Vibrio aestuarianus	35048
Vibrio carchariae	35084
Vibrio harveyi	700106
Vibrio natriegens	14058
Vibrio proteolyticus	53559
Microalgae	**Algobank code**
Cylindrotheca closterium	AC 170
Exanthemachrysis gayraliae	AC 15
Halamphora coffeaeformis	AC 713
Pleurochrysis roscoffensis	AC 32
Porphyridium purpureum	AC 122

3.3.3 Marine Antibacterial assay

The bacteria were grown at 26 °C in a marine medium, composed of 0.5% peptone (neutralised bacteriological peptone, Oxoid LTD) in filtered (Whatman 1001-270, pore size 11 µm) natural seawater from the Solent (UK). The studied compounds were dissolved in 100% methanol (general purpose grade, Fisher Chemical), and transferred to clear polystyrene 96-well plates (Fisher Scientific), dried under vacuum and sterilised under UV illumination to afford wells containing 0.01, 0.1, 1 and 10 µg/mL of each compound. Each concentration was replicated six times (Bressy *et al*., 2010). Both the bacterial growth and adhesion inhibition were studied. No viable cells at 0.01 µg/mL is reported as "<0.01 µg/mL" (only seen for the SEA-NINE reference).

3.3.3.1 Growth inhibition experiments

100 µL of bacterial culture (2×10^8 colony forming units (CFU) mL^{-1}) were added to each well under aseptic conditions and incubated at 26 °C for 48 h. The minimum inhibitory concentration (MIC) was defined as the minimum concentration resulting in no change in optical density at 630 nm after incubation for 48 h (Thabard *et al*., 2011).

3.3.3.2 Adhesion inhibition experiments

Microplates were prepared and inoculated as described above. After 48 h incubation, wells were emptied and rinsed once with 100 µL of sterile seawater to remove the non-attached cells and air-dried at room temperature. The remaining bacterial biofilm was stained with 100 µL of 0.3% (v/v) aqueous crystal violet and the OD was measured at 595 nm (Thabard *et al*., 2011). Media was used as blank and MIC was defined as the lowest concentration that produced a reduction in adhesion.

3.3.4 Microalgal assay

The microalgal strains were grown at 20 °C in F/2 medium for five days prior to use. The microalgal concentration was assessed after five days via analysis of the chlorophyll *a* content (Chambers *et al*., 2011) and dilutions of the stock culture were made accordingly to generate stock solutions of each microalgae containing 0.1 mg/L chlorophyll *a*. 100 µL of the stock solutions were transferred to the wells of black 96-well plates prepared with ranging concentrations of compounds as described above. Both adhesion and growth inhibition were studied.

3.3.4.1 Growth inhibition experiments

The inoculated plates were grown at 20 °C for 5 days under constant light exposure (140 µmol m^{-2} s^{-1}). After the incubation, the microplates were centrifuged at 4100 rpm for 10 min at 4 °C using a Beckman Coulter Allegra 25R centrifuge and subsequently emptied. Chlorophyll *a* was liberated by the addition of 100 µL of 100% methanol to each well. The pigment concentration was quantified employing the fluorimetric method (Chambers *et al*., 2011). MIC values were calculated as explained for bacteria (based on OD measurements).

3.3.4.2 Adhesion inhibition experiments

Black microplates were prepared and inoculated as stated above. Media was used as blank. After 5 days of incubation, the medium was gently removed using a multichannel pipette to eliminate all the non-attached cells. 100 µL of 100% methanol was then added to each well to liberate chlorophyll *a*. The pigment concentration was quantified employing the fluorimetric method explained above.

3.3.5 Balanus cyprid settlement

Cyprid larvae of *B. improvisus* were reared in a laboratory cultivating system at Tjärnö Marine Biological Laboratory, as described by Berntsson (Berntsson *et al*., 2000). The settlement assays with *B. improvisus* cyprids were conducted in non-treated polystyrene Petri dishes (Ø 48 mm, Nunc #150340) containing 10 mL filtered (0.2 µm) seawater. The compounds were dissolved in DMSO and further serially diluted with DMSO to yield the desired concentration series of which 10 µL was added to each test dish. A total of 18–22 newly moulted cyprids were added to each test dish which were incubated at ambient temperature (20–25 °C) for five days. At the end of the experiment, the number of metamorphosed juvenile barnacles, as well as live and dead cyprids, was assessed under a dissection microscope. Each test concentration of replicated four times (n = 4) and dishes with 10 µL DMSO served as controls.

3.3.6 Synthesis

2-((3,5-dibromo-4-methoxybenzoyl)oxy)-N,N,N-trimethylethanaminium chloride (**3**)

The compound was prepared according to Tadesse *et al*. in 4% yield[25]. Spectral data matched those previously reported.

5-(2-(3,5-dibromo-4-methoxyphenyl)acetamido)-N,N,N-trimethylpentan-1-aminium iodide (**4)**

The compound was prepared according to Tadesse *et al*. in 13% yield (Tadesse *et al*., 2014). Spectral data matched those previously reported.

(2-amino-3-(3,5-dibromo-4-methoxyphenyl)propanoyl)arginine (**5**)

Methyltyrosine (195 mg, 1 mmol) was dissolved in aqueous HCl (6 M, 4 mL) and cooled to 0 °C before bromine (105 µL, 4.1 mmol) was added. The reaction was run for 30 minutes at 0 °C and then allowed to reach room temperature.

After 30 minutes the excess Br_2 was removed by flushing the reaction mixture with nitrogen and dibromomethyltyrosine precipitated as white crystals. The solvent was removed under reduced pressure and the precipitate was dissolved in a water: dioxane solution (1:1, 25 mL) before $NaHCO_3$ (252 mg, 3 mmol) and Boc_2O (437 mg, 2 mmol) were added. The reaction was stirred for 16h at ambient temperature. The solvent was evaporated and the residue dissolved in ethyl acetate. The organic layer was washed with $NaHSO_4$ (5%) and dried over $NaSO_4$, before the removal of the solvent to afford the product. Spectral data matched those previously reported by Stewart *et al.* (Steward *et al.*,2004) and the product was used without further purification. The *N*-boc-dibromo methyl tyrosine (450 mg, 1 mmol) and L-arginine methyl ester (188 mg, 1 mmol) were dissolved in DMF (5 mL). HBTU (758 mg, 2 mmol) and 830 µL DIPEA (4.8 mmol) was added and the reaction was stirred at ambient temperature for 16 h. The ester protecting group was saponified for 10 min in 5 % NaOH at rt and the free acid was collected by filtration and dried under vacuum after addition of 5% $NaHSO_4$. The boc-group was removed by treatment with 4 M HCl in dioxane (10 mL) for 16h at rt. The crude peptide was collected after solvent evaporation and purified using HPLC (isolated yield 39%). IR ν_{max} 3208, 1659, 1477, 1428 1138 cm^{-1}; ^{1}H-NMR (400 MHz; CD_3OD): δ= 7.57 (s, 2H), 4.44 (dd, *J* = 8.0, 5.1 Hz, 1H), 4.17-4.14 (m, 1H), 3.85 (s, 3H), 3.26-3.18 (m, 3H), 3.02 (dd, *J* = 14.4, 8.0 Hz, 1H), 2.00-1.93 (m, 1H), 1.80-1.67 (m, 3H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ= 172.8 (C), 167.8 (C), 157.2 (C), 153.7 (C), 133.7 (CH), 133.1 (C) 118.9 (C), 53.8 (CH_3), 53.8 (CH), 52.0 (CH), 40.4 (CH_2), 35.6 (CH_2), 28.6 (CH_2), 24.8 (CH_2) ppm. ESIMS *m/z* 507.8 $[M + H]^+$ (calcd. for $C_{16}H_{24}Br_2N_5O_4$, 508.02).

1-(3-((2S,5S)-5-(3,5-dibromo-4-methoxybenzyl)-3,6-dioxopiperazin-2-yl)propyl)guanidine (**4**)

Dipeptide **V** (507 mg, 1 mmol) was dissolved in 0.15 M acetic acid (20 mL) in 1-butanol before addition of *N*-methylmorpholine (0.22 mL). The reaction mixture was heated to 120 °C for 1 h in a microwave synthesizer and the ring closed 2,5-diketopiperazine was isolated after solvent removal and HPLC purification (isolated yield 33%). IR ν_{max} 3208, 1666, 1458, 1424, 1145 cm^{-1}; ^{1}H-NMR (400 MHz; CD_3OD): δ= 7.45 (s, 2H), 4.36 (t, *J* = 3.5 Hz, 1H), 3.91 (m, 1H), 3.85 (s, 3H), 3.26 (dd, *J* = 14.0, 3.8 Hz, 1H), 3.01-2.98 (m, 2H), 2.90 (dd, *J* = 14.0, 4.7 Hz, 1H), 1.32-1.13 (m, 4H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ= 169.5 (C), 168.5 (C), 158.6 (C), 154.7 (C), 136.4 (C), 135.9 (CH), 118.9 (C), 61.3 (CH_3), 56.9 (CH), 55.0 (CH), 41.9 (CH_2), 38.3 (CH_2), 31.9 (CH_2), 24.8 (CH_2) ppm. ESIMS *m/z* 489.8 $[M + H]^+$ (calcd. for $C_{16}H_{21}Br_2N_5O_3$, 490.00).

1-(3-((2S,5S)-5-(4-methoxybenzyl)-3,6-dioxopiperazin-2-yl) propyl) guanidine (**7**)

The compound was prepared employing methodology analogous to the synthesis of **6** aside from omission of the bromination step starting from methyltyrosine. Isolated yield 47%. IR ν_{max} 3186, 1663, 1518, 1466, 1138 cm^{-1}; ^{1}H-NMR (400 MHz; CD_3OD): δ= 7.22 (d, *J* = 8.6 Hz, 2H), 6.90 (d, *J* = 8.6 Hz, 2H), 4.43 (dd, *J* = 8.2, 5.3 Hz, 1H), 4.11 (dd, *J* 8.4, 5.4 Hz, 1H) 3.78 (s, 3H), 3.25-3.20 (m, 3H), 2.98 (dd, *J* = 14.5, 8.4 Hz, 1H), 2.05-1.92 (m, 1H), 1.82-1.65 (m, 3H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ= 172.9 (C), 168.4 (C), 159.4 (C), 157.2 (C), 130.2 (CH), 125.7 (C), 114.1 (CH), 54.3 (CH_3), 54.3 (CH), 52.1 (CH), 40.5 (CH_2), 36.3 (CH_2), 28.5 (CH_2), 24.9 (CH_2) ppm. ESIMS *m/z* 334.1 $[M + H]^+$ (calcd. for $C_{16}H_{23}N_5O_3$, 334.18).

1-(3-((2S,5S)-5-((1H-indol-3-yl) methyl)-3,6-dioxopiperazin-2-yl) propyl) guanidine (**8**)

The compound was prepared according to the protocol of Li *et al.* in 55% yield[47]. Spectral data matched those previously reported.

3.3.7 Peptide purification

The crude peptides were purified by reversed phase HPLC (RP-HPLC) on a Delta-Pak (Waters) C18 column (100 Å, 15 µm, 25 × 100 mm) employing mixtures of water and acetonitrile (both containing 0.1% TFA) as eluent. The purified peptides were further analysed by RP-HPLC using an analytical Delta-Pak (Waters) C18 column (100 Å, 5 µm, 3.9 × 150 mm). To ensure positive identification of the desired product, positive ion electrospray mass spectrometry was performed on a Waters Quattro micro quadrupole mass spectrometer and subsequently confirmed qualitatively by NMR.

3.4 Results and discussion

Biofouling represents a global challenge due to the increased costs and environmental issues connected with chemical countermeasures implemented to prevent reduced boat speeds and increased maintenance costs caused by biofouling on man-made marine structures (Yebra *et al.*, 2004; Fitridge *et al*., 2012). Historically, biofouling has been successfully prevented by employing protective marine paints incorporating organotin derivatives (mainly tributyl tin, TBT) and/or copper oxide (Almeida *et al*., 2007). These compounds were shown to be both toxic towards non-target organisms and persistent within the sediment (Okoro *et al*., 2011; Kotake *et al*., 2012). This has resulted in the ban of TBT formulations by the International Maritime Organisation with a complete stop in 2008 (Yebra *et al*., 2004). Marine antifouling natural products are now being intensively investigated as environmentally benign alternatives to develop green antifouling solutions (Qian *et al*., 2010; Marechal and Hellio, 2009; Dobretsov *et al*., 2006).

In the current study, 16 different fouling species involved in marine surface colonisation are included to generate a broad estimation of the bioactivities of the studied

compounds. The organism panel comprises both microfouling species, such as marine bacterial strains and microalgae and macrofouling barnacles (hard macrofoulers). Diatoms represent particularly relevant microfouling strains as they rapidly form resilient slimy layers on marine surfaces (Molino and Wetherbee, 2008). The diatoms *Cylindrotheca closterium* and *Halamphora coffeaeformis* were therefore also included as target organisms. Compounds were studied at concentrations up to 10 µg/mL (presented as in µM in the tables) to remain within a high activity range (Hanssen *et al.*, 2014). The US Office of Naval Research considers a compound concentration <25 µg/mL as relevant for the prevention of growth on static marine structures. The antifouling bioactivity of synoxazolidinone A (1), synoxazolidinone C (2), pulmonarin A (3) and pulmonarin B (4) is summarised in Table 3.2.

Table 3.2: The effect of synoxazolidinone A (1), synoxazolidinone C (2), pulmonarin A (3) and pulmonarin B (4) on growth (Gr) and adhesion (Ad) of fouling marine bacteria, microalgae and barnacles. Results are expressed as MIC (µM).

Organism	MIC (µM)							
	1		2		3		4	
	Ad[1]	Gr[2]	Ad	Gr	Ad	Gr	Ad	Gr
Marine bacteria								
Halomonas aquamarina	20	-[3]	-	2	3	-	-	-
Polaribacter irgensii	-	20	20	2	-	0.2	-	-
Pseudoalteromonas elyakovii	-	0.02	-	20	-	0.2	-	-
Roseobacter litoralis	-	0.02	2	0.2	0.03	-	20	-
Shewanella putrefaciens	-	0.2	-	20	-	-	-	-
Vibrio aestuarians	-	0.02	2	0.2	0.03	-	20	-
Vibrio carchariae	-	2	20	2	3	-	20	-
Vibrio harveyi	-	-	2	0.02	-	-	-	-
Vibrio natriegens	-	0.02	20	2	0.03	-	20	-
Vibrio proteolyticus	-	0.02	2	0.2	-	-	-	-
Microalgae								
Cylindrotheca closterium	20	20	2	0.2	-	-	-	-
Exanthemachrysis gayraliae	20	20	2	0.2	-	-	-	-
Halamphora coffeaeformis	20	20	2	2	30	-	-	-
Pleurochrysis roscoffensis	20	20	2	2	-	-	-	-
Porphyridium purpureum	-	20	0.2	0.02	-	0.2	-	-
Crustacean settlement								
Balanus improvisus (IC_{50})	15		2		-		-	-

[1]Adhesion inhibition

[2]Growth inhibition

[3]Compound inactive at ≤10 µg/mL concentrations

Synoxalidinone A was virtually inactive towards the adhesion of bacteria at the tested concentrations (only one strain inhibited at 20 µM) but inhibited the settlement of four of the five microalgal species, including the two diatoms, at 20 µM. Prevention of bacterial growth already at 20 nM concentrations against 50% of the strains tested indicate that synoxalidinone A display potent antibacterial properties also against marine bacteria (Tadesse *et al*., 2010).

Synoxalidinone A was further active against the adhesion of all of the included microalgal species at 20 µM. The settlement of barnacle larvae was inhibited at a 15 µM concentration, which is higher than barettin (Sjogren *et al*., 2009) (0.9 µM), bastadin-9 (1.0 µM) (Ortlepp *et al*., 2007) and ianthelline (6.3 µM) (Hanssen *et al*., 2014) but lower than for oroidin (49 µM) (Tsukamoto *et al*., 1996) and similar to 5,5'dibromohemibastadin-1 (10 µM) (Ortlepp *et al*., 2007). Synoxalidinone C, which is a bicyclic derivative of synoxalidinone A possessed higher anti-adhesive properties towards both bacteria and microalgae with MIC-values ranging between 0.2 and 20 µM. Synoxalidinone C was further shown to effectively prevent the growth of all included bacteria and microalgal strains at low concentrations. An IC_{50}-value of 2 µM towards the settlement of *B. improvisus* larvae for synoxalidinone C indicates a high activity against barnacles as displayed in Fig. 3.4.

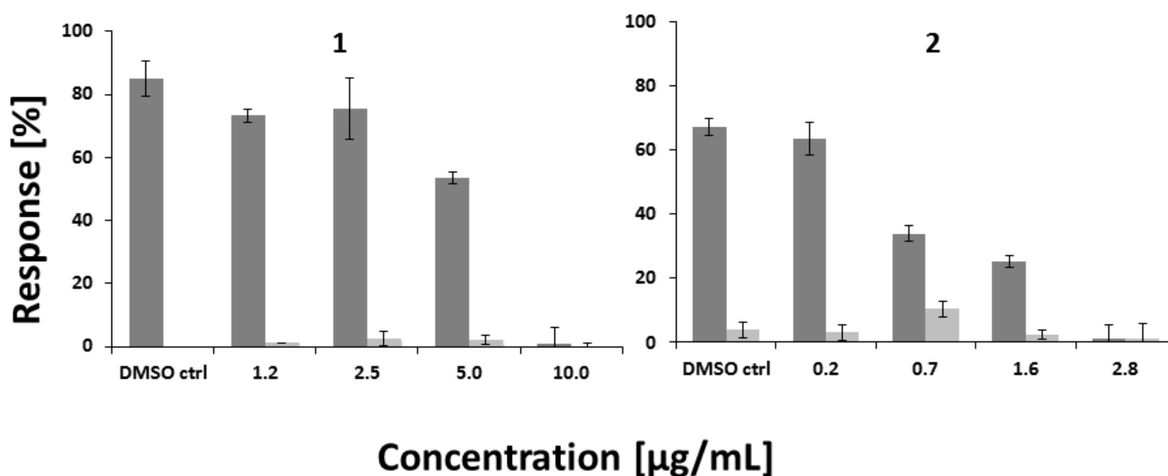

Figure 3.4: Effect of synoxalidinone A and synoxalidinone C on the settlement of *B. improvisus* cyprid larvae as presented percentages of settled (dark grey columns), and dead cyprids (light grey columns) and given as means ± SE (n = 4).

Remaining larvae were free swimming. IC_{50} was determined to be 15 µM and 2.0 µM respectively for synoxalidinone A and synoxalidinone C. DMSO (0.1%, v/v) in FSW was used as negative control.

The two included synoxazolidinones represent novel bromotyrosine scaffolds. Neither the 4-oxazolidinone core of synoxalidinone A, or the unique bicyclic core of synoxalidinone C has been studied before as links between the brominated tyrosine part of the molecule and the arginine-like cationic side bearing the guanidine group. Both synoxalidinone A and synoxalidinone C are powerful antifouling compounds and the overall inhibitory activities encountered in this study are comparable to or significantly lower than for previously studied bromotyrosine derivatives such as the bastadins (Bayer *et al*., 2011), ianthelline (Hanssen *et al*., 2014), barettin (Sjogren *et al*., 2004) and oroidin (Tsukamoto *et al*., 1996).

While the promising anti-barnacle data for synoxalidinone A and synoxalidinone C is comparable to other highly active marine antifouling compounds, the antibacterial and

antialgal activities are superior. In comparison with e.g. Ianthelline (Kelly *et al*., 2005; Hanssen *et al*., 2014) and barettin (Sjogren *et al*., 2004; Hanssen *et al*., 2014), that both have been extensively studied, anti-microfouling activity is seen primarily towards bacterial growth and it is only pronounced against *V. aestuarians*. Ianthelline inhibits the growth of *P. roscoffensis* at 20 µM while barettin inhibits the growth and adhesion of both *C. closterium* and *P. purpureum* at 0.2 µM (Hanssen *et al*., 2014). With low or submicromolar MIC values against the growth and adhesion of all included microalgal strains, including the diatoms, synoxalidinone C represent a very potent inhibitory compound towards both microfouling organisms and macrofouling barnacles at low concentrations. Synoxalidinone A demonstrates similar overall activities as synoxalidinone C but has a weaker effect on the adhesion of microorganisms. It appears that the additional pyrrolidine ring of synoxalidinone C increases the bioactivity of the compound in relation to synoxalidinone A. In the papers describing their discovery, both synoxalidinone A and synoxalidinone C were shown to be effective antibacterial compounds at 20-60 µM against both Gram-positive and Gram-negative human pathogenic bacteria (Tadesse *et al*., 2010; Tadesse *et al*., 2011).

The lowest MIC-values of the synoxazolidinones against marine bacteria is, however, three orders of magnitude lower with the growth of several strains being inhibited at 20 nM. This significant difference indicates an increased antibiotic effect in the marine environment and suggests that these compounds have been tuned to target marine bacteria and that the high antibacterial effect is not directly transferable to terrestrial bacteria. Whether the differences in activity between the marine and terrestrial bacteria also relies on differences in mode of action or longer generation times for marine bacteria is unclear but it is apparent that the effect is general. Suboptimal growth conditions for the marine bacteria could however also make them particularly

susceptible in the assays (Gilbert *et al*., 1990; Svenson, 2013). The cytotoxicity of the compounds has been studied and toxic effects against human fibroblasts at 60 µM have been reported for synoxalidinone C (Tadesse *et al*., 2011). No toxicity towards balanide larvae is seen, suggesting a deterring rather than a toxic mechanism at the tested concentrations.

The pulmonarins are structurally less elaborate in comparison to synoxalidinone A and synoxalidinone C and they contain a positively charged quaternary ammonium group instead of the guanidine of the synoxazolidinones, implying a different biosynthetic origin. Pulmonarin A was very active towards the adhesion of *V. aestuarians*, *V. natriegens* and *R. littoralis* at 30 nM and against *H. coffeaeformis* and *H. aquamarina* at 3 µM. No other bacterial strains were affected. Interestingly the influence of pulmonarin A on the bacterial growth was lower than for the adhesion and only two bacterial strains were sensitive to pulmonarin A at the concentrations employed. The effect on microalgae was not pronounced for pulmonarin A and only single species were sensitive in each assay. No activity against barnacle settlement was detected. Pulmonarin B was substantially less active and it was inactive towards both the microalgae and the balanid attachment.

A weak inhibitory effect at 20 µM was seen for bacterial adhesion towards four bacterial strains while the growth remained unaffected.

The pulmonarins display competitive AChE inhibition and no other bioactivities (no antibiotic or cytotoxic activity) were discovered during the initial comprehensive screens of the compounds (Tadesse *et al*., 2014). Nevertheless, as for the synoxazolidinones, it appears that the marine bacteria are more sensitive than the terrestrial ones towards these compounds and pulmonarin A and **p**ulmonarin C were shown to be active against the adhesion and to some extent the growth of bacteria at low concentrations. The main

structural differences between the highly active synoxazolidinones and the less active pulmonarins lie in the constrained 4-oxazolidinone core of synoxazolidinones A and C and the ammonium group in pulmonarin A and B. Many potent antifouling bromotyrosine derivatives have a cationic guanidine or guanidine-like group. The lowered basicity and strongly reduced capacity for hydrogen bonding of the tetraalkyl ammonium groups in pulmonarin A and B, combined with an increased molecular flexibility, may be factors influencing the bioactivities of the Pulmonarins unfavourably. While both families of compounds are structurally similar they may serve different purposes in the organism or reflect products of essential processes (Haslam, 1994).

A small library of synthetic analogs of synoxazolidinone A employing the 2,5-diketopiperazine central core found in barettin as replacement for the synthetically more challenging 4-oxazolidione core was prepared based on the high activities of synoxazolidinone A and synoxazolidinone C. The diketopiperazine mimic allows for a rapid assessment of the chemical and structural requirements for activity. The total synthesis of synoxazolidinone A was recently reported by Shymanska *et al*. (Shymanska *et al*., 2014). Peptide mimics **5**-**8** were synthesised via solution phase peptide synthesis and the cyclic compounds were ring-closed via microwave assisted synthesis (Fig. 3.5).

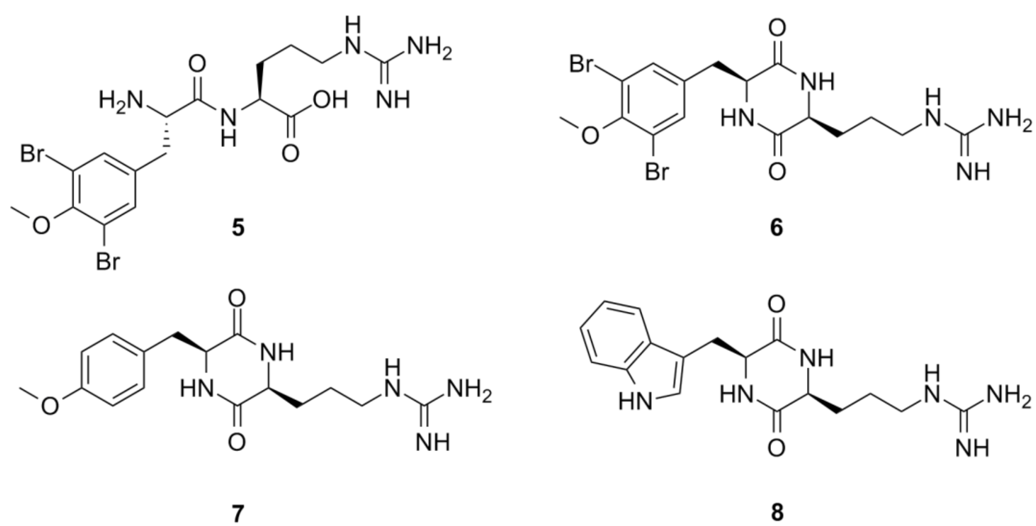

Figure 3.5: Chemical structures of compounds **5**, **6**, **7** and **8**

Compounds **5** and **6** were prepared via bromination of methyltyrosine (Steward *et al.*, 2014) and subsequent amide coupling with arginine methyl ester employing HBTU as the coupling reagent. Several activation strategies were investigated for the amide coupling but only HBTU gave significant yields. This corresponds well with coupling of related bromotyrosine derivatives which can be surprisingly challenging and low yielding (Tadesse *et al.*, 2014). The linear **5** was isolated after deprotection and **6** could be isolated after ring-closure. Compound **7** was generated in a similar fashion starting from methyltyrosine and **8** was prepared according to Li *et al.* from tryptophan (Li *et al.,* 2013). The synthesis of **5** and **6** is outlined below (Fig. 4.6) and their bioactivities are summarised in Table 2.

Synthesis of **5** and **6**

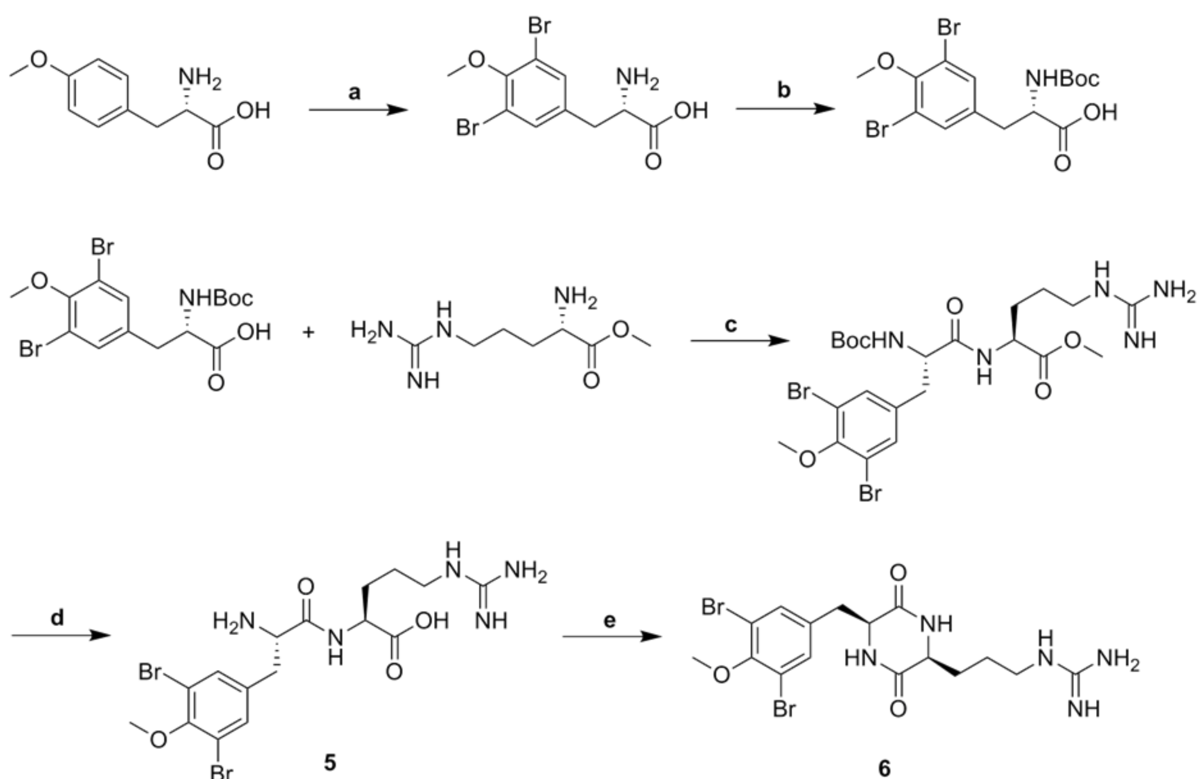

Figure 3.6: Synthesis of compounds **5** and **6**.

Reagents, conditions, isolated yield (a) Br_2, HCl (aq), 0 °C, 30 min; (b) Boc_2O, H_2O/dioxane, rt, 16 h; (c) HBTU, DIPEA, DMF, rt, 16 h; (d) 5% NaOH (aq), rt, 10 min, 4M HCl/dioxane, rt, 16 h, 39%; (e) AcOH, 1-butanol, NMM, microwave 120 °C, 1 h, 33%

Table 3.3: The effect of synthetic **5-8** on growth (Gr) and adhesion (Ad) of fouling marine bacteria, microalgae and barnacles. Results are expressed as MIC (µM)

Organism	MIC (µM)							
	5		6		7		8	
	Ad[1]	Gr[2]	Ad	Gr	Ad	Gr	Ad	Gr
Marine bacteria								
Halomonas aquamarina	0.2	-[3]	0.2	-	0.03	-	0.3	-
Polaribacter irgensii	0.02	0.2	20	0.02	0.03	30	0.3	0.03
Pseudoalteromonas elyakovii	-	0.02	-	0.2	-	0.3	-	0.03
Roseobacter litoralis	-	-	-	0.02	0.3	-	30	-
Shewanella putrefaciens	-	0.2	-	0.02	0.3	30	-	-
Vibrio aestuarians	-	2	-	0.02	-	0.03	-	0.03
Vibrio carchariae	0.02	-	-	-	0.03	-	0.3	-
Vibrio harveyi	-	20	-	20	-	-	30	0.03
Vibrio natriegens	0.2	-	-	-	0.3	-	30	-
Vibrio proteolyticus	-	0.02	-	0.02	0.03	-	0.3	0.03
Microalgae								
Cylindrotheca closterium	0.02	-	-	20	-	-	-	-
Exanthemachrysis gayraliae	20	-	20	20	-	-	30	-
Halamphora coffeaeformis	20	0.2	20	20	3	30	30	30
Pleurochrysis roscoffensis	-	-	-	20	-	-	-	-
Porphyridium purpureum	-	0.02	-	0.2	-	-	-	-
Crustacean settlement								
Balanus improvisus (IC_{50})	-		-		-		-	

[1]Adhesion inhibition

[2]Growth inhibition

[3]Compound inactive at ≥10 µg/mL concentrations

The linear **5** displays high activity against the adhesion of four of the ten bacterial strains and three of the microalgae. Compound **5** also affects the growth of eight of the bacterial and microalgal strains. No activity towards *B. improvisus* was detected for **5**. Microwave assisted cyclisation of **5** generated **6** which exhibits near identical bioactivities as **1**, mainly towards the bacterial growth, which suggests close structural mimicry. Again, no effect on barnacles was seen.

Both the constrained synoxazolidinone A and synoxazolidinone C are active inhibitors of the microfouling species, and it is interesting to observe the high activity of **5**, which is a flexible molecule. However neither **5** nor the constrained **6** affected the barnacle settlement. To evaluate the role of the halogen substituents, compound **7** was prepared. Compound **7** displayed the broadest observed activity against bacterial adhesion with MIC-values down to 30 nM against several bacterial strains. Bacterial growth was less affected and the inhibitory effect on microalgae was low. Barnacle settlement was unaffected by **7**. Seeing that significant activity could be obtained for a compound lacking halogens spurred the preparation of compound **8** where the brominated tyrosine element is replaced with an indole, thus also effectively generating a debrominated analog of 8,9-dihydrobarettin (Sjogren *et al*., 2004). It has previously been shown by Bohlin and coworkers that 8,9-dihydrobarettin is inferior to barettin in balanide larvae settlement assays (Sjogren *et al*., 2004). Compound **8** was an active inhibitor of both bacterial adhesion and growth, with a greater potency towards growth. It exhibited a weak effect on microalgae and no effect on barnacles as illustrated in Fig. 3.7.

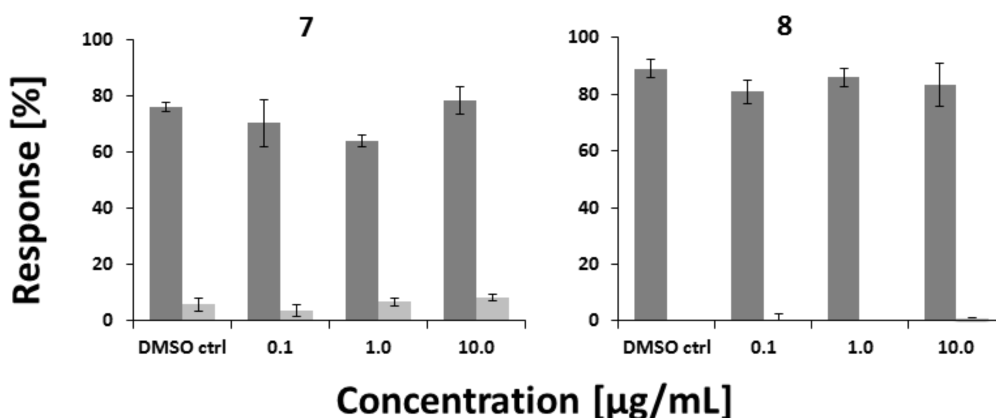

Figure 3.7: Effect of mimics **7** and **8** on the settlement of *B. improvisus* cyprid larvae presented as percentages of settled (dark grey columns), and dead cyprids (light grey columns) and given as means ± SE (n = 4).

Remaining larvae were free swimming. None of the compounds were active at the tested concentrations. DMSO (0.1%, v/v) in FSW was used as negative control.

Their biological activity of the synthetic mimics data indicate that it is possible to generate highly effective antifouling compounds by employing the marine bromotyrosine pharmacophore as a template (Hanssen *et al*., 2014). In comparison with synoxazolidinone A and C, the effect on microalgae is lower for the mimics, and it is shown that removal of the bromine atoms in **7** and **8** renders the compounds almost inactive against microalgal growth and adhesion. None of the synthetic compounds are able to interfere with the *Balanus* larvae indicating that a certain degree of structural integrity, not present in the analogues, is needed for that particular bioactivity. Most effective small natural inhibitors of barnacle settlement display an element of structural rigidity as a link between the brominated hydrophobic part of the molecule and the cationic moiety.

For ianthelline, the bastadins and synthetic derivatives thereof, the structural constraint is provided by an α-oxo-oxime moiety postulated to be essential for the bioactivity, mainly via phenoloxidase inhibition (Ortlepp *et al*., 2007; Bayer *et al*., 2011; Hanssen *et al*., 2014;). Barettin is a potent antifouling agent and its effect on barnacles has been assigned to a combination of the 2,5-diketopiperazine core and an exocyclic double bond to the brominated indole (Sjogren *et al*., 2004). Reduction of the double bond, to generate 8,9-dihydrobarretin, leads to a clear reduction in antifouling activity toward balanide cyprid settlement (Sjogren *et al*., 2004). For the synoxazolidinones, the 4-oxazolidinone core structure is linked, in analogy with barettin, via an exocyclic double bond to the hydrophobic brominated moiety (Tadesse *et al*., 2010; Tadesse *et al*., 2011). This structural motif has only been reported once before in a natural product, the antibacterial lipoxazolidinones isolated from a marine actinomycete (Macherla *et al*., 2007). The study also described the importance of the constrained scaffold for a maintained activity. As the use of a saturated 2,5-diketopiperazine scaffold in mimics **6**-**8** seems insufficient to fulfil the essential requirements also for activity towards barnacles it appears that the double bond is a crucial structural feature needed to generate high inhibitory activity towards both barnacles and microfouling species. The rotational freedom of the hydrophobic residues in the mimics appears to have little influence on the antibacterial effect, but it clearly reduces the inhibitory activity towards barnacle settlement. Introducing an exocyclic bond as that found in barettin and synoxazolidinone A and C is possible, but also makes the synthesis much more complicated (Bergman, 2013). For a compound to be regarded as a feasible candidate for inclusion in paint formulations on large scale, structural simplicity, low cost and ease of preparation is paramount in addition to high activity and low toxicity.

The total synthesis of synoxazolidinone A was recently reported (Shymanska *et al*., 2014) while the synthesis of the bicyclic synoxazolidinone C, which is the most potent compound in the study, has yet to be achieved.

From the extensive biological data it is clear that the four natural compounds and the synthetic mimics are potent antifouling compounds. The activity is dictated by the structural features displayed by the tested compounds but other aspects also influence the outcome. The sensitivity towards the compounds differs not only between types of organisms but also within each group. For the bacteria, which are all Gram-negative, it is clear that some species are less sensitive against these types of antifouling compounds. The adhesion of *P. elyakovii, V. harveyi* and *S. putrefaciens* were virtually unaffected by exposure to any of the eight compounds studied. Their growth on the other hand is highly affected by the compounds and *P. elyakovii* is the most sensitive of all the bacterial strains in the growth inhibition assay. The opposite is seen for *H. aquamarina* where the growth is only affected by synoxazolidinone C while the adhesion is effectively inhibited by all other included compounds but synoxazolidinone **C**. For the microalgae it is obvious that the diatom *H. coffeaeformis* is particularly sensitive. *P. purpureum* on the other hand represents a microalgae where the growth is strongly affected by the compounds while the adhesion is not. The potential underlying mechanisms (cellular membrane composition, metabolism, generation time etc.) responsible for the differences in sensitivities between the species lie outside the scope of this study.

In comparison with the commercial antifouling compound 4,5-dichloro-2-octylisothiazol-3-one, marketed by Dow as SEA-NINE, several of the tested compounds display comparable bioactivities towards the adhesion and growth of bacteria.

The same bacterial species as discussed in the previous section displayed lower sensitivity towards the commercial product indicating a general species resilience as opposed to a specific resistance towards compounds **1-8**. The adhesion and growth of the microalgae were consistently more sensitive to the commercial product. SEA-NINE displays an LC_{50} of 0.34 µg/mL against the settlement of *B. amphitrite* (see supporting information), which is lower than all the included compounds.

Synoxazolidinone A, synoxazolidinone C, pulmonarin A and pulmonarin B have all originally been isolated from an extract of *S. pulmonaria* and it is clear that the organism is able to generate a strong chemical defence with broad activity against a range of epibionts. In particular, synoxazolidinone A and C were found in relatively high amounts in the extract and the biological role of the synoxazolidinones is likely a defensive one. Based on the substantially weaker antifouling properties of pulmonarin A and pulmonarin B, and their lower concentrations (in the original extract), it appears unlikely that they are produced for a general defensive purpose.

3.5 Conclusions

Four members of the synoxazolidinones and the pulmonarins have been evaluated as inhibitors of the biofouling process. A total number of 16 known biofouling species of marine bacteria, microalgae and crustaceans have been included in the test panel to generate a comprehensive overview of the effects of the compounds. The synoxazolidinones display a broad and high activity towards the growth and adhesion of all tested species. Synoxazolidinone C is the most active compound in the study and represent a compound superior in bioactivity compared to most reported antifouling bromotyrosine derivatives in the literature. Its antifouling activity is comparable to that of the commercial product SEA-NINE against marine bacteria. The pulmonarins were generally only active against the growth and adhesion of bacteria. Four simplified mimics of the synoxazolidinones were prepared to evaluate the structure activity relationship. It was shown that the effect on microfouling can be mimicked using a 2,5-diketopierazine scaffold while the inhibitory effect on balanid settlement could not be repeated with the synthetic mimics.

Chapter 4

Chapter 4: Evaluation of cationic micropeptides derived from the innate immune system as inhibitors of marine biofouling

4.1 Abstract

The search for environmentally friendly alternatives to toxic antifouling paints has spurred research into compounds or natural origin. In the current study, 13 short synthetic amphiphilic cationic micropeptides have been evaluated for their capacity to inhibit the marine fouling process. The peptides were derived from the antimicrobial iron-binding innate defence protein lactoferrin found in breast milk and tear fluid. The whole biofouling process was studied and microfouling organisms such as marine bacteria and microalgae were included as well as the macrofouling barnacle *Balanus improvisus*. In total 19 different marine biofouling organisms were included and both the adhesion and growth of the microfoulers was investigated. The antifouling effects of the peptides were compared to the commercial antifouling herbicide SEA-NINE. It was shown that the majority of the peptides were active against the settlement of barnacle cyprids with IC_{50} values down to 0.5 µg/mL. Six peptides were active against the adhesion and growth of the microorganisms. Two peptides were particularly active against the microfoulers with MIC-values ranging 1-0.01 µg/mL and comparable to the antifouling activities of the commercial reference SEA-NINE.

This work has been published in a refereed journal, Biofouling, and is presented below.

The citation for the original publication is:

Rozenn Trepos, Gunnar Cervin, Claire Pile, Henrik Pavia, Claire Hellio, Johan Svenson. (2015). Evaluation of cationic micropeptides derived from the innate immune system as inhibitors of marine biofouling. Biofouling, Vol. 31, Iss. 4, 393-403.

Author contributions:

The author designed the experiments, performed the antibacterial and antimicroalgal assays with the peptides and the commercial herbicide SEA-NINE. The co-authors design the peptides library and performed the *Balanus* cyprids settlement assays. The manuscript was written in collaboration with the co-authors.

4.2 Introduction

The economical and environmental costs associated with the unwanted accumulation of organisms on marine structures are enormous (Townsin, 2003; Yebra *et al.*, 2004, Callow *et al.*, 2011). The settlement of marine organisms on submerged surfaces is known as biofouling and the natural process affects all marine activities. The uncontrolled growth of organisms on the hull of a boat requires a significant increase in propulsive power and leads to subsequent increases in fuel consumption and CO_2 emission (Schultz, 2007; Schultz *et al*., 2010). Taking into account that 90% of the global trade of goods is via the world trading fleet it has been estimated that effective antifouling (AF) measures saves approximately 150 billion dollars globally each year and a further 450 million tonnes of CO_2 from being emitted to the atmosphere (Schultz, 2007; Trepos *et al*, 2014). Biofouling is in addition a phenomenon that is important to control in areas such as offshore industry, aquaculture and energy production (Braithwaite *et al.*, 2004; Apolinario *et al*., 2009; Langhamer *et al*., 2010; ; Adams *et al*., 2011). It has in particular recently been pointed out that biofouling has deterring effects on important renewable energy technologies such as offshore wind power and wave energy plants (Wilhelmsson *et al*., 2008; Langhamer *et al*., 2010) .

The biocide tributyltin (TBT) has been used in paints for the last four decades to keep the biofouling under control on the majority of the world's vessels (Almeida *et al*., 2007). With the international ban on the use of TBT by and organotin compounds in 2008 a void, in which no equally efficient AF countermeasures are available, has been created (Yebra *et al*., 2004). Copper containing AF paints have been used as alternatives but have also been shown to have detrimental environmental effects (Brooks *et al*., 2009). The new legislation states that AF solutions can no longer be general biocides and researchers are now turning to Nature and compounds of natural origin in search of

environmentally friendly biomimetic settling deterrents (Dobretsov *et al*., 2006; Qian *et al*., 2010; Callow and Callow, 2011).

Several marine natural products produced by sessile marine organisms and marine microorganisms have been identified as antifoulants and are currently under investigation (Burgess *et al.,* 2003; Ortlepp *et al.,* 2007; Qian *et al.,* 2010; Qian *et al*., 2015). barettin (Sjogren *et al.,* 2004), oroidin (Tsukamoto *et al*., 1996a), ianthelline (Hanssen *et al.,* 2014), the synoxazolidinones (Trepos *et al.,* 2014) and bastadins (Ortlepp *et al*., 2007) are all examples of well-studied natural marine AF compounds. Natural compounds of terrestrial origin have also been evaluated and a compound such as the sesquiterpene polygodial found in several plants, represent a promising lead towards natural AF solutions (Cahill *et al.,* 2013; Cahill *et al*., 2014).

Lactoferrin is an antibacterial innate defense protein found mucosal secretions, milk, and tears in many mammals (Arnold *et al*., 1977; Gifford *et al*., 2005; González-Chávez *et al*., 2009). It is the second most abundant protein in milk after casein (Conneely, 2001). The protein is digested into lactoferricin upon passage though the gastrointestinal tract and the resultant cationic 25 amino acid residue peptide display interesting antimicrobial bioactivities (Tomita *et al*., 1991; Bellamy *et al*., 1992; Kuwata *et al.*, 1998).

Truncation of the native lactoferricin peptides and introduction of synthetic amino acid replacements have enabled the generation of highly antibacterial micropeptides comprising only three amino acids (Strøm *et al.*, 2002; Strøm *et al*., 2003; Vogel *et al*., 2002; Haug *et al*., 2004). These small peptides are generally C-terminally modified with a bulky amide for activity and stability reasons and can be tuned to display high bioactivities at low micromolar minimum inhibitory concentrations (MIC) against bacteria resistant to conventional antibiotics (Svenson *et al*., 2008; Karstad *et al*., 2012).

The synthesis of these small peptides is straightforward and most peptides can be both rapidly and economically produced. The strong effect of this class of micropeptides on biofilm forming bacteria such as *Staphylococcus epidermidis* and *Staphylococcus aureus* served as inspiration for evaluating them in an aqueous environment to assess their potential AF effect on the different stages of the biofouling process (Flemming *et al*., 2009; Jorge *et al*., 2012). These amphipilic peptides, initially originating from natural innate immune peptides represents a new class of bioactive compounds that have not previously been evaluated in a marine setting before.

The peptides can be tuned to display powerful antimicrobial and cytotoxic activities towards human pathogenic bacteria and certain types of cancer and are under investigation in a number of clinical trials (Fadnes *et al*., 2011; Saravolatz *et al*., 2012). The mode of action is on the cell membrane level which allows for significant freedom in the design of bioactive compounds as is obvious from the library included in the present study. The peptide sequence is not crucial for activity and compounds can be designed with ranging basicity, stability and hydrophobicity, not only for optimized activity but also for potential formulation purposes (Karstad *et al*., 2012). As such they represent an interesting class of compounds to evaluate for AF applications.

The currently studied peptide library was composed of antibacterial cationic tripeptides designed to range from inactive to highly active and with ranging structural cationic and hydrophobic features (Karstad *et al.*, 2012). Most peptides where chosen to remain within the pharmacophore needed for terrestrial antimicrobial activity, to allow for a comprehensive evaluation of the structural contributions to activity and for comparison between the different environments (Strøm *et al.*, 2003).

Other interesting alternative approaches to AF involve nontoxic polymer coatings that either deter the settling organism from adhering or weakens the interactions between

the organism and the surface to allow removal via hydrodynamic shear forces (Callow and Callow , 2011). The recent development of promising amphilipic polymers made from copolymerized peptoids as fouling release coatings and other hydrophobic coatings displaying linked quaternary ammonium groups suggest that the concept of cationic amphiphilic peptides may be display interesting AF properties in several areas (Majumdar *et al*., 2008a; Majumdar *et al*., 2008b; Martinelli *et al.*, 2011; Lejars *et al*., 2012; van Zoelen *et al*., 2014).

A significant difference in antibacterial effect towards terrestrial human pathogenic bacteria and fouling marine bacteria has been seen for several isolated marine secondary metabolites and the studied peptides offers a relevant comparison for further studies into the underlying mechanisms (Hanssen *et al*. 2014; Trepos *et al*. 2014). The current investigation represents the first study of the effects of compounds initially derived from the innate immune system on marine biofouling and on organisms in a marine setting.

4.3 Materials and methods

4.3.1 Materials

4.3.1.1 Marine biofouling organisms

19 different marine organisms previously shown to be involved in marine biofouling in estuarine and marine environments were included in the present study and presented below in Table 3.1 (Hanssen *et al*., 2014; Trepos *et al*., 2014). The 10 marine bacterial strains were obtained from the ATCC bacterial collection. 8 pure, but non-axenic, marine microalgal strains involved in surface colonisation and biocorrosion (obtained from Algobank, Caen, France) were also evaluated together with reared *Balanus improvisus* cyprids. The included microfouling organisms are compiled in Table 4.1.

Table 4.1: Biofouling organisms studied in the present study

Species	Code
Marine bacteria	**ATCC**[1]
Halomonas aquamarina	14400
Polaribacter irgensii	700398
Pseudoalteromonas elyakovii	700519
Roseobacter litoralis	49566
Shewanella putrefaciens	8071
Vibrio aestuarianus	35048
Vibrio carchariae	35084
Vibrio harveyi	700106
Vibrio natriegens	14058
Vibrio proteolyticus	53559
Microalgae	**Algobank code**
Cylindrotheca closterium	AC 170
Exanthemachrysis gayraliae	AC 15
Halamphora coffeaeformis	AC 713
Pleurochrysis roscoffensis	AC 32
Porphyridium purpureum	AC 122
Hymenomonas coronate	AC 115
Rhodosorus marinus	AC 119
Pleurochrysis carterae	AC 1

[1]American tissue culture code

4.3.1.2 Peptide library design

Peptides were selected based on their ranging antimicrobial bioactivity towards human pathogenic bacteria and also their chemical diversity (Flaten *et al*., 2011; Karstad *et al*., 2012) (Fig. 4.1). Four inactive, structurally related reference peptides (**1**-**4**) were also included. The synthesis and purification of the peptides used in this study have been previously reported (Karstad *et al*., 2012)

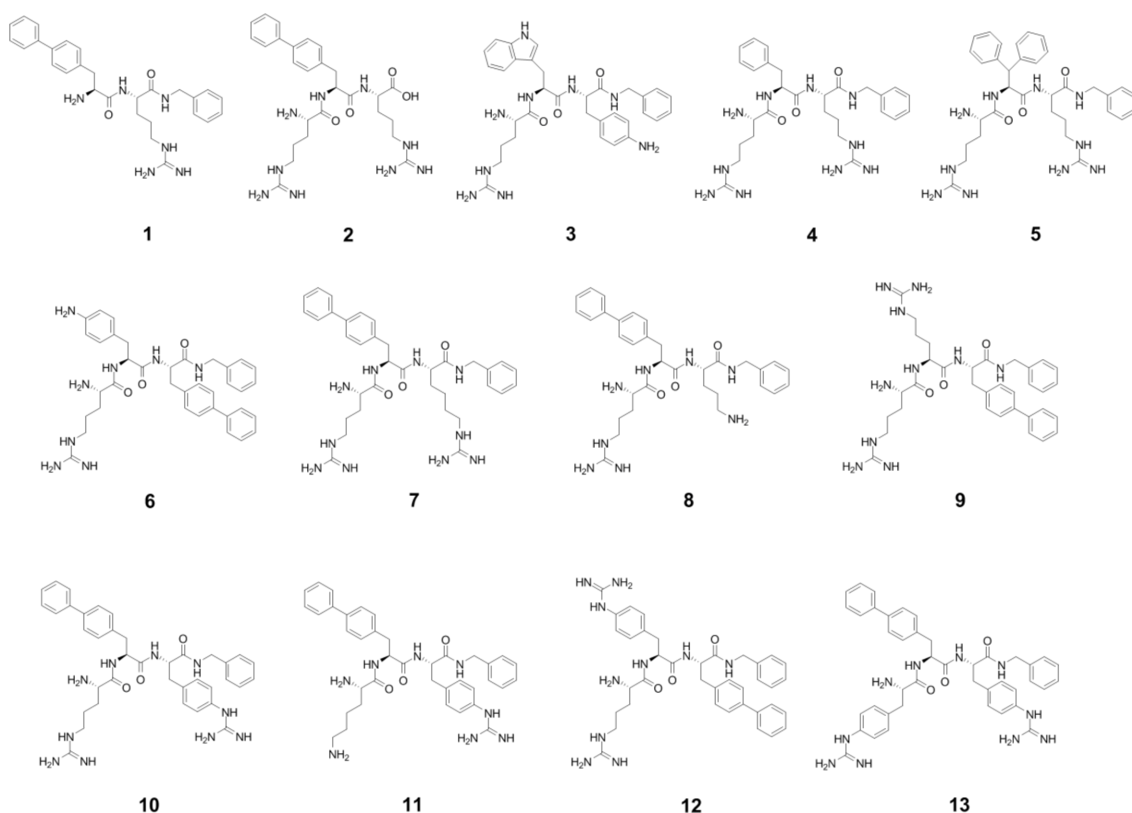

Figure 4.1: Chemical structures of the cationic micropeptides evaluated in the current study each comprising combinations of natural and unnatural cationic and hydrophobic residues

4.3.2 Methods

4.3.2.1 Balanus cyprid settlement

Cyprid larvae of *B. improvisus* were reared in a laboratory cultivating system at Tjärnö Marine Biological Laboratory, as described by Berntsson (Berntsson *et al*., 2000a). The settlement assays with *B. improvisus* cyprids were conducted in non-treated polystyrene Petri dishes (Ø 48 mm, Nunc #150340) containing 10 mL filtered (0.2 µm) seawater. All peptides were dissolved in DMSO and then serially diluted with DMSO to give the desired concentration series (ranging from 0.1 to 5 µg/mL) of which 10 µL was added to each test dish. A total of 18–22 newly moulted cyprids were added to each test dish which were incubated at room temperature (20–25 °C) for five days. At the end of the experiment, the number of metamorphosed juvenile barnacles, as well as live and dead cyprids, was assessed under a dissection microscope. Each peptide concentration was replicated four times (n = 4) and dishes with 10 µL DMSO served as controls. The concentration of a peptide leading to 50% inhibition of the settlement compared to the control was reported as the IC_{50} value (Trepos *et al*., 2014) .

4.3.2.2 Antibacterial assays

The effect of the peptides on the growth and adhesion of bacteria and microalgae was studied as described before (Hanssen *et al*., 2014; Trepos *et al*., 2014). Briefly, the bacteria were grown at 26 °C in a marine medium, composed of 0.5% peptone (neutralised bacteriological peptone, Oxoid LTD) in filtered (Whatman 1001-270, pore size 11 µm) natural seawater from the Solent (UK).

The peptides were dissolved in 100% methanol (general purpose grade, Fisher Chemical), and transferred to clear polystyrene 96-well plates (Fisher Scientific), dried under vacuum and sterilised under UV illumination. Wells were prepared using

concentrations of 0.01, 0.1, 1 and 10 µg/mL and each concentration was replicated six times (Bressy *et al*., 2010) . For the growth inhibition experiments 100 µL of bacterial culture (2 × 10^8 colony forming units (CFU) mL^{-1}) were added to each well under aseptic conditions and incubated for 48 h at 26 °C. The minimum inhibitory concentration (MIC) was defined as the minimum concentration resulting in no change in optical density at 630 nm after incubation for 48 h (Thabard *et al.,* 2011). The bacterial adhesion was evaluated in the microplates by similar inoculation as described above. After 48 h incubation, wells were emptied and rinsed once with 100 µL of sterile seawater to remove the non-attached cells and air-dried at room temperature. The remaining bacterial biofilm was stained with 100 µL of 0.3% (v/v) aqueous crystal violet and the OD was measured at 595 nm (Sonak *et al*., 1995). Media was used as blank and MIC was defined as the lowest concentration that produced a reduction in adhesion. Peptides displaying MIC-values >10 µg/mL were considered inactive in this study.

4.3.2.3 Microalgal assay

Each algal strain was grown for five days prior to use at 20 °C in F/2 medium. After five days, microalgal concentration was assessed via analysis of the chlorophyll *a* content and dilutions of the stock culture were made accordingly to generate stock solutions of each microalgae containing 0.1 mg/L chlorophyll *a*. 100 µL of the stock solutions were transferred to the wells of black 96-well plates prepared with ranging concentrations of the peptides as described above. In the growth inhibition experiment the inoculated plates were grown for 5 days under constant light exposure (140 µmol m^{-2} s^{-1}) at 20 °C. After the incubation, the microplates were centrifuged at 4100 rpm for 10 min at 4 °C using a Beckman Coulter Allegra 25R centrifuge and subsequently emptied. 100 µL of 100% methanol was added to each well to liberate chlorophyll *a*. The pigment

concentration was quantified employing the fluorimetric method (Chambers *et al*., 2011) and MIC values were calculated, based on OD measurements, as explained for bacteria. The adhesion inhibition was assessed in black microplates that were prepared and inoculated as stated above. Media was used as blank. After 5 days of incubation, the medium was gently removed using a multichannel pipette to eliminate all the non-attached cells. 100 µL of 100% methanol was then added to each well to liberate chlorophyll *a*. The pigment concentration was quantified employing the fluorimetric method explained above. Peptides displaying MIC-values >10 µg/mL were considered inactive in this study.

4.4 Results

4.4.1 Balanus cyprid settlement

The majority of the investigated peptides demonstrated a high settling deterring effect on *B. improvises* cyprids. All peptides, apart from **1** and **4**, were active in the screening and displayed IC_{50} values ≤5 µg/mL. Peptide **6** and **13** were the most active peptide in the study with IC_{50} values of 1.0 and 0.5 µg/mL respectively as shown in Fig. 4.2.

The peptides displayed no apparent toxicity in the employed concentration range. The highly active peptides **6** and **10**-**13** induced an inactivation of the cyprid swimming while the less active and highly polar peptide **2** increased the swimming of the cyprids. The results of the settlement data are compiled in Table 4.2 together with the physicochemical properties of the peptides.

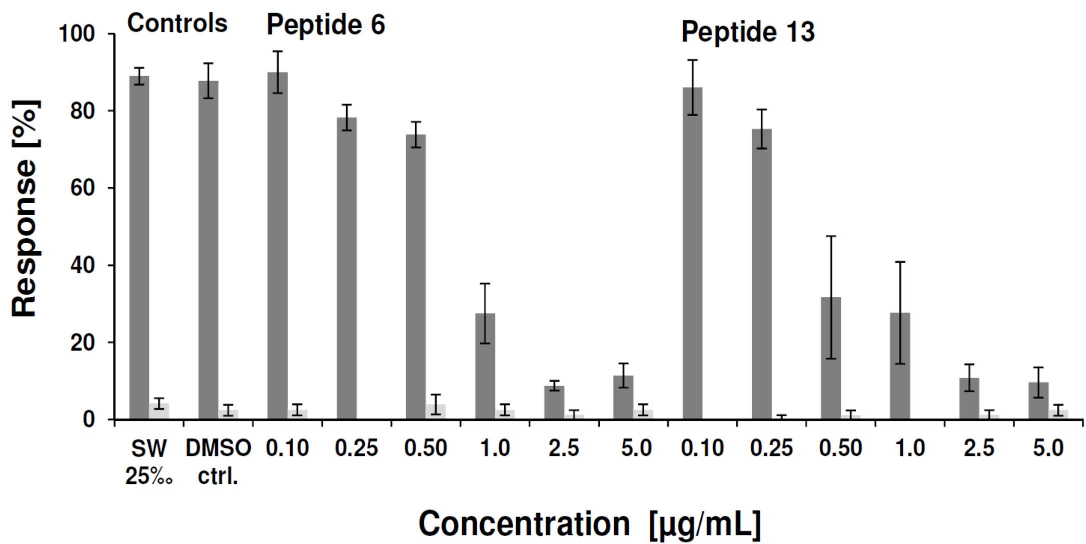

Figure 4.2: Effects of **6** and **13** on the settlement of *B. improvisus* cyprid larvae as presented percentages of settled (dark grey columns), and dead cyprids (light grey columns) and given as means ± SE (n = 4).

Remaining larvae were free swimming. IC_{50} values were determined to be 1.0 µg/mL and 0.5 µg/mL respectively for **6** and **13**. DMSO (0.1%, v/v) in filtered seawater was used as the negative control.

Table 4.2: The effects of 13 peptides on the settlement and behavior of *B. improvisus* cyprid larvae (*n*=4).

Peptide	Sequence[1]	M_w	IC_{50} Balanus (µg/mL)	MIC *S. aureus* (µg/mL)[2]	CLog P[3]	Cyprid observations
1	Bip-R-NHBn	486.6	>5	n.d.	1.9642	
2	R-Bip-ROH	553.7	5	n.d.	-3.3760	Very active
3	R-W-AppNHBn	611.7	5	>150	-0.0004	
4	R-F-RNHBn	566.7	>5	150	-1.8504	
5	R-Dip-RNHBn	642.8	5	25	-0.4124	
6	R-App-BipNHBn	648.8	1	15	1.8976	Inactive
7	R-Bip-HarNHBn	656.8	5	10	0.5666	
8	R-Bip-OrnNHBn	600.8	5	10	0.8776	
9	R-R-BipNHBn	642.8	5	5	0.0376	
10	R-Bip-GppNHBn	690.8	2.5	5	1.4976	Inactive
11	K-Bip-GppNHBn	662.8	1.5	5	2.8666	Inactive
12	R-Gpp-BipNHBn	690.9	2.5	2.5	1.4976	Inactive
13	Gpp-Bip-GppNHBn	738.9	0.5	<2.5	1.6470	Inactive
	Seanine[4]	282.2	0.3	n.a.	4.8990	

[1] Unnatural modification: Bip – Biphenylalanine, Har – Homoarginine, App – 4-aminophenylalanine, Dip – Diphenylalanine, Gpp – 4-guanidionophenylalanine, NHBn – Benzylated C-terminus, Orn – Ornithine

[3]Calculated using Chemoffice 10.0

[4]From DOW: http://www.dow.com/microbial/applications/ma_ma_products.htm

4.4.2 Growth inhibition

The growth inhibiting effect of the peptides on microfouling bacteria and microalgae was investigated and the data from the active peptides is summarised in Table 3.3 Peptides **1**, **2**, **4**, **5**, **7**, **10** and **12** displayed MIC-values >10 µg/mL and were considered inactive. Peptides **6** and **13** exhibited low MIC values against growth down to 0.01 µg/mL against several bacteria and down to 0.1 µg/mL against selected microalgae.

Table 4.3: Growth inhibition[1]

Organism	MIC (µg/mL)						
	3	**6**	**8**	**9**	**11**	**13**	Seanine
Marine bacteria							
Halomonas aquamarina	10	0.1	-[2]	-	1	0.1	0.1
Polaribacter irgensii	10	0.1	10	-	1	0.01	1
Pseudoalteromonas elyakovii	1	1	10	-	10	0.1	0.1
Roseobacter litoralis	1	0.01	-	-	-	0.01	1
Shewanella putrefaciens	1	0.1	-	-	-	0.01	1
Vibrio aestuarianus	10	0.01	10	-	10	0.01	<0.01
Vibrio carchariae	10	0.1	10	-	-	0.01	<0.01
Vibrio harveyi	1	0.1	-	-	-	0.1	1
Vibrio natriegens	1	0.1	-	-	-	0.1	1
Vibrio proteolyticus	10	0.1	10	-	10	0.1	0.01
Microalgae							
Cylindrotheca closterium	0.1	0.1	1	-	-	1	<0.01
Exanthemachrysis gayraliae	0.1	1	1	-	-	0.1	<0.01
Halamphora coffeaeformis	1	0.1	0.1	-	-	1	<0.01
Pleurochrysis roscoffensis	1	0.1	0.1	-	-	1	<0.01
Porphyridium purpureum	1	1	1	10	-	0.1	<0.01
Hymenomonas coronate	1	1	1	10	-	0.1	
Rhodosorus marinus	0.1	0.1	1	1	-	1	
Pleurochrysis carterae	0.1	0.1	0.1	10	-	1	

[1]Only peptides displaying MIC values ≤10 µg/mL are included.

[2]Inactive

4.4.3 Adhesion inhibition

With the exception of peptide **9**, the same peptides that were active against the growth of test organisms also inhibited the adhesion of the microfoulers as presented in Table 4.4. Peptides **6** and **13** displayed superior inhibitory effect towards adhesion in analogy to their growth inhibitory effects.

Table 4.4: Adhesion inhibition[1]

Organism	MIC (µg/mL)					
	3	**6**	**8**	**11**	**13**	Seanine
Marine bacteria						
Halomonas aquamarina	10	0.01	-[2]	-	0.01	<0.01
Polaribacter irgensii	10	0.1	-	-	0.01	0.1
Pseudoalteromonas elyakovii	10	0.1	-	-	0.1	<0.01
Roseobacter litoralis	10	0.01	-	-	0.01	<0.01
Shewanella putrefaciens	10	0.01	-	-	0.01	1
Vibrio aestuarianus	10	0.01	-	10	0.01	1
Vibrio carchariae	10	0.01	-	-	0.01	<0.01
Vibrio harveyi	10	0.01	-	-	0.01	1
Vibrio natriegens	10	0.01	-	-	0.1	<0.01
Vibrio proteolyticus	10	0.01	10	-	0.1	0.01
Microalgae						
Cylindrotheca closterium	0.1	0.1	0.1	-	0.1	<0.01
Exanthemachrysis gayraliae	0.1	0.1	0.1	-	0.1	<0.01
Halamphora coffeaeformis	0.1	0.1	0.1	-	0.1	<0.01
Pleurochrysis roscoffensis	0.1	0.1	0.1	-	0.1	<0.01
Porphyridium purpureum	1	1	1	-	1	<0.01
Hymenomonas coronate	1	1	1	-	1	
Rhodosorus marinus	1	1	1	-	1	
Pleurochrysis carterae	1	1	1	-	1	

[1]Only peptides displaying MIC values ≤10 µg/mL are included.

[2]Inactive

4.5 Discussion

Biofouling is often regarded as a stepwise process where the adsorption or organic molecules to an immersed surface is followed by settlement of microorganisms such as bacteria and microalgae (Roberts *et al.*, 1991; Qian *et al*., 2007). Subsequent settlement of higher organisms, for example bivalves, crustaceans and microalgae is referred to as macrofouling and has in several studies been shown to be facilitated and dictated by the already established microfouling community on the surface (Beech *et al,*. 2005; Huggett *et al*., 2006; Qian *et al*., 2007). The actual biofouling process is however not as linear and most macrofoulers are also able to rapidly settle on a clean immersed surface within hours (Roberts *et al*., 1991; Callow *et al*., 1997) . The complex scenario in which several types of organisms are involved is difficult to mimic in a single assay and it is necessary to include both micro- and macrofoulers in a study when assessing the complete potential AF properties of a compound (Qian *et al.,* 2010; Bernbom *et al*., 2013). The 19 different organisms included in the present investigation provide a comprehensive overview of the AF effect of the investigated peptides, both on the growth and adhesion (Trepos *et al.*, 2014).

The current study included peptides that are pharmaceutically relevant. The peptides are all fulfilling the pharmacophore model described by Strøm and colleagues, which dictate that at least two units of hydrophobic bulk in conjunction with two positive charges are needed for activity (Strøm *et al*., 2003). The peptides may, at glance, appear structurally and chemically similar but subtle structural features dictate whether the peptides are inactive (MIC ≥150 µg/mL) or highly active (MIC ≤5 µg/mL) (Karstad *et al.*, 2012) .

Both the bulk and the cationic charge are provided by a range of different natural and unnatural amino acid subunits and these substitutions create peptides with improved pharmaceutical properties (Svenson *et al.*, 2009; Karstad *et al.*, 2010; Karstad *et al.*, 2012).

The fact that the peptides range from inactive to highly active by design is further reflected in their differences in overall hydrophobicity as described by the theoretical ClogP value (Table 4.2) which correlate well with their retention times on a C_{18} column which often serves as an experimental estimate of their hydrophobicity (Flaten *et al.*, 2011) . It has previously been shown that for this type of peptides there is a close correlation between antimicrobial activity and a certain degree of C_{18} retention (Flaten *et al.*, 2011) . These amphipilic peptides, initially originating from natural innate immune peptides represents a new class of bioactive compounds that have not previously been evaluated in a marine setting before. They can be tuned to display powerful antimicrobial and cytotoxic activities towards human pathogenic bacteria and certain types of cancer and are under investigation in a number of clinical trials (Saravolatz *et al.*, 2012**;** Fadnes *et al.*, 2011). The mode of action is on the cell membrane level which allows for significant freedom in the design of bioactive compounds as is obvious from the library included in the present study. The peptide sequence is not crucial for activity and compounds can be designed with ranging basicity, stability and hydrophobicity, not only for optimized activity but also for potential formulation purposes. As such they represent an interesting class of compounds to evaluate for AF applications. The macrofoulers represent the main culprit in the biofouling community responsible for the majority of the accumulated mass and increased resistance (Lejars *et al.*, 2012). Barnacle cyprids generally settle rapidly on an immerged substrate (Roberts *et al.*, 1991) and provide a relevant model organism for AF investigations into calcareous macrofoulers.

B. improvises was exposed to varying concentrations of the peptides for five days before the extent of metamorphosis and settlement of juvenile barnacles was assessed. Several peptides displayed high settling deterring effects at 5 µg/mL and below (Table 3.2). Peptide **13** displayed a MIC value of 0.5 µg/mL (0.7 µM), which is lower than for many studied natural AF compounds (Burgess *et al*., 2003; Hanssen *et al*., 2014; Trepos *et al*., 2014; Fusetani *et al*., 2011). Peptide **6** and **10**-**12** also represent highly active compounds and a clear link between the antibacterial activity and the hydrophobicity of the most active peptides and the settling deterring effect is seen. Peptides containing one or two artificial Phe derivatives as mimics of Arg and Lys (Gpp or App, peptides **2**, **6** and **10**-**13**) are also the most active ones suggesting that these two similar synthetic amino acids play a beneficial role for the balanide activity. The difference between Gpp and App lies in the guanidine in Gpp which is charged at physiological conditions (pKa ~10.8) in contrast to the neutral App (pKa ~4.9) (Svenson *et al*., 2009). Both sidechains contain an additional phenyl ring which makes these sidechains more hydrophobic compared to Arg or Lys, which they are designed to mimic. The beneficial effect of the uncharged App is significant enough to make the inactive (towards pathogenic bacteria) peptide **2** active in the balanide screen. Spectroscopic structure activity studies of similar short antimicrobial peptides in various environments have illustrated the preference of the C-terminal benzyl cap to group with the central bulky hydrophobic group to minimize surface expose to the surrounding aqueous environment (Isaksson *et al*., 2011). In the peptides containing App or Gpp the option of forming similar bonds between the aromatic sidechains of Gpp and App and the benzylamide in solution exists and appears to be preferred and this may generate additional bioactive conformations. The two guanidines, in peptides containing two arginines in the right arrangement, can π-stack to further reinforce an amphiphilic structure (Isaksson *et al*., 2011).

Energy minimized model of peptides **6** and **7** illustrating the different calculated solution structures are shown in Figure 4.3.

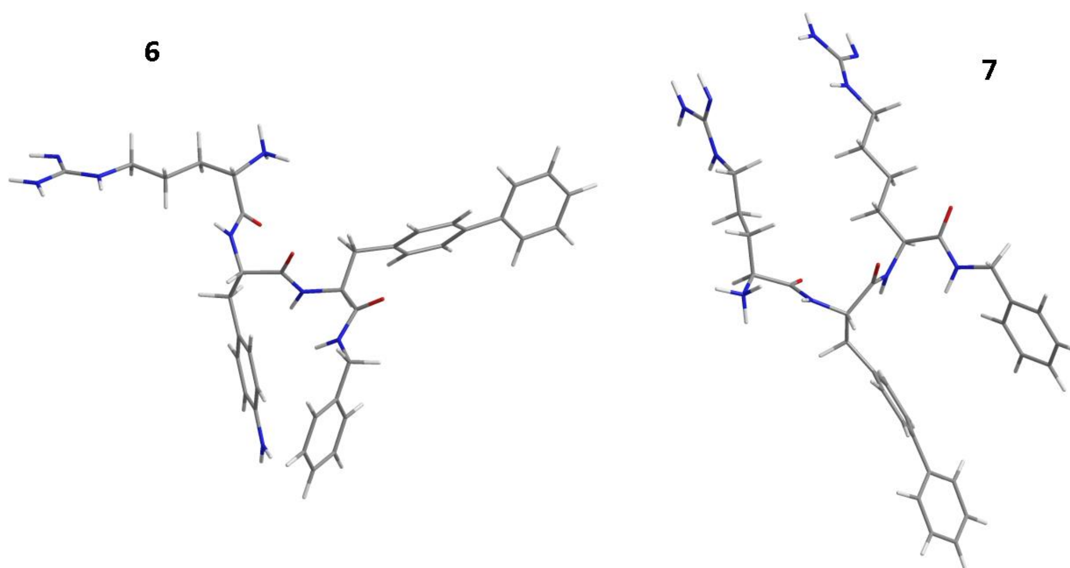

Figure 4.3: Energy minimized (Chem3D Ultra 10.0) conformations (carbon-grey, oxygen-red, nitrogen-blue and hydrogen-white) of peptide **6** and **7**.

Illustration show the App sidechain in **6** can form interactions with the benzyl amide while it interacts with the Bip sidechain in peptide **7**. The arginine sidechains in **7** are lined up for π-stacking interactions. Both peptides can form amphiphilic structures.

The dependence on a balance between cationic charge and hydrophobicity, needed for antibacterial activity against human pathogenic bacteria, is clearly not as defined for the AF activity as the two inactive peptides **2** and **3** display MIC values of 5 µg/mL toward *B. improvises*. Peptides **2** and **3** are significantly more polar that the most active peptides and they were included originally as negative control peptides. A more pronounced hydrophobicity is nevertheless needed for a more prominent activity and the most active peptides all display similar hydrophobicities with ClogP values between (1.5-2.9).

A charge/hydrophobicity balance is needed for activity and a high hydrophobicity is not enough as illustrated by the dipeptide **1** (ClogP of ~2.0) which apparently lacks a cationic residue for activity. Of interest is also the activity of peptide **2** which lack the essential C-terminal hydrophobic modification and represent the most polar compound in the study. Peptide **2** induced an increased cyprid swimming in contrast to peptides **6** and **10-13** that appears to inactivate the cyprids suggesting two different modes of action. The activating effect on cyprids by AF compounds has recently been used to develop successful green AF counter measures as exemplified medetomidine (Selektope) developed by I-tech which induce reversible, increased cyprid swimming upon interaction with the active compound. None of the peptides appeared to display any toxic effects on the cyprids within the tested concentration range (Figure 4.2).

Microfouling by bacteria and microalgae leads to a slimy layer on marine surfaces that may attract larger organisms (Qian *et al*., 2007) and also leads to increased biocorrosion (Dexter *et al*., 1993) and this process is thus also of great importance to control. Peptides **3**, **6**, **8**, **9**, **11** and **13** displayed ranging activities towards the growth and adhesion of both marine bacteria and microalgae.

The remaining peptides were considered inactive with MIC-values >10 µg/mL. Peptides **3**, **6** and **13** being the most notably active ones displayed MIC-values as low as 0.01 µg/mL against several bacteria and down to 0.1 µg/mL against microalgae. The observed activities are superior to the several marine antifoulants such as Ianthelline and Barettin and similar to the highly active Arctic secondary AF metabolite Synoxazolidine C (Hanssen *et al*., 2014; Trepos *et al*., 2014; Qian *et al*., 2015). The activity of the peptides was generally higher towards bacteria where peptide **6** and **13** clearly are as potent as the commercial positive control against bacterial growth and adhesion.

The activity against growth and adhesion was similar overall for the active compounds and no clear distinction is seen even if both **6** and **13** may be considered more active towards adhesion. The positive control SEA-NINE-is superior against the microalgae, which is to be expected as it is also used as an herbicide (Gardiola *et al*., 2012). The two diatoms *Cylindrotheca closterium* and *Halamphora coffeaeformis*, which represent particularly difficult organism to target with any AF approach (Molino *et al*., 2008), are as sensitive towards the peptides as the other studied microalgae. No apparent difference in species sensitivity towards the peptides, which has been reported for other compounds (Trepos *et al*., 2014), could be observed.

It was anticipated that most of the included antimicrobial peptides would be active against the marine microflora based on previous observations on isolated antibacterial marine secondary metabolites (Trepos *et al*., 2014). While selected peptides indeed are very efficient at preventing both growth and adhesion of the studied bacteria it is interesting to see that the effect is not entirely general. Several peptides, active against human pathogenic bacteria and *B. improvises* settling, such as peptides **5**, **7**, **10** and **12** did not display any AF bioactivity towards the microfoulers at concentrations ≤10 µg/mL. Higher concentrations were not studied within a relevant bioactivity range for commercial AF-products (Ritschoff *et al*., 2001; Trepos *et al*., 2014). For **10** and **12** that suggest that the marine bacteria are less sensitive than the human pathogens, which was unexpected. It should be noted that all the included marine bacteria are Gram-negative and that these peptides usually display a more pronounced preference for Gram-positive bacteria where the anionic bacterial membrane is easier to access (Haug *et al*., 2008).

Peptides **10** and **12** are structurally similar to the very active **13** and they both contain the same structural features but in a different arrangement and illustrate that the link between physicochemical properties, amphiphilicity and activity is not as clear for the microfoulers as against the cyprids or human pathogenic bacteria. Removal of the charged guanidine from the Gpp moiety in the inactive **12** transforms it into the highly active **6** which share the localisation of charge and bulk with **3**, also containing App. Peptide **3** is inactive against pathogenic bacteria (MIC >150 µg/mL) but display AF-activity against all the included marine species. The cationic charge (+2) in **3** and **6** is grouped towards the N-terminus which appears to be beneficial when combined with three hydrophobic residues, and illustrate how the distribution of charge and bulk plays a role for the bioactivity. These and similar short cationic peptides generally possess no defined secondary solution structure even if it has been shown that an amphiphilic solution structure appears to facilitate membrane insertion via reduce solubility (Isaksson *et al.,* 2011). Upon interaction with the bacterial cell membrane, it is believed they all adopt a bioactive amphiphilic conformation which allows for membrane insertion. The distribution of the functional groups in peptides **3** and **6**, as illustrated in Fig. 4.3 for peptide **6**, is a likely explanation for their high AF bioactivity, given a similar mode of action against the marine bacteria and microalgae.

4.6 Conclusion

Thirteen synthetic micropeptides derived from the innate immune system have been evaluated for their influence on the marine biofouling process. Eleven peptides displayed high activities against barnacles at low micromolar concentrations. Six peptides were further active against the adhesion and growth of both marine bacteria and microalgae.

Collectively it is shown that peptides **6** and **13** are powerful, general AF compounds against both micro- and macrofouling species with bioactivities comparable to the commercial product SEA-NINE. A new AF solution should ideally be deterring and nontoxic which makes these compounds interesting to study further. The data in the current paper indicate that these short peptides can be designed to demonstrate powerful AF activities via ranging assemblies and choice of building blocks. This observation is envisaged to allow for freedom in the design of effective compounds within this structural realm that can also be tuned, with maintained AF activity, to display beneficial formulation properties for potential paint incorporation in the future. The combinations of functionalities displayed by the active peptides in the current study may also serve as inspiration for the development of fouling release coatings.

Chapter 5

Chapter 5: Evaluation of the impact of five biocides on *Symbiodinium microadriaticum* and *Symbiodinium voratum*.

5.1 Abstract

Most reef building corals rely on symbiotic microalgae (genus *Symbiodinium*) to supply a substantial proportion of their energy requirements. Coral bleaching is defined as either a decrease in *Symbiodinium* density and/or a reduction in photosynthetic pigments, which alters the light scattering and absorption characteristics. Most of the maritime trading routes go via tropical waters and the contamination of tropical marine environment (water column and sediment) by leachate from antifouling paints has led concerns regarding the effects on corals and their symbionts. Furthermore, concerns over the presence of herbicides in the aquatic environment at relatively low concentration and the coral-bleaching have grown recently. Due to the difficulty in culturing *Symbiodinium sp.* in laboratories, these ecologically important symbionts have not yet been used extensively in toxicological experiments. The aim of the study consisted of evaluating the potential toxicity of five biocides used as AF. Two commercial biocides (SEA-NINE and Irgarol), one synthetic biocides (thiram) and two biocides of natural origin (myristic acid and Totarol) will be tested at environmental concentrations, toward the survival of two species of *Symbiodinum sp*.: *Symbiodinium microadriaticum* CCMP 2467 and *Symbiodinium voratum* AC 561. In addition, we exposed the two species of *Symbiodinium sp*. to a thermal stress to investigate the influence of the biocides on thermal susceptibility. Decrease in growth was observed after exposing the two strains to the two commercial biocides (SEA-NINE and Irgarol). The thermal stress was an aggravating factor in delaying the growth. myristic acid, thiram and Totarol have shown no toxic effect on *S.microadriaticum* and *S.voratum*.

This work was presented as a poster during the 17th International Conference of Marine Corrosion and Fouling at Singapore in 2014.

The author designed the experiments and performed the toxicity assays.

5.2 Introduction

Models predict that reef ecosystems will decrease in coral cover, diversity, and/or undergo phase shifts away from coral-dominated reefs in the coming decades (Hughes *et al.* 2003, Donner *et al*. 2005, 2007; Hoegh-Guldberg *et al.* 2007). Already, corals have declined an estimated 80% in the Caribbean since the 1970's (Gardner *et al*. 2003), and 1-2% annually in the Indo-Pacific over the past 30 years (Bruno and Selig, 2007; De'ath *et al*. 2012). Coral decline has been due to many local and regional factors, including disease outbreaks (Lessios *et al*. 1984, Brandt *et al*. 2009), overfishing (Jackson *et al*. 2001, Pandolfi *et al*. 2003), hurricanes (Manzello et al., 2007), pollution (Wooldridge, 2009) and climate change (Hoegh-Guldberg *et al*. 1999, Hughes *et al*. 2003). The distribution of *Symbiodinium* variants has been correlated with different environmental conditions, usually temperature or light (Warner *et al*., 1996; Rowan *et al.,* 1998; Glynn *et al*., 2001; Baker, 2003; Rowan, 2004; Goulet, 2006; Berkelmans and van Oppen, 2006; Abrego *et al*., 2008; Sampayo *et al*. 2008; Jones *et al*., 2008; Thornhill *et al*., 2008). Diversity among *Symbiodinium* variants likely contributes to the ability of a coral host to thrive in a variety of environmental conditions, such as gradients in depth, latitude, irradiance and temperature (Rowan and Knowlton, 1995; Rowan *et al*. 1997; Baker, 2001; Rodriguez Lanetty *et al.,* 2001; MacDonald *et al*., 2008; Thornhill *et al*., 2008; Sampayo *et al*., 2008). Temperature has been shown to be the key determining factor in limiting species abundances and ranges (Hofmann and Todgham, 2010).

Healthy coral reefs are among the most biologically diverse and economically valuable ecosystems on earth. Coral reefs are biogenic structures that often contribute significantly to the seaward section of tropical shorelines, buffering the coast from wave action and erosion (Peters *et al*., 1997). Coral ecosystems are hotspots of marine biodiversity, they are a source of food, protect coastlines from storms and erosion, provide habitat and spawning and nursery grounds for economically important fish species. Coral reefs provide jobs and income to local economies from fishing, recreation and tourism and are a source of new medicines (Cesar *et al,* 2003). The coral reefs are crucial to tropical fisheries and tourism and provide many island populations with primary building materials (Van Dam *et al*., 2011). Estimated potential net benefits of global coral reefs are about 29.8 billion of dollars (Cesar *et al*., 2003). The importance of the coral bleaching should be more supported regarding the importance and economic value of the coral ecosystem.

The importance of the genus *Symbiodinium* is considerable because their relationship with corals is of fundamental importance for the survival of the reef (Hoegh-Guldberg *et al*., 1999). As coral symbiosis based upon algal primary production is the engine driving coral reef ecosystems, stressors that interfere with photosynthetic processes could undermined the basis of this biologically and economically important marine habitat with serious consequences (Lesser and Farrell, 2004). Coral bleaching is defined as either a decrease in *Symbiodinium* density and/or a reduction in photosynthetic pigments (Coles and Jokiel, 1978; Warner *et a*l., 1996; Hoegh-Guldberg, 1999; Roth *et al*., 2012; Roth, 2014), which alters the light scattering and absorption characteristics. Reef corals are able to build productive ecosystems largely due to their mutualistic symbiosis with dinoflagellate algae of the genus *Symbiodinium*. *Symbiodinium* species are genetically diverse, with nine sub-generic clades (named A-I) currently recognized

(Pochon and Gates, 2010). Reef-building scleractinian corals most commonly associate with *Symbiodinium* is clades A-D (Baker, 2003) although members of clades F and G have also been reported (Rodriguez-Lanetty *et al*., 2004; LaJeunesse *et al*., 2010).

Most of the maritime trading routes go via tropical waters (Hellio *et al*., 2010). Moreover, the contamination of tropical marine environment (water column and sediment) by leachate from antifouling paints (Carberry *et al*., 2006; Konstantinou *et al*., 2004; Sapozhnikova *et al.,* 2007) has led concerns regarding the effects on corals and their symbionts. Therefore, it is of high importance to evaluate the impact of antifouling compound on key coral reef organisms and develop new eco-friendly antifouling solution (Marechal *et al*., 2009; Trepos *et al*., 2014). Within this paper, we assessed the impact of five commercial biocides in use or previously used in antifouling formulation (SEA-NINE, Irgarol, myristic acid, Totarol, and thiram) on two species of *Symbiodinium sp*.. The severity and frequency of sea surface temperature anomalies has been increasing over recent decades due to anthropogenic climate change (Hughes *et al.* 2003, Hoegh-Guldberg *et al.* 2007) and due to global warming mass bleaching events (Plass-Jonhson *et al*., 2015). Nevertheless, it remains unknown whether corals will be able to recover between annual bleaching events (Shoepf *et al*., 2015) and what will be the combined effect of thermal stress and biocides on strains of *Symbiodinium sp*. We investigated the effect of the combined stress of global warming (via the thermal stress) and biocides exposure.

5 3 Materials and Methods

5.3.1 Preparation of biocide solutions

Irgarol, myristic acid, thiram and Totarol were purchased from Sigma-Aldrich, UK. SEA-NINE was purchased from Dow, UK. Biocides' stock solutions of 1 mg/L were prepared for each product. Totarol was dissolved in methanol, while all the four other biocides were dissolved in DMSO. Prior to the assays dilutions were performed to obtain solutions with final concentrations of 0.1, 1, 10 and 100 µg/ml. In order to ensure mortality was not due to the residual carrier solvents, methanol and DMSO were tested at the same concentration range.

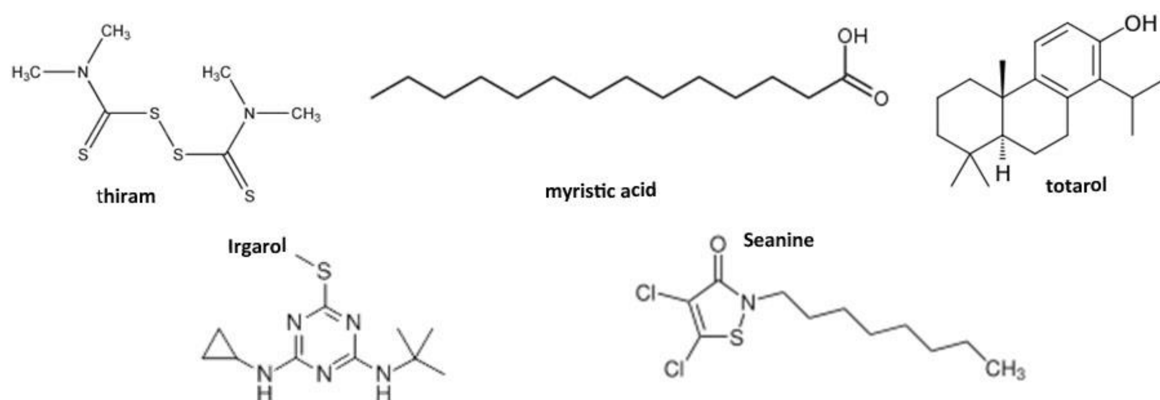

Figure 5.1: chemical structures of thiram, myristic acid, Totarol, Irgarol and SEA-NINE.

5.3.2 Species of *Symbiodinium sp*.

Two species of *Symbiodinium sp* were selected for their diversity of clade and origin.

S. microadriaticum (CCMP 2467) was obtained from the National Center for Marine algae and Microbiota (USA). *S. microadriaticum* belongs to the clade A. This specie is present in the Gulf of Aquaba (Red Sea) and has been isolated from the scleractinian coral *Stylophora pistillata*.

S. voratum (AC 561) was obtained from Algobank (France). The specie belongs to the clade E and is found in the English Channel.

The species were maintained on liquid media F/2 (Guillard *et al.* 1962) under a light cycle of 12h light (100 µmol/m/s) /12h darkness, at a temperature of 24°C for *S. microadriaticum* and 20°C for *S. voratum*.

V.3.3 Cell density monitoring

Prior to the experiments the microalgal concentration was assessed via analysis of the chlorophyll α content (Chambers *et al*., 2011), and dilutions of the stock culture were made accordingly to generate stock solutions of each microalgae containing 0.1 mg/L chlorophyll α. One hundred microliters of the stock solutions were transferred to the wells of 96-well plates prepared with ranging concentrations of biocides (0.01-10 µg/ml) as described above.

The plates were incubated at 24°C for *S. microadriaticum* and 20°C for *S. voratum*. Media only was used as control. Each treatment and the control were replicated six times. The number of cells/ml was determined using a haemocytometer after 72 hours (D3), 1 week (D7), 2 weeks (D14) and 3 weeks (D21). The percentage of inhibition was determined by comparing the total number of cells per ml of each biocide solution to the control.

V.3.4 Toxicity on photosystems

One hundred microliters of the 0.1 mg/L chlorophyll α stock solutions (see 5.3.1) were transferred to the wells of black 96-well plates prepared with ranging concentrations of biocides as described above.

The plates were incubated respectively at 24°C for *S. microadriaticum* and 20°C for *S.voratum*. Media only was used as control. Each treatment and the control were replicated six times. The inoculated plates were grown for 72 hours (D3), 1 week (D7), 2

weeks (D14) and 3 weeks (D21) under constant light exposure (140 µmol m^{-2} s^{-1}) at 20 °C and 24°C.

After the incubation, the microplates were centrifuged at 4,100 rpm for 10 min at 4 °C using a Beckman Coulter Allegra 25R centrifuge. The supernatant was carefully discarded and one hundred microliters of 100 % methanol was added to each well to liberate chlorophyll α. The pigment concentration was quantified employing the fluorimetric method (Chambers *et al.,* 2011). The percentage of inhibition was determined by comparing the pigment concentration of each biocide solution to the control.

5.3.5 Thermal Stress

The species were maintained on liquid media F/2 (Guillard *et al.* 1962) under a light cycle of 12 hours light (100 µmol/m/s) /12h darkness, at a temperature of 24°C for *S. microadriaticum* and 20°C for *S. voratum*. Prior to the experiment, the species were acclimated at a temperature of 26°C for *S. microadriaticum* and 22°C for *S. voratum*. The temperature was gradually increased by 0.4°C per week until the target temperature in the heat stress treatments was achieved. After acclimation, but prior to the start of the experiment, the microalgal concentration was assessed via analysis of the chlorophyll α content (Chambers *et al*., 2011).

The experiments of cell density monitoring (see section 5.3.3) and toxicity on photosystems (see section 5.3.4) were repeated with the thermal stress of 2°C.

5.4 Results

5.4.1 Evaluation of the impact of SEA-NINE and Irgarol on two species of *Symbiodinium sp*.

5.4.1.1 Cell density

The results are presented in Figures 5.2 and 5.3. The results are expressed as percentage of decrease of cell density. A decrease of the cell density was observed at all the concentration tested for SEA-NINE-and Irgarol for *S. microadriaticum*. The decrease in cell density of *S. microadriaticum* was up to 81% at the highest concentration tested (IC=10µg/ml) for SEA-NINE and up to 79% for Irgarol (IC=10µg/ml). A decrease of the cell density of *S. voratum* was observed at all the concentration tested for SEA-NINE with a decrease up to 92% at the highest concentration tested (IC=10µg/ml) and at concentration >0.1µg/ml for Irgarol. After three weeks the decrease in cell density remained.

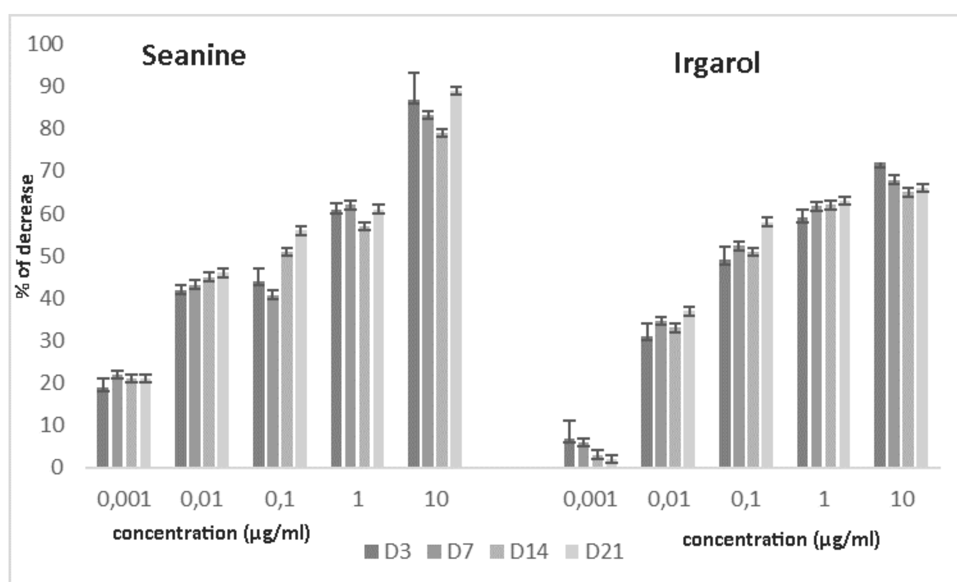

Figure 5.2 Impact of five concentrations (0.001-10µg/ml) of SEA-NINE- and Irgarol, monitored during 21 days (D3, D7, D14 and D21), on the cell density of *S. voratum*. The results are expressed as percentage of decrease of cell density and given as means ±SE (*n*=6).

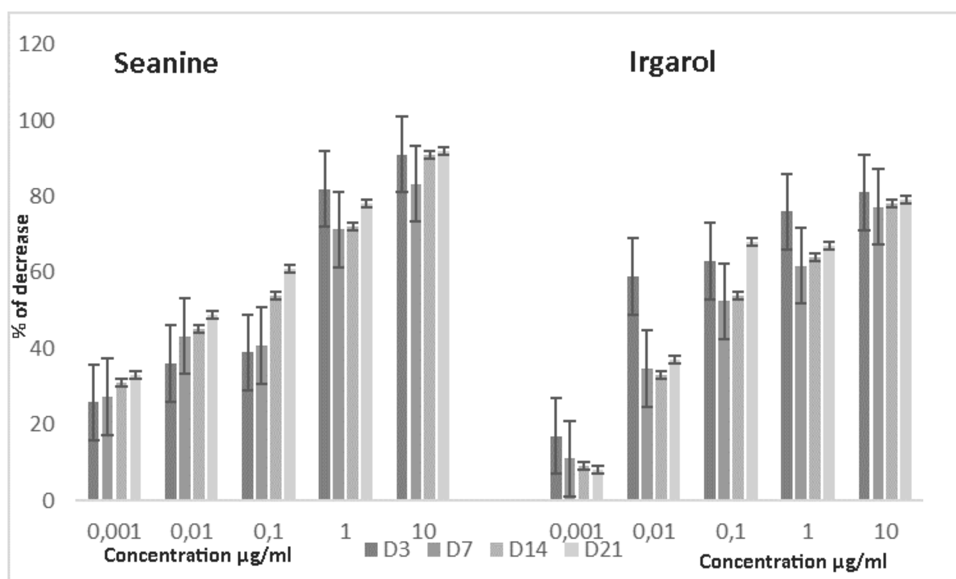

Figure 5.3 Impact of five concentrations (0.001-10µg/ml) of SEA-NINE-and Irgarol, monitored during 21 days (D3, D7, D14 and D21), on the cell density of *S. microadriaticum*. The results are expressed as percentage of decrease of cell density and given as means ±SE (*n*=6)

5.4.1.2 Toxicity of photosystems

The results are presented in Table 5.1 and expressed as the minimum concentration where a significant decrease of chlorophyll was seen. A decrease of the chlorophyll α concentration was observed for SEA-NINE and Irgarol at all the concentration tested and at all the time for both species.

	Biocides	Minimum inhibition concentration (µg/ml)			
organisms	Time	D3	D7	D14	D21
S.voratum	Seanine	IC<0.01	<0.01	IC<0.01	<0.01
	Irgarol	IC>0.01	<0.01	<0.01	<0.01
S.microadriaticum	Seanine	IC<0.01	<0.01	IC<0.01	<0.01
	Irgarol	IC>0.01	<0.01	<0.01	<0.01

Table 5.1 : Minimum inhibition concentration (µg/ml) of SEA-NINE and Irgarol on the toxicity of photosystems of *S. voratum* and *S. microadriaticum* over a period of 21 days (D3, D7, D14, D21).

5.4.2 Evaluation of the impact of thiram, Totarol and myristic acid on two species of *Symbiodinium sp.*

5.4.2.1 Cell density

The results are shown in Figures 5.4 and 5.5. The results are expressed as percentage of decrease in cell density. Myristic acid inhibit the growth of *S. voratum* at all the concentration tested at D3 but after D7 no significative difference is seen. Thiram has shown an inhibitive activity on *S. voratum* at concentration>1µg/ml, the maximum decrease in cell density was 21 %. After D3 no decrease in cell density was shown for myristic acid and thiram. None of the concentration tested of Totarol inhibit significatively the growth of *S. voratum nor S. microadriaticum*. Myristic acid inhibit the growth of *S. microadriaticum* at concentration>10 µg/ml at D3. Thiram has shown an inhibitive activity on *S. microadriaticum* at concentration>1µg/ml, the maximum inhibition of growth was > 44 %. None of the concentration tested of Totarol affect *S. microadriaticum* after D7. After D3 no activity was observed for Totarol.

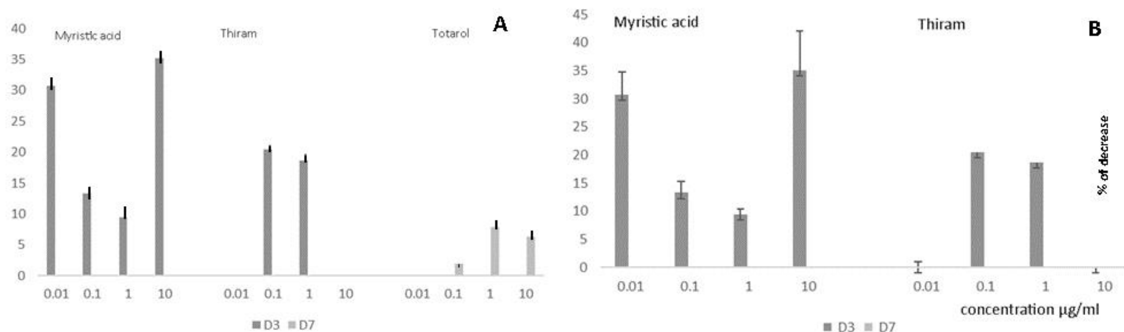

Figure 5.4. Inhibition of the growth of *S. voratum* (A) and *S. microadriaticum* (B) exposed to five concentrations (range from 0.001 to 10µg/ml) of myristic acid, thiram and Totarol during 21 days (D3, D7, D14 and D21). The results are expressed as percentage of decrease of the cell density and given as means ±SE (*n*=6).

5.4.2.2 Toxicity of photosystems

Myristic acid, thiram and Totarol did not affect the chlorophyll α concentration of the culture of *S. voratum* and *S. microadriaticum* at any of the concentration tested. Myristic acid, thiram and Totarol have no long-term toxic effect on the photosystems of *S. voratum* and *S. microadriaticum*.

5.4.3 Evaluation of the impact of biocides and thermal stress on two species of *Symbiodinium sp.*

5.4.3.1 Cell Density

The results are presented below in Figure 5.6 and Figure 5.7. The results are expressed as percentage of decrease of cell density. A decrease of the cell density was observed at all the concentration tested for SEA-NINE and Irgarol for *S. microadriaticum* and *S. voratum* . The decrease in cell density of *S. microadriaticum* was up to 100% at the IC> 0,1 µg/ml for SEA-NINE and up to 100% for Irgarol at IC>1µg/ml.

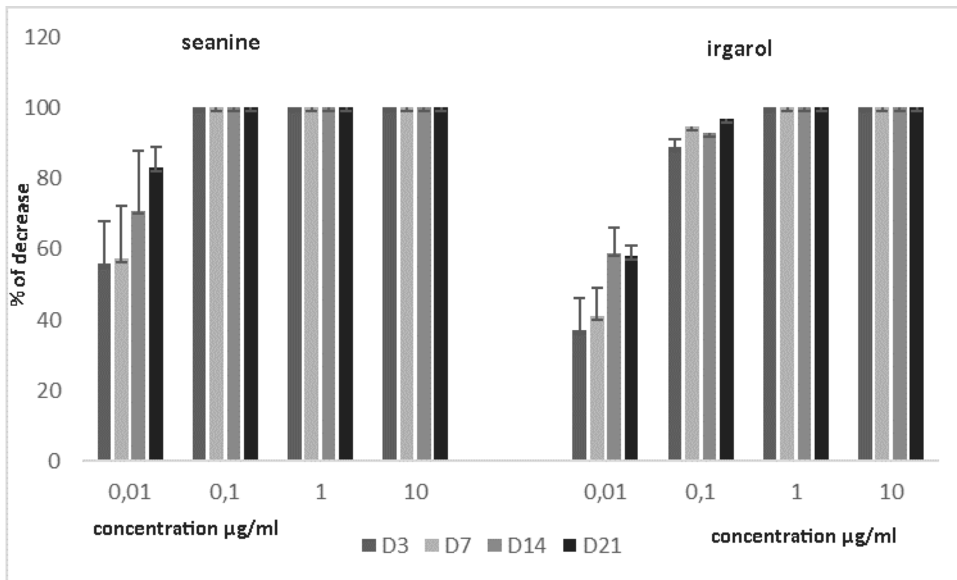

Figure 5.5 Impact of five concentrations (0.001-10µg/ml) of SEA-NINE and Irgarol and thermal stress, monitored during 21 days (D3, D7, D14 and D21), on the cell density of *S. microadriaticum* . The results are expressed as percentage of decrease of cell density and given as means ±SE (*n*=6).

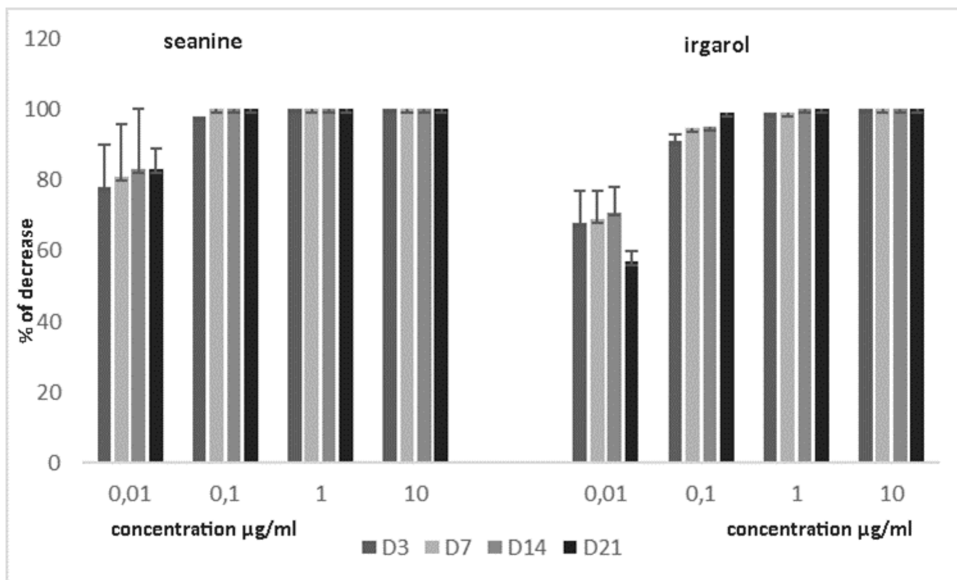

Figure 5.6: Impact of five concentrations (0.001-10µg/ml) of SEA-NINE and Irgarol and thermal stress, monitored during 21 days (D3, D7, D14 and D21), on the cell density of *S. voratum*. The results are expressed as percentage of decrease of cell density and given as means ±SE (*n*=6).

5.4.3.2 Toxicity on photosystems

The results are shown in the Table 5.2 below.

For SEA-NINE-and Irgarol a decrease of chlorophyll α concentration is observed at all the concentration tested and remained until the end of the experimentation (21 days). There is a significant increase of the inhibition of the growth and a significant decrease of the chlorophyll α concentration when the temperature increase. The IC_{50} is very low (IC_{50}>0,01 µg/ml) for both species and at all time.

Biocides	Time	Organisms	
		S.voratum IC50 (µg/ml)	*S.microadriaticum* IC50 (µg/ml)
Sea nine	D3	0,0092	0,025
	D7	0,0094	<0 ,01
	D14	0,0083	0,0083
	D21	0,0069	0,0069
Irgarol	D3	0,0062	0,039
	D7	0,0069	0,062
	D14	0,0064	0,0079
	D21	0,00095	0,0087

Table 5.2: Decrease of the chlorophyll concentrations in *S.voratum* and *S. microadriaticum* after exposure to SEA-NINE and Irgarol during a period of 21 days (D3, D7, D14,D21). Results are expressed as IC_{50} (µg/ml)

5.5 Discussion

5.5.1 Evaluation of the impact of the five biocides on two species of *Symbiodinium sp*.

For SEA-NINE and Irgarol a decrease of chlorophyll α concentration and cell density was observed at all the concentration tested for *S. microadriaticum* and *S. voratum*. Irgarol is highly toxic for algae (Hellio and Yebra, 2009). Irgarol inhibits the photosystems II by binding to the D1 protein thus affecting the photosynthesis (Hall *et al*., 1999; Arrhenius *et al*., 2006; Hellio and Yebra ,2009). D1 are rapid-turnover proteins that bind the components of the reaction centers and electron transport chain together, and are part of the water-splitting complex in photosystems II (Miyao *et al.* 1995). They are a vulnerable component of photosystems II. SEA-NINE quickly penetrate the cell membrane and react with intracellular thiols thus inhibiting specific enzymes (Collier *et al*., 1990; Hellio and Yebra; 2009). SEA-NINE also seems to be able to affect more than one thiol group by generating a cascade of intracellular radicals (Arrhenius *et al*., 2006; Hellio and Yebra, 2009). Irgarol and SEA-NINE primary target in corals is likely to be *Symbiodinium spp*. (Jones, 2003; Negri *et al*., 2005). In the present study, these two commercial biocides presumably inhibited photosynthesis of *S. microadriaticum* and *S. voratum* at very low concentration. Furthermore, *Symbiodinium* spp. in culture are likely to optimize their physiology to culture conditions (Slavov *et al*., 2016), the impact of these biocides on the field could be higher. The results of this study suggest the possibility that in some coastal areas SEA-NINE and Irgarol could affect coral health.

5.5.2 Evaluation of the impact of biocides and thermal stress on two species of *Symbiodinium sp*.

The thermal stress increases the toxicity of SEA-NINE- and Irgarol (Figure 5.7).

According to Hoegh *et al.* (1999) and Lough *et al*. (2007) based on how corals respond to increased temperatures today, an increase of 2°C in the average sea temperature in tropical and subtropical Australia will lead to annual bleaching. This study show that a thermal stress associated with biocide at low concentration will lead to an increase of mortality of *S. microadraticum* and *S. voratum*. The ability to associate with diverse symbionts has been suggested as one mechanism by which corals might be able to respond rapidly to environmental change (Buddemeier and Fautin 1993; Baker, 2001; Baker, 2003; Fautin and Buddemeier, 2004; Berkelmans and van Oppen, 2006; Jones *et al.* 2008, Baskett *et al.* 2009, Correa and Baker, 2011). However, symbiont-mediated acclimatisation is not possible for all coral species (Hoegh-Guldberg *et al.* 2002, LaJeunesse *et al*. 2003, Goulet *et al*., 2006; Baird *et al.* 2007, Correa *et al*. 2009). It remains unknown whether corals will be able to recover between bleaching events.

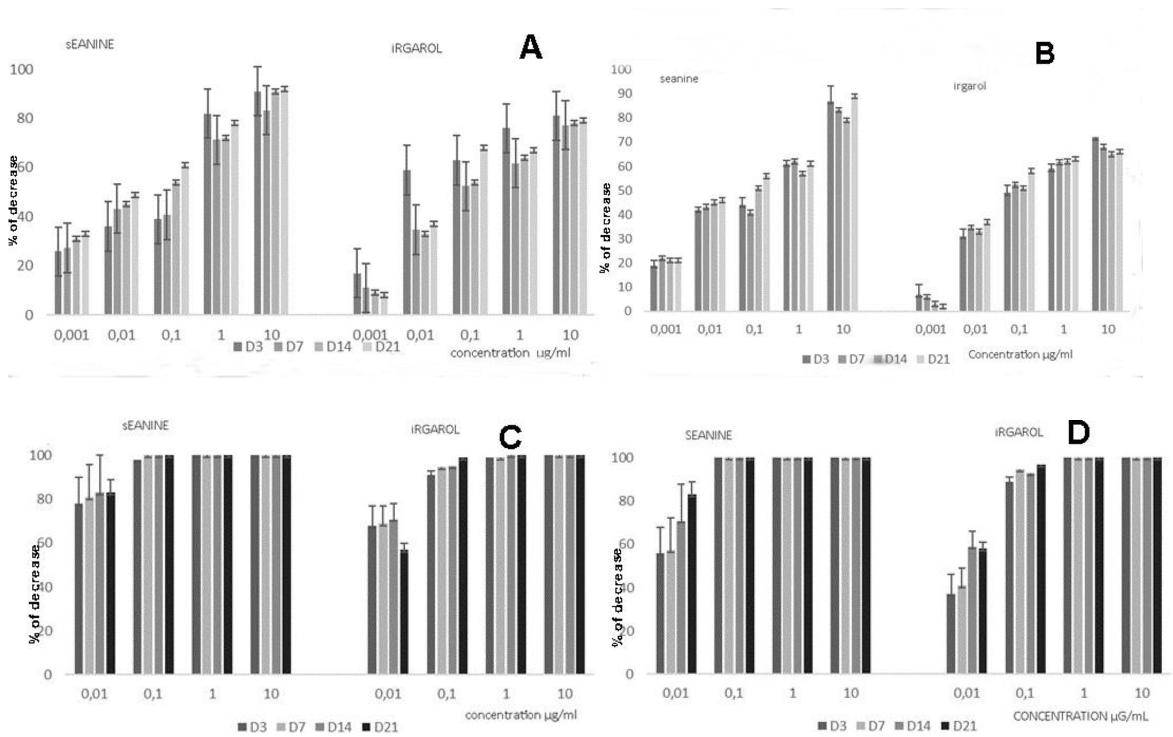

Figure 5.7: Impact of SEA-NINE (**A**), Irgarol (**B**) combined with thermal stress (**C** and **D**) on the cell density of *S. voratum* and *S. microadriaticum* over a period of 21 days (D3, D7, D14, D21). The results are expressed as percentage of inhibition and given as means ±SE (*n*=6).

5.6 Conclusion

A decrease of the chlorophyll α concentration and the cell density was observed for SEA-NINE and Irgarol at all the concentration tested and at all the time for all the strains of *Symbiodinium.* Thermal stress increases the toxicity of SEA-NINE and Irgarol.

Myristic acid, thiram and Totarol have no long term toxic effect on the species of *Symbiodinium* sp. tested. These biocides are good candidates for a new eco-friendly anti-fouling compounds.

Our experiments have highlighted the importance of evaluating the impact of biocides used as antifouling compound on key coral reef organisms for developing new eco-friendly anti-fouling solution.

Chapter 6

Chapter 6: Conclusion

The overall aims of this thesis were to find novel naturally occurring compounds with AF properties and investigate a new bioassay for evaluating the toxic impact of AF compounds on *Symbiodinium* spp.

This research covered the following topics:

- Bromotyrosine derivative ianthelline isolated from the Arctic marine sponge *Stryphnus fortis*. This compound was shown to be a powerful micro- and macro-AF compound, mainly against marine bacteria and the settlement of barnacles (chapter 2). In addition, this compound was found to be active against all human pathogenic bacterial strains included in the current study, both Gram positive and Gram negative.
- Antifouling compounds from the Sub-Arctic Ascidian *Synoicum pulmonaria* and synthetic analogs. Four members of the synoxazolidinones display a broad and high activity towards the growth and adhesion of 16 key marine species involved in marine fouling. Compound 2 is the most active compound in the study and represents a compound with an anti-bacterial activity comparable to the commercial product SEA-NINE-against marine bacteria (chapter 3).
- Peptides derived from the innate immune system as new AF compounds. These peptides initially originating from the innate immune system represent a new class of bioactive compounds that have not previously been evaluated in a marine setting (chapter 4). Three peptides were found to be active against the adhesion and growth of marine bacteria and microalgae.
- Development of a laboratory-based bioassay against the ecologically important symbionts of coral *Symbiodinium s*pp. for assessing the potential toxicity of biocides used as AF agents (chapter 5). Irgarol and SEA-NINE-were shown to affect the symbiont growth

and photosynthesis.

- * Investigate the influence of biocides and thermal stress on two species of *Symbiodium* coral symbionts. (Chapter 5). Decrease in growth was observed after exposing the two strains to both the commercial biocides SEA-NINE-and Irgarol. The thermal stress was clearly an aggravating factor.

6.1 General discussion

6.1.1 Novel compounds with AF properties

S. fortis is rich in ianthelline, and approximately 0.05 % of the organism wet weight is ianthelline, indicating that it is an important secondary metabolite. Ianthelline has a pronounced inhibitory effect on marine bacterial growth. All the marine bacteria this compound was tested against are Gram-negative, and most display MIC values below 10 µg/mL, some as low as 0.1 µg/mL, illustrating a strong and broad antibacterial activity. In the bacterial adhesion studies, the MIC values were higher. The antibacterial activity of ianthelline is similar to that of the bromotyrosine derivative zamamistatin isolated from the Okinawan sponge *Pseudoceratina purpurea* by Takada *et al*. (2001). In comparison to barettin, which is both structurally related and a thoroughly studied marine AF compound, ianthelline displays a higher antibacterial activity. Microalgae forms slimy layers on marine surfaces that are generally challenging to prevent (Molino and Wetherbee, 2008), however it was shown that ianthelline was active against half of the tested microalgal strains. *Hymenomonas coronata*, *Rhodosorus marinus* and *Pleurochrysis carterae* were all sensitive to ianthelline in solution with MIC values ranging from 0.1 to 1 µg/mL while *Pleurochrysis roscoffensis* displayed a MIC value

of 10 µg/mL. No effect on algal adhesion was seen for ianthelline. Assays on *B. improvisus* larvae were also performed to assess the potential effects ianthelline may specifically have on barnacles. As evident from the settlement and metamorphosis inhibition data, Ianthelline represents a potent AF compound against *B. improvisus* cyprid larvae. An IC_{50} of 3.0 µg/mL indicates a high deterring effect and makes ianthelline significantly more active against balanide settlement than most reported bromotyrosine derived sponge metabolites and their synthetic analogues (Tsukamoto *et al.,* 1996. Ortlepp *et al*., 2007). Ianthelline is four times as active as 5,5'-dibrolohemibastadin-1, and no apparent coupling between the moderate phenoloxidase inhibition and *Balanus* settlement is seen for Ianthelline, as has been previously reported for the synthetic hemibastadins (Bayer *et al*., 2011). Ianthelline displays activity similar to ceratinamide B but is one order of magnitude less active than ceratinamide A, a highly potent AF bromotyrosine derivate with low toxicity isolated from the marine sponge *P. purpurea* (Tsukamoto *et al*., 1996). Even at the highest concentration tested (20 µg/mL), ianthelline exhibited low toxicity. Strains resistant to conventional antibiotics and biofilm forming bacteria were included in the test panel of seven bacterial strains. The Gram-positive bacteria displayed sensitivities towards ianthelline ranging from 2.5 to 50 µg/mL while the two Gram-negative strains were both highly sensitive with MIC values of 7.5 µg/mL. *S. aureus* displayed the highest sensitivity towards ianthelline at 2.5 µg/mL, and the methicillin-resistant strain was slightly more tolerant. These activities are similar to those reported for synoxazolidinone A (see section VI.1.1.2) which is a closely structurally related marine compound isolated from the Arctic ascidian *S. pulmonaria* (Tadesse *et al.,* 2010). In this molecule, the central oxime is replaced with an oxazolidinone core. Previous cytotoxicity studies indicate that ianthelline does not act via cellular membrane disruption against mammalian cells (Hanssen *et al*., 2012).

Instead, the kill kinetics suggests intracellular targets as no biological effect was seen until cells were incubated with ianthelline for extended periods and at higher concentrations. It appears that the antibacterial effect is primarily induced at the bacterial membrane interface. The rapid killing, once a threshold concentration on ianthelline has been reached, is typical for a compound acting by a non-specific membrane depolarisation mechanism (Shai, 1999). The lipid composition of the bacterial cell membrane differs significantly from the neutral mammalian cell membrane (Lohner *et al*., 2008), and a different mode of action on the anionic bacterial cell membrane is suspected. Cationic amphiphilic antimicrobial peptidic derivatives, which ianthelline can be regarded as, are generally active at the bacterial membrane interfaces via several mechanisms (Melo *et al*., 2009), and a pronounced antibacterial effect can be seen even for small di- and tri-peptidomimetics (Flaten *et al*., 2011). Ianthelline was shown to be a powerful micro- and macro-AF compound, mainly against marine bacteria and the settlement of barnacles. A lower activity is seen against microalgae, and a moderate bioactivity is reported for blue mussel phenoloxidase inhibition. Furthermore, ianthelline was active against all tested human pathogenic bacterial strains included in the current study, both Gram positive and Gram negative.

Synoxalidinone A was virtually inactive towards the adhesion of bacteria at the tested concentrations (only one strain inhibited at 10 µg/mL) but inhibited the settlement of four of the five microalgal species, including the two diatoms, at 10 µg/mL. Prevention of bacterial growth already at 0.01µg/mL concentrations against 50% of the strains tested indicate that synoxalidinone A also displays potent antibacterial properties against marine bacteria (Tadesse *et al*., 2010). Synoxalidinone A was further active against the adhesion of all of the included microalgal species at 10 µg/mL. The settlement of

barnacle larvae was inhibited at a 7.5 µg/mL concentration, which is higher than barettin (Sjogren *et al.*, 2009) (0.9 µg/mL), bastadin- (1 µg/mL) (Ortlepp *et al.*, 2007) and ianthelline (6.3 µg/mL) (see section VI.1.1.1, Hanssen *et al.*, 2014) but lower than for oroidin (49 µg/mL) (Tsukamoto *et al.*, 1996) and similar to 5,5'dibromohemibastadin-1 (Ortlepp *et al.*, 2007). Synoxalidinone C, which is a bicyclic derivative of synoxalidinone A possessed higher anti-adhesive properties towards both bacteria and microalgae with MIC-values ranging between 0.1 and 10 µg/mL. Synoxalidinone C was further shown to effectively prevent the growth of all included bacteria and microalgal strains at low concentrations. An IC_{50}-value of 1 µg/mL towards the settlement of *B. improvisus* larvae for synoxalidinone C indicates a high activity against barnacles. The two included synoxazolidinones represent novel bromotyrosine scaffolds. Neither the 4-oxazolidinone core of synoxalidinone A, or the unique bicyclic core of synoxalidinone C has been studied before as links between the brominated tyrosine part of the molecule and the arginine-like cationic side bearing the guanidine group. Both synoxalidinone A and synoxalidinone C are powerful antifouling compounds and the overall inhibitory activities are comparable to or significantly lower than for previously studied bromotyrosine derivatives such as the bastadins (Bayer *et al.*, 2011), ianthelline (Hanssen *et al.*, 2014), barettin (Sjogren *et al.*, 2004) and oroidin (Tsukamoto *et al.*, 1996).While the promising anti-barnacle data for synoxalidinone A and synoxalidinone C is comparable to other highly active marine antifouling compounds, the antibacterial and antialgal activities are superior. In comparison with e.g. ianthelline (see section 6.1.1, Kelly *et al.*, 2005; Hanssen *et al.*, 2014) and Barettin (Sjogren *et al.*, 2004; Hanssen *et al.*, 2014), that both have been extensively studied, anti-microfouling activity is seen primarily towards bacterial growth and it is only pronounced against *V. aestuarians*. Ianthelline inhibits the growth of *P. roscoffensis* at 10 µg/mL while barettin inhibits the

growth and adhesion of both *C. closterium* and *P. purpureum* at 0.1 µg/mL (Hanssen *et al*., 2014). With low or submicromolar MIC values against the growth and adhesion of all included microalgal strains, including the diatoms, synoxalidinone C represents a very potent inhibitory compound towards both microfouling organisms and macrofouling barnacles at low concentrations. Synoxalidinone A demonstrates similar overall activities as synoxalidinone C but has a weaker effect on the adhesion of microorganisms. It appears that the additional pyrrolidine ring of synoxalidinone C increases the bioactivity of the compound in relation to synoxalidinone A. The lowest MIC-values of the synoxazolidinones against marine bacteria are, however, three orders of magnitude lower with the growth of several strains being inhibited at 0.01 µg/mL. This significant difference indicates an increased antibiotic effect in the marine environment and suggests that these compounds have been tuned to target marine bacteria and that the high antibacterial effect is not directly transferable to terrestrial bacteria. Whether the differences in activity between the marine and terrestrial bacteria also rely on differences in mode of action or longer generation times for marine bacteria is unclear but it is apparent that the effect is general. Suboptimal growth conditions for the marine bacteria could however also make them particularly susceptible in the assays (Gilbert *et al*., 1990; Svenson, 2013). The cytotoxicity of the compounds has been studied and toxic effects against human fibroblasts at 30 µg/mL have been reported for synoxalidinone C (Tadesse *et al*., 2011). No toxicity towards balanide larvae is seen, suggesting a deterring rather than a toxic mechanism at the tested concentrations. The pulmonarins are structurally less elaborate in comparison to synoxalidinone A and synoxalidinone C and they contain a positively charged quaternary ammonium group instead of the guanidine of the synoxazolidinones, implying a different biosynthetic origin. Pulmonarin A was very active towards the adhesion of 5

strains of bacteria. Interestingly the influence of pulmonarin A on the bacterial growth was lower than for the adhesion and only two bacterial strains were sensitive to pulmonarin A at the concentrations employed. The effect on microalgae was not pronounced for pulmonarin A and only single species were sensitive in each assay. No activity against barnacle settlement was detected. Pulmonarin B was substantially less active and it was inactive towards both the microalgae and the balanid attachment. The pulmonarins display competitive AChE inhibition and no other bioactivities (no antibiotic or cytotoxic activity) were discovered during the initial comprehensive screens of the compounds (Tadesse *et al.*, 2014). Nevertheless, as for the synoxazolidinones, it appears that the marine bacteria are more sensitive than the terrestrial ones towards these compounds and pulmonarin A and pulmonarin C were shown to be active against the adhesion and to some extent the growth of bacteria at low concentrations. The main structural differences between the highly active synoxazolidinones and the less active pulmonarins lie in the constrained 4-oxazolidinone core of synoxazolidinones A and **C** and the ammonium group in pulmonarin A and B. Many potent antifouling bromotyrosine derivatives have a cationic guanidine or guanidine-like group. The lowered basicity and strongly reduced capacity for hydrogen bonding of the tetraalkyl ammonium groups in pulmonarin A and B, combined with an increased molecular flexibility, may be factors influencing the bioactivities of the pulmonarins unfavourably. While both families of compounds are structurally similar they may serve different purposes in the organism or reflect products of essential processes (Haslam, 1994). A small library of 4 synthetic analogs of synoxazolidinone A employing the 2,5-diketopiperazine central core found in Barettin as replacement for the synthetically more challenging 4-oxazolidione core was prepared based on the high activities of synoxazolidinone A and synoxazolidinone C. The linear **5** displays high activity against

the adhesion of four of the ten bacterial strains and three of the microalgae. Compound **5** also affects the growth of eight of the bacterial and microalgal strains. No activity towards *B. improvisus* was detected for **5**. Compound **7**, which was prepared to evaluate the role of the halogen substituents, displayed the broadest observed activity against bacterial adhesion with MIC-values down to 30 nM against several bacterial strains. Bacterial growth was less affected and the inhibitory effect on microalgae was low. Barnacle settlement was unaffected by **7**. Compound **8** was an active inhibitor of both bacterial adhesion and growth, with a greater potency towards growth. It exhibited a weak effect on microalgae and no effect on barnacles. The biological activity of the synthetic mimics data indicate that it is possible to generate highly effective antifouling compounds by employing the marine bromotyrosine pharmacophore as a template (Hanssen *et al.*, 2014). In comparison with synoxazolidinone A and C, the effect on microalgae is lower for the mimics, and it is shown that removal of the bromine atoms in **7** and **8** renders the compounds almost inactive against microalgal growth and adhesion. The use of a saturated 2,5-diketopiperazine scaffold in mimics **6**-**8** seems insufficient to fulfil the essential requirements also for activity towards barnacles it appears that the double bond is a crucial structural feature needed to generate high inhibitory activity towards both barnacles and microfouling species None of the synthetic compounds are able to interfere with the *Balanus* larvae indicating that a certain degree of structural integrity, not present in the analogues, is needed for that particular bioactivity. Most effective small natural inhibitors of barnacle settlement display an element of structural rigidity as a link between the brominated hydrophobic part of the molecule and the cationic moiety. For Ianthelline and synthetic derivatives thereof, the structural constraint is provided by an α-oxo-oxime moiety postulated to be essential for the bioactivity, mainly via phenoloxidase inhibition (Ortlepp *et al.*, 2007;

Bayer *et al*., 2011; Hanssen *et al*., 2014;).For the synoxazolidinones, the 4-oxazolidinone core structure is linked, via an exocyclic double bond to the hydrophobic brominated moiety (Tadesse *et al*., 2010; Tadesse *et al*., 2011). This structural motif has only been reported once before in a natural product, the antibacterial lipoxazolidinones isolated from a marine actinomycete (Macherla *et al*., 2007). The 4 natural compounds and the synthetic mimics are potent antifouling compounds. In comparison with the commercial antifouling SEA-NINE, several of the tested compounds display comparable bioactivities towards the adhesion and growth of bacteria. Synoxazolidinone A, synoxazolidinone C, pulmonarin A and pulmonarin B have all originally been isolated from an extract of *S. pulmonaria* and it is clear that the organism is able to generate a strong chemical defence with broad activity against a range of epibionts. In particular, synoxazolidinone A and C were found in relatively high amounts in the extract and the biological role of the synoxazolidinones is likely a defensive one. Based on the substantially weaker antifouling properties of pulmonarin A and pulmonarin B, and their lower concentrations (in the original extract), it appears unlikely that they are produced for a general defensive purpose. The synoxazolidinones display a broad and high activity towards the adhesion and growth of all tested species. Synoxazolidinone C possessed higher anti-adhesive properties towards both bacteria and microalgae with MIC-values ranging between 0.1 and 10 µg/mL. Synoxazolidinone C was further shown to effectively prevent the growth of all included bacteria and microalgal strains at low concentrations. An IC_{50}-value of 1 µg/mL towards the settlement of *B. improvisus* larvae indicates a high activity against barnacles. Synoxalozidone C represents a compound superior in bioactivity to most reported antifouling bromotyrosine derivatives in the literature. Its antifouling activity is comparable to that of the commercial product SEA-NINE against marine bacteria. The amphipilic peptides, initially originating from natural innate

immune peptides represents a new class of bioactive compounds that have not previously been evaluated in a marine setting before. The peptides may appear structurally and chemically similar but subtle structural features dictate whether the peptides are inactive (MIC ≥150 µg/mL) or highly active (MIC ≤5 µg/mL) (Karstad *et al.*, 2012). A more pronounced hydrophobicity is needed for a more prominent activity and the most active peptides all display similar hydrophobicities. A charge/hydrophobicity balance is needed for activity and a high hydrophobicity alone is not sufficient as illustrated by the dipeptide **1** which apparently lacks a cationic residue for activity. Peptides **3**, **6** and **13** are the most notably active ones displaying MIC-values as low as 0.01 µg/mL against several bacteria and down to 0.1 µg/mL against microalgae. The observed activities are superior to several marine antifoulants such as ianthelline (chapter 2, see section 4.1.1) and similar to the highly active Arctic secondary AF metabolite synoxazolidine C (see section 6.1.1, Chapter 3; Hanssen *et al.*, 2014; Trepos *et al.*, 2014; Qian *et al.*, 2015). The activity of the peptides was generally higher towards bacteria where peptide **6** and **13** clearly are as potent as the commercial positive control against bacterial growth and adhesion. The activity against growth and adhesion was similar overall for the active compounds and no clear distinction is seen even if both **6** and **13** may be considered more active towards adhesion. It was anticipated that most of the included antimicrobial peptides would be active against the marine microflora based on previous observations on isolated antibacterial marine secondary metabolites (Trepos *et al.*, 2014). While selected peptides are indeed very efficient at preventing both growth and adhesion of the studied bacteria it is interesting to see that the effect is not entirely general. Several peptides, active against both human pathogenic bacteria and *B. improvises* settling, such as peptides **5**, **7**, **10** and **12,** did not display any AF bioactivity towards the microfoulers at

concentrations ≤10 µg/mL. It should be noted that all the included marine bacteria are Gram-negative and that these peptides usually display a more pronounced activity towards Gram-positive bacteria where the anionic bacterial membrane is easier to access (Haug *et al.*, 2008). Peptides **10** and **12** are structurally similar to the very active **13** and they both contain the same structural features but in a different arrangement and illustrate that the link between physicochemical properties, amphiphilicity and activity is not as clear for the microfoulers as against the cyprids or human pathogenic bacteria. Removal of the charged guanidine from the Gpp moiety in the inactive **12** transforms it into the highly active **6** which share the localisation of charge and bulk with **3**, also containing App. Peptide **3** is inactive against pathogenic bacteria (MIC >150 µg/mL) but displays AF-activity against all the included marine species. The cationic charge (+2) in **3** and **6** is grouped towards the N-terminus which appears to be beneficial when combined with three hydrophobic residues, and illustrate how the distribution of charge and bulk plays a role for the bioactivity. These and similar short cationic peptides generally possess no defined secondary solution structure even if it has been shown that an amphiphilic solution structure appears to facilitate membrane insertion via reduced solubility (Isaksson *et al.,* 2011). Upon interaction with the bacterial cell membrane, it is believed they all adopt a bioactive amphiphilic conformation which allows for membrane insertion. The distribution of the functional groups in peptides **3** and **6**, is a likely explanation for their high AF bioactivity, given a similar mode of action against the marine bacteria and microalgae. The mode of action is on the cell membrane level. The fact that the peptides range from inactive to highly active is further reflected in their differences in overall hydrophobicity as has previously been shown for this type of peptide. Several peptides displayed high settling deterring effects at ≤5 µg/mL against *B. improvises*. Peptides, **2, 6** and **10-13**, containing one or two

artificial Phe derivatives as mimics of Arg and Lys are also the most active ones suggesting that these two similar synthetic amino acids play a beneficial role for the anti-balanide activity. The difference between Gpp and App lies in the guanidine in Gpp which is charged at physiological conditions in contrast to the neutral App (Svenson *et al.*, 2009). Both sidechains contain an additional phenyl ring which makes these sidechains more hydrophobic compared to Arg or Lys, which they are designed to mimic. The beneficial effect of the uncharged App is significant enough to make the inactive (towards pathogenic bacteria) peptide **2** active in the balanide screen. Of interest is also the activity of peptide **2** which lack the essential C-terminal hydrophobic modification and represents the most polar compound in the study. Peptide **2** induced an increased cyprid swimming in contrast to peptides **6** and **10-13** that appears to inactivate the cyprids suggesting two different modes of action. The activating effect on cyprid swimming by AF compounds has recently been used to develop successful green AF counter measures as exemplified by medetomidine (Selektope) developed by I-tech which induces reversible, increased swimming upon cyprid interaction with the active compound. None of the peptides appeared to display any toxic effects on the cyprids within the tested concentration range (Figure 6.3). Peptides **6** and **13** are powerful, general AF compounds against both micro- and macrofouling species with bioactivities comparable to the commercial product SEA-NINE. Among all the compounds tested, four compounds were selected for being highly active against fouling and non-toxic.

- Ianthelline was shown to be a powerful micro- and macro-AF compound, mainly against marine bacteria and the settlement of barnacles. A lower activity is seen against microalgae, and a moderate bioactivity is reported for blue mussel phenoloxidase inhibition. Furthermore, ianthelline (Figure 6.1) is active against all tested human pathogenic bacterial strains included in the current study, both Gram positive and Gram negative.

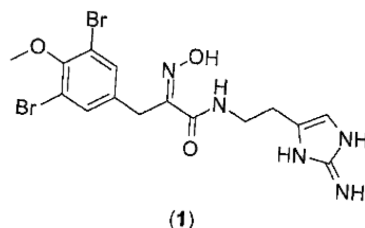

Figure 6.1: Chemical structure of ianthelline

- The synoxazolidinones display a broad and high activity towards the adhesion and growth of all tested species. Synoxazolidinone C (Figure.6.2) possessed higher anti- adhesive properties towards both bacteria and microalgae with MIC-values ranging between 0.1 and 10 µg/mL. Synoxazolidinone C was further shown to effectively prevent the growth of all included bacteria and microalgal strains at low concentrations. An IC_{50}- value of 1 µg/mL towards the settlement of *B. improvisus* larvae indicates a high activity against barnacles. Synoxalozidone C represents a compound superior in bioactivity compared to most reported antifouling bromotyrosine derivatives in the literature. Its antifouling activity is comparable to that of the commercial product SEA-NINE against marine bacteria.

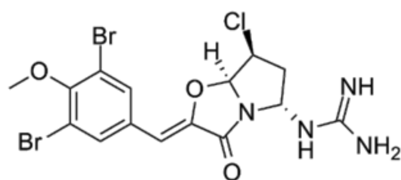

Figure 6.2: Chemical structure of synoxalozidone C

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- Among the thirteen synthetic micropeptides derived from the innate immune system evaluated for their influence on the marine biofouling process, eleven peptides displayed high activities against barnacles at low micromolar concentrations and six peptides were further active against the adhesion and growth of both marine bacteria and microalgae. Peptides **6** and **13** (Figure 6.3) are powerful, general AF compounds against both micro- and macrofouling species with bioactivities comparable to the commercial product SEA-NINE.

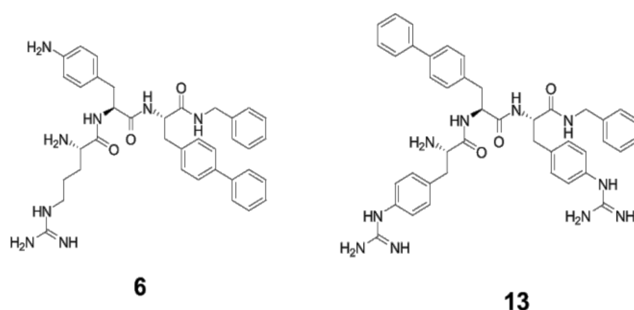


Figure 6.3: Chemical structures of compound **6** and **13** derived from the innate immune system

The four compounds were selected for being both highly active against fouling and non-toxic. Furthermore, Synoxalozidone C and synthetic micropeptides **6** and **13** derived from the innate immune system have bioactivities comparable to the commercial product SEA-NINE.

As a new AF solution should ideally be deterring and non-toxic, these six compounds are very good candidates for an AF application.

6.1.2 Evaluations of the toxicity of biocides on *Symbiodinium spp*.

Concerns over the presence of herbicides in the aquatic environment at relatively low concentration and coral-bleaching have grown recently (Van Dam *et al*., 2015; Slavov *et al*., 2016). The aim of the study was to investigate a laboratory-based bioassay with the ecologically important symbionts *Symbiodinium spp*. for assessing the potential toxicity of biocides used as AF agents. For SEA-NINE and Irgarol a decrease of chlorophyll α concentration and cell density was observed at all the concentration tested for both species. Irgarol is highly toxic for algae (Hellio and Yebra, 2009). Irgarol inhibits the photosystems II by binding to the D1 protein thus affecting the photosynthesis (Hall *et al*., 1999; Arrhenius *et al*., 2006; Hellio and Yebra ,2009). D1 are rapid-turnover proteins that bind the components of the reaction centers and electron transport chain together, and are part of the water-splitting complex in photosystems II (Miyao *et al.* 1995). They are a vulnerable component of photosystems II. SEA-NINE quickly penetrates the cell membrane and reacts with intracellular thiols thus inhibiting specific enzymes (Collier *et al*., 1990; Hellio and Yebra; 2009). SEA-NINE also seems to be able to affect more than one thiol group by generating a cascade of intracellular radicals (Arrhenius *et al*., 2006; Hellio and Yebra, 2009). Irgarol and SEA-NINE 's

primary target in corals is likely to be *Symbiodinium spp*. (Jones, 2003; Negri *et al*., 2005). In the present study, these two commercial biocides presumably inhibited photosynthesis by *S.microadriaticum* and *S.voratum* at very low concentrations. Furthermore, *Symbiodinium* spp. in culture are likely to optimize their physiology to culture conditions (Slavov *et al*., 2016), the impact of these biocides in the field could therefore be higher. The results of this study suggest the possibility that in some coastal areas SEA-NINE-and Irgarol could adversely affect coral health.

Thermal stress increases the toxicity of SEA-NINE and Irgarol. According to Hoegh *et al.* (1999) and Lough *et al*. (2007) based on how corals respond to increased temperatures today, an increase of 2°C in the average sea temperature in tropical and subtropical Australia will lead to annual bleaching. This study show that a thermal stress in association with biocide at low concentration will lead to an increase of mortality of *S.microadraticum* and *S.voratum*. The ability to associate with diverse symbionts has been suggested as one mechanism by which corals might be able to respond rapidly to environmental change (Buddemeier and Fautin 1993; Baker, 2001; Baker, 2003; Fautin and Buddemeier, 2004; Berkelmans and van Oppen, 2006; Jones *et al.* 2008, Baskett *et al.* 2009, Correa and Baker, 2011). However, symbiont-mediated acclimatisation is not possible for all coral species (Hoegh-Guldberg *et al.* 2002, LaJeunesse *et al*. 2003, Goulet *et al*., 2006; Baird *et al.* 2007, Correa *et al*. 2009). It remains unknown whether corals will be able to recover between bleaching events.

6.2 Conclusion and recommendations for future research

6.2.1 New antifouling compounds

Among all the compounds tested, four compounds were selected for being both highly active against fouling and non-toxic. Furthermore, synoxalozidone C and synthetic micropeptides **6** and **13** derived from the human innate immune system have bioactivities comparable to the commercial product SEA-NINE. As a new AF solution should ideally be deterring and non-toxic, these six compounds are very good candidates for an AF application. The activity of the peptides derived from the innate immune system can be correlated to their structure and overall to their hydrophobicity as it has been shown that for this type of peptide there is a close correlation between antimicrobial activityand hydrophobicity (Flaten *et al*., 2011). This structure-activity relationship could be used to design new molecules with AF activity.

Four compounds have displayed high AF activities during laboratory assays and have shown no toxicity against the key marine fouling species tested. The impact of these compounds against *Symbiodinium sp*. should be assessed. Field assays in different conditions will be needed to confirm the AF activity of the molecules. The compounds will need to be incorporated into paints and then applied to static panels suspended from rafts. The physical distribution of the compound within the coating is likely to be crucial to the functioning, longevity and application of the coating (Lejars et al., 2012). A natural product with AF properties that is poorly compatible with its binder or solvent may rise to the top or sink to the bottom of the drying film. If the AF effects are confirmed test patches on ships' hulls will be applied

6.2.2 Evaluations of the toxicity of biocides on *Symbiodinium spp.*

The specific conclusions of the current research are as following:

- Myristic acid, thiram and Totarol have shown no long term effect on *Symbiodinium spp*.
- SEA-NINE and Irgarol affect the growth and survival rate of the two species of *Symbiodinium spp*.

-This is the first indication that the presence of low level of Irgarol or SEA-NINE can impact the coral health.

-The high sensitivity of the free-living *S. voratum to* low levels of SEA-NINE and Irgarol is of high concern as these free-living species are vital for infection of aposymbiotic coral larvae and facilitating restocking of bleached colonies.

The results presented here were obtained with the use of chlorophyll α fluorescence techniques which have been widely applied in algal and coral ecotoxicology (Ralph *et al*.,2005; Van Dam., 2015) and a haemocytometer for monitoring the growth. The method is accurate for monitoring the cell density and toxicity against photosystems II, two factors involved in coral bleaching. The use of Pulse Amplitude Modulated (PAM)-fluorometry could be another option as several studies suggest that PAM fluorometry correlates well with more established toxicity measures such as growth rates and biomass increase in microalgae (Küster and Altenburger, 2007; Magnusson *et al*., 2008), and bleaching in corals (Negri *et al*., 2011; van Dam *et al*., 2012).

The experiments have been conducted with two species of *Symbiodinium sp*. belonging to the clade A and the clade E. It will be of interest to perform the

experiments with strains of symbionts belonging to different clades and especially *Symbiodinium* sp. belonging to the clade D. Indeed, the adaptive strategy of corals associating with clade D to survive projected increases in ocean temperature due to global warming has been seen as “a nugget of hope” (Berkelmans and van Oppen, 2006; Stat and Gates, 2011). This strategy could be impacted at sites with a potential for biocide contamination. Furthermore, corals in their natural environment can simultaneously host a variety of *Symbiodinium* genotypes (Carlos *et al*., 2000; Rowan and Knowlton, 1995) and may regulate the dominating fractions to complement prevailing environmental conditions (Baker *et al*., 2004; Berkelmans and van Oppen, 2006).

The thermal stress experiments have been performed with an increase of 2°C. It will be of interest to conduct the experiments with an extended range of temperature as 2°C is the most optimistic increase of temperature agreed during the recent COP 21 in Paris in December 2015. We suggest that this laboratory-based bioassay could be included as a routine procedure while assessing the AF activity of a new compound.

References

Abarzua, S.; Jakubowski, S.; Eckert, S.; Fuchs, P. (1999). Biotechnological investigation for the prevention of marine biofouling. II. Blue-green algae as potential producers of biogenic agents for the growth inhibition of microfouling organisms. Botanica Marina, 42, 459–465.

Abrego, D.; Ulstrup, K.E.; Willis, B.L.; Van Oppen, M.J.H. (2008). Species-specific interactions between algal endosymbionts and coral hosts define their bleaching response to heat and light stress. Proceed of the Royal Society of Biology, 275, 2273–2282.

Acevedo, M.S; Puentes, C.;Carreno, K.;Leon, J.G.;Stupak, M.;Garcia, M;Perez, M.;Blustein, G. (2013). Antifouling paints based on marine natural products from Colombian Caribbean. International Biodeterioration and Biodegradation, 83, 97-104.

Adams, C. M.; Shumway, S. E.; Whitlatch, R. B.; Getchis, T. (2011). Biofouling in Marine Molluscan Shellfish Aquaculture: A Survey Assessing the Business and Economic Implications of Mitigation. Journal of the World Aquaculture Society, 42 (2), 242-252.

Ainsworth, T. D., Thurber, R. V., and Gates, R. D. (2010). The future of coral reefs: a microbial perspective. Trends in Ecology and Evolution, 25, 233–240

Aldred, N. & Clare, A. S. (2008). The adhesive strategies of cyprids and development of barnacle-resistant marine coatings. Biofouling, 24(5), 351–63.

Aldred, N.; Phang, I.Y; Conlan, S.L, Clare, A.S, Vancso, G.J. (2008) The effects of a serine protease, Alcalase, on the adhesives of adhesives of barnacle cyprids (*Balanus amphitrite*). Biofouling, 24(2),97–107.

Almeida, E.; Diamantino, T. C.; de Sousa, O. (2007). Marine paints: The particular case of antifouling paints. Progress in Organic Coatings, 59 (1), 2-20.

Alzieu, C.; Sanjuan, J.; Michel, P.; Borel, M.; Dreno, J.P. (1989). Monitoring and assessment of butyltins in Atlantic coastal waters. Marine Pollution Bulletin, 20, 22–26.

Andrews, J.M. (2001) Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy,48, suppl. S1, 5-16.

Anon (1952). Marine fouling and its prevention, US Naval Institute, Annapolis, US.

Apolinario, M.; Couthino, R.; Hellio, C.; Yebra, D. (2009). Understanding the biofouling of offshore and deep-sea structures. Woodshead Publishing: Cambridge, UK: 2009.

Arnold, R.; Cole, M.; McGhee, W. (1977). A bactericidal effect for human lactoferrin. Science**,** 197 (4300), 263-265.

Autumn, K.; Liang, Y.A; Hsieh, S.T.; Zesch, W.; Chan, W.P.; Kenny, T.W.; Fearing, R. and Full, R.J.(2000) Adhesive force of a single gecko foot-hair., Nature ,405, 681.

Arrhenius, A., Backhaus, T., Grönvall, F., Junghans, M., Scholze, M., & Blanck, H. (2006). Effects of three antifouling agents on algal communities and algal reproduction: mixture toxicity studies with TBT, Irgarol, and SEA-NINE. Archives of Environmental Contamination and Toxicology, 50(3), 335–45.

Baird, A.H.; Cumbo, V.R.; Leggat, W. and Rodriguez-Lanetty, M. (2007). Fidelity and Flexibility in Coral Symbioses. Marine Ecology Progress, 347, 307–309.

Baldwin, D.H; Sandahl, J.F.; Labenia, J.S.; Scholz N.L. (2003). Sublethal effects of copper on soho salmon: Impacts on non over lapping receptor pathways in the peripheral olfactory nervous system. Environmental Toxicology and Chemistry, 22, 10, 2266-2274.

Baker, A.C.; Rowan, R. (1997). Diversity of symbiotic dinoflagellates (zooxanthellae) in scleractinian corals of the Caribbean and eastern Peastern Pacific. Proceed of International Coral Reef Symposium, 8th, Panama, 2, 1301-1305.

Baker, A.C. (2001). Ecosystems: Reef Corals Bleach to Survive Change. Nature, 411,6839, 765–66.

Baker, A.C. (2003). Flexibility and Specificity in Coral-Algal Symbiosis: Diversity, Ecology, and Biogeography of *Symbiodinium*. Annual Review of Ecology, Evolution, and Systematics, 34 (1), 661–689.

Baker AC, Glynn PW, Riegl B (2008). Climate change and coral reef bleaching: an ecological assessment of long-term impacts, recovery trends and future outlook. Estuarine, Coastal and Shelf Science, 80, 435-471.

Barrios, C.A.; Xu, Q.W.; Cutright, T; Newby, B.M.Z. (2005). Incorporating zosteric acid into silicone coatings to achieve its slow release while reducing fresh water bacterial attachment. Colloids and Surfaces – Biointerfaces, 41, 83-93.

Barry, S.; Hayes, K. R.; Barry, S. C.; Hayes, K. R.; Hewitt, C. L.; Behrens, H. L., Bakke, S. M. (2008). Ballast water risk assessment: Principles, processes, and methods Ballast water risk assessment, International Council for the Exploration of the Sea, Oxford Journals, 120-131.

Baskett, M.L.; Gaines, S.D. and Nisbet, R.M. (2009). Symbiont Diversity May Help Coral Reefs Survive Moderate Climate Change. Ecological Applications, 19 (1), 3–17.

Bayer, M.; Hellio, C.; Marechal, J.P.; Frank, W.; Lin, W.H.; Weber, H.; Proksch, P. (2011). Antifouling Bastadin Congeners Target Mussel Phenoloxidase and Complex Copper(II) Ions. Marine Biotechnology, 13, 1148-1158.

Beech WB, Sunner J. (2004). Biocorrosion: towards understanding interactions between biofilms and metals. Current Opinion in Biotechnology, 15, 181-186.

Beech, I. B.; Sunner, J. A.; Hiraoka, K., (2005). Microbe-surface interactions in biofouling and biocorrosion processes. International Microbiology, 8 (3), 157-168.

Bellamy, W.; Takase, M.; Wakabayashi, H.; Kawase, K.; Tomita, M. (1992). Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. Journal of Applied Bacteriology, *73*, 472-479.

Bengtsson, B. E.; Tarkpea, M. (1995). A 96-h Acute Toxicity Test with the Brackish Water Crustacean *Nitocra spinipes* Boeck: Assessment of Chemicals, Products and Effluents. Environmental Toxicology and Water Quality, 10, 147-150.

Ben-Haim, Y., Banim, E., Kushmaro, a, Loya, Y., & Rosenberg, E. (1999). Inhibition of photosynthesis and bleaching of zooxanthellae by the coral pathogen *Vibrio shiloi*. Environmental Microbiology, 1(3), 223–229.

Bergman, J. (2013). Synthesis and Studies of Two Marine Indole Alkaloids, Barettin and Caulersin. Phytochemistry Reviews,12,3, 487–494.

Berkelmanns R. and van Oppen , M.J.H. (2006). Flexible partners in coral symbiosis: a 'nugget of hope' for coral reefs in an era of climate change. Proceed of the Royal Society of Biological Sciences, 273, 2305-2312.

Bernbom, N.; Ng, Y.Y.; Olsen, S.M.; Gram, L. (2013). *Pseudoalteromonas spp.* serve as initial bacterial attractants in mesocosms of coastal waters but have subsequent antifouling capacity in mesocosms and when embedded in paint. Applied Environmental Microbiology. 79, 6885–6893.

Berntsson, K. M.; Jonsson, P. R.; Lejhall, M.; Gatenholm, P. (2000a). Analysis of behavioural rejection of micro-textured surfaces and implications for recruitment by the barnacle *Balanus improvisus*. Journal of Experimental Marine Biology and Ecology, 251 (1), 59-83.

Berntsson, K.M.; Andreasson, H.; Jonsson, P.R.; Larsson, L.; Ring, K.; Petronis, S.; Gatenholm, P. (2000b). Reduction of barnacle recruitment on micro-textured surfaces: analysis of effective topographic characteristics and evaluation of skin friction. Biofouling, 16, 245-261.

Bers, A.V.; Wahl, M. (2004). The influence of natural surface microtopographies on fouling. Biofouling, 20, 43-51.

Besemer, K. (2015). Biodiversity, community structure and function of biofilms in stream ecosystems. Research in Microbiology, 166(10), 774–81

Bixler, G. D. and Bhushan, B. (2012). Biofouling: lessons from nature. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences, 370(1967), 2381–2417.

Bhadury P, Wright P.C. (2004). Exploitation of marine algae: biogenic compounds for potential antifouling applications. Planta, 219,561-578.

Blank, R.J.; Trench, R.K. (1985). Speciation and symbiotic dinoflagellates. Science, 229,656-658.

Blank, R.J.; Trench, R.K. (1986). Nomenclature of endosymbiotic dinoflagellates. Taxon 35,286-94.

Booij, P.; Sjollema, S.B.; Van der Geest, H.G; Leonards, P.E.G; Lamoree, M.J.; De Voogt, P.W.; Admiraal, W.; Laane, R.W.P.M; Vethaak, A.D. (2014). Toxic Pressure of Herbicides on Microalgae in Dutch Estuarine and Coastal Waters. Journal of Sea Research,5, 1085-1105.

Braithwaite, R. A.; McEvoy, L. A. (2004). Marine Biofouling on Fish Farms and Its Remediation. In Advances in Marine Biology, Academic Press,47, 215-252.

Brandt, M.E.; McManus, J.W. (2009). Disease Incidence Is Related to Bleaching Extent in Reef-Building Corals. Ecology, 90, 10, 2859–2567.

Briand, JF. (2009). Marine antifouling laboratory bioassays: an overview of their diversity. Biofouling 25:297-311

Bressy, C.; Hellio, C.; Marechal, J. P.; Tanguy, B.; Margaillan, A. (2010). Bioassays and field immersion tests: a comparison of the antifouling activity of copper-free poly(methacrylic)-based coatings containing tertiary amines and ammonium salt groups. Biofouling, 26 (7), 769-777.

Brooks, S.J. and Waldock, M. (2008). Copper as an antifouling biocide. Antifouling Biocides, Springer publishing.

Brooks, S. and Waldock, M. (2009). Copper Biocides in the Marine Environment. In Ecotoxicology of Antifouling Biocides, Eds. Springer Japan, 413-428.

Brown B.E, (1997). Coral bleaching: causes and consequences. Coral Reefs. 16, 129-138.

Brown B.E, (2000) The significance of pollution in eliciting the ‘bleaching’ response in symbiotic cnidarians. International Journal of Environmental Pollution 13,392-415.

Brugnoni, L. I., Lozano, J. E. and Cubitto, M. A. (2007) Potential of yeast isolated from apple juice to adhere to stainless steel surfaces in the apple juice processing industry. International Journal of Food Research,40, 332-340.

Bruno, J.F.; Selig, E.R.; Casey, S.K.; Page, C.A.; Willis, B.L.; Harvell, C.D.; Sweatman, H. and Melendy, A.M. (2007). Thermal Stress and Coral Cover as Drivers of Coral Disease Outbreaks, 5,6, 1220-1227.

Buddemeier, R. W. and Fautin, D. G. (1993). Coral bleaching as an adaptive mechanism. BioScience, 43, 320, 6.

Burgess, J. G.; Boyd, K. G.; Armstrong, E.; Jiang, Z.; Yan, L. M.; Berggren, M.; May, U.; Pisacane, T.; Granmo, A.; Adams, D. R. (2003). The development of a marine natural product-based antifouling paint. Biofouling, 19, 197-205.

Butler, A.J.; Van Altena, I.A.; Dumme, J.J. (1996). Antifouling activity of Lyso-platelet-activating factor extracted from Australian sponge *Crella incrustans*. Journal of Chemical Ecology, 22, 11, 2041-2061.

Cao, Z., Brault, N., Xue, H., Keefe, A., & Jiang, S. (2011). Manipulating Sticky and Non-Sticky Properties in a Single Material. Angewandte Chemie International Edition, 50 (27), 6102–6104.

Cahill, P. L.; Heasman, K.; Jeffs, A.; Kuhajek, J. (2013) Laboratory assessment of the antifouling potential of a soluble-matrix paint laced with the natural compound polygodial. Biofouling**,** 29 (8), 967-975.

Cahill, P. L.; Kuhajek, J. M. (2014). Polygodial a contact active antifouling biocide. Biofouling, 30 (9), 1035-1043.

Callow, M., (1990). Ship fouling: problems and solutions. Chemistry and Industry (London), (5), 123-127.

Callow, M.E.; Callow, J.A.; Pickett-Heaps, J.D.; Wetherbee, R. (1997). Primary adhesion of *Enteromorpha* (Chlorophyta, Ulvales) propagules: quantitative settlement studies and video microscopy. Journal of Phycology. 33,938–947.

Callow, M. E.; Callow, J. A.; Pickett-Heaps, J. D.; Wetherbee, R. (2007). Primary adhesion of *Enteromorpha* (Chlorophyta, Ulvales) propagules: quantitative settlement studies and video microscopy. Journal of Phycology, 33 (6), 938-947.

Callow, J. A.; Callow, M. E. (2011). Trends in the development of environmentally friendly fouling-resistant marine coatings. Nature Communication *,*2011 *; 2*, 244.

Caquet, T., Roucaute, M., Mazzella, N., Delmas, F., Madigou, C., Farcy, E., Gabellec, R. (2013). Risk assessment of herbicides and booster biocides along estuarine continuums in the Bay of Vilaine area (Brittany, France). Environmental Science and Pollution Research, 20(2), 651–666.

Carbery, K., Owen, R., Frickers, T., Otero, E., Readman, J., (2006). Contamination of Caribbean coastal waters by the antifouling herbicide Irgarol 1051. Marine Pollution Bulletin,52, 635-643.

Carpentier B, Cerf O. (1993). Biofilm and their consequences with particular reference to hygiene in the food industry. Journal of Applied Bacterioly 75:499-511.

Chambers LD, Stokes KR, Walsh FC, Wood RJK. (2006). Modern approaches to marine antifouling coatings. Surface Coating Technology ,201, 3642-3652.

Chambers, L. D.; Hellio, C.; Stokes, K. R.; Dennington, S. P.; Goodes, L. R.; Wood, R. J. K.; Walsh, F. C. (2011). Investigation of *Chondrus crispus* as a potential source of new antifouling agents. International Biodeterioration & Biodegradation**,** 65 (7), 939-946.

Chambers, L.D.; Wharton, J.A.; Wood, R.J.K.; Walsh, F.C.; Stokes, K.R. (2014). Techniques for the measurement of natural product incorporation into an antifouling coating. Progress in organic coatings, 77, 2, 473-484.

Chapman, J., Hellio, C., Sullivan, T., Brown, R., Russell, S., Kiterringham, E., Regan, F. (2013). Bioinspired synthetic macroalgae: Examples from nature for antifouling applications. International Biodeterioration and Biodegradation, 86, part A, 1–8.

Chaw, K. C., & Birch, W. R. (2009). Quantifying the exploratory behaviour of Amphibalanus amphitrite cyprids. Biofouling, *25*(7), 611–9.

Cheng S, Tian JT, Chen SG, Lei YH, Chang XT, Liu T, Yin YS. (2009). Microbially influenced corrosion of stainless steel by marine bacterium *Vibrio natriegens*: (i) corrosion behavior. Math Science Engineer C-Biol 29,751-755.

Chiovitti, A.; Dugdale, T.M.; Wetherbee, R. (2006). Diatom Adhesives: molecular and mechanical properties. Biological adhesives, Springer Berlin Heidelberg, 79-103.

Cesar, H., Pet-soede, L., & Pet-soede, L. (2003). The Economics of Worldwide Coral Reef Degradation.

Clare, A. S.; Marechal, J.-P.; Da Gama, B. A. P.; Pereira, R. C.; Hellio, C. (2009). Natural

marine products with antifouling activities. In Advances in Marine Antifouling Coatings and Technologies , 572–622.

Coles, S.L.; Jokiel, P.L. (1978) Synergistic effects of temperature, salinity and light on the hermatypic coral *Montipora verrucosa*. Marine Biology, 49,187–195

Coles, S.L.; Brown, B.E. (2003) Coral bleaching--capacity for acclimatization and adaptation. Advances Marine Biology, 46,183-223.

Collier, P.J.; Ramsey, A.; Waigh, R.D.; Douglas, K.T.; Austin, P.; Gilbert, P. (1990) Chemical reactivity of some isothiazolone biocides. Journal of Applied Bacterioly, 69,578– 584

Conneely, O. M. (2001). Anti-inflammatory Activities of Lactoferrin. Journal of the American College of Nutrition, 20 (sup5), 389-395.

Cooper, T. F.; Berkelmans, R.; Ulstrup, K. E.; Weeks, S.; Radford, B.; Jones, A. M.; Van Oppen, M. J. H. (2011). Environmental factors controlling the distribution of symbiodinium harboured by the coral *acropora millepora* on the great barrier reef. PLoS ONE, 6(10).

Correa, A. M. S.; Brandt, M. E.; Smith, T. B.; Thornhill, D. J.;Baker, A. C. (2009). Symbiodinium associations with diseased and healthy scleractinian corals. Coral Reefs, 28(2), 437–448.

Correa, A. M. S.; Baker, C.A. (2011). Disaster Taxa in Microbially Mediated Metazoans: How Endosymbionts and Environmental Catastrophes Influence the Adaptive Capacity of Reef Corals. Global Change Biology, 17 (1), 68–75.

Costerton, J.W.; Cheng, K.-J.; Geesey, G.G.; Ladd, T.I.; Nickel, J.C.; Dasgupta, M.; Marrie, T.J. (1987). Bacterial biofilms in nature and disease. Annual Review of Microbioly. 41, 435–464

Costerton, J.W.; Lewandowski, Z.; Caldwell, D.E. (1995) Microbial biofilms. Annual Review of Microbiology, 49, 711–745.

Culioli, G.; Ortalo-Magne, A.; Hellio, C.; Clare, A.S. and Piovetti, L. (2008). Anti-fouling activity of merodisterpenoids from the marine brown algae *Halydris siliquosa.* Journal of Natural Product, 71, 1121-1126.

Da Gama Perez, B. A; Plouguerné, E.; Crespo Pereira , R. (2014). The antifouling defence mechanisms of marine macroalgae*:* Advances in Botanical Research, 71, 14, 413-440.

De'ath, G.; Fabricius, K.E.; Sweatman, H.; Puotinen, M. (2012). The 27-Year Decline of Coral Cover on the Great Barrier Reef and Its Causes. Proceedings of the National Academy of Sciences, 109, 44, 17995–99.

De Brouwer, J.F.C; Cooksey, K.E.; Wigglesworth-Cooksey, B.; Staal, M.J.; Stal, L.J.; Avci, R. (2006) Time of flight-secondary ion mass spectrometry on isolated extracellular fractions and intact biofilms of three species of benthic diatoms. Journal of Microbiological Methods ,65 ,562–72

De Messano, L. V. R.; Sathler, L.; Reznik L.Y.; Coutinho, R. (2009). The effect of biofouling on localized corrosion of the stainless steels N08904 and UNS S32760. International Biodeterioration & Biodegradation, 63, 5, 607-614.

De Nys, R.; Steinberg, P.D; Willemsen, P.; Dworjanyn, S.A; Gabelish, C.L; King, R.J. (1995). Broad-spectrum effects of secondary metabolites from the red alga *Delisea pulchra* in antifouling assays. Biofouling, 8, 259-271.

De Nys R, Dworjanyn SA, Steinberg PD.(1998) A new method for determining surface concentrations of marine natural products on seaweeds. Marine Ecology Progress Serie, 162, 79–87

De Nys, R. ; Guenther, J. ; Uriz, M. J. (2010). Natural Control of Fouling. In S. Durr & J. C. Thomason (Eds.), Biofouling , 109–120.

Dafforn, K.A.; Lewis,J.A and Johnston E.J. (2011) .Antifouling Strategies: History and Regulation, Ecological Impacts and Mitigation. Marine Pollution Bulletin 62 (3), 453-465.

Dahms, H-U.; Dobretsov, S; Qian, P-Y. (2004). The effect of bacterial and diatom biofilms on the settlement of the bryozoan *Bugula neritina*. Journal of Experimental Marine Biology and Ecology, 313,191– 209

Dahms, H.U.; Hellio, C. (2009). Laboratory bio-assays for screening marine antifouling compounds. In: Hellio C, Yebra DM; Advances in marine antifouling coatings and technologies. Woodhead Publishing, New York.

Dahlstrom, M.; Martensson, L. G. E.; Jonsson, P. R.; Arnebrant, T.; Elwing, H. (2000). Surface active adrenoceptor compounds prevent the settlement of cyprid larvae of *Balanus improvisus*. Biofouling, 16 (2-4), 191-203.

Darrigran, G.; Damborenea, C. (2015). Strategies and Measures to Prevent Spread of Invasive Species Strategies and Measures to Prevent Spread, Invading Nature - Springer Series in Invasion Ecology, 357-371

Davies, I. M.; Bailey, S. K.; Harding, M. J.C. (1998). Tributyltin inputs to the North Sea from shipping activities, and potential risk of biological effects, Journal of Marine Science Vol. 55, 34–43.

Dexter, S. C. (1993). Role of microfouling organisms in marine corrosion. Biofouling, 7, (2), 97-127.

Diyabalanage, T.; Amsler, C.D.; McClintock,J.B.; Baker, B.J. (2006). Palmerolide A, a Cytotoxic Macrolide from the Antarctic Tunicate *Synoicum adareanum*. Journal of the American Chemical Society 128 ,17, 5630–5631.

Dobretsov, S.; Quian, P.Y. (2002). Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. Biofouling; 18:217–28.

Dobretsov, S.; Dahms, H. U.; Qian, P. Y. (2006). Inhibition of biofouling by marine microorganisms and their metabolites. Biofouling**,** 22 (1), 43-54.

Dobretsov, S.; Hairong Xiong, Y.X., Levin,L.A.;Qian, P-Y. (2007). Novel Antifoulants: Inhibition of Larval Attachment by Proteases. Marine Biotechnology 9 (3): 388–97.

Donner, S.D.; Skirving, W.J.; Little, C.M.; Oppenheimer, M.; Hoegh-Guldberg, O. (2005). Global Assessment of Coral Bleaching and Required Rates of Adaptation under Climate Change." Global Change Biology ,11 ,12, 2251–2265.

Donner, S.D. and Potere, D. (2007). The Inequity of the Global Threat to Coral Reefs. BioScience, 57, 3, 214-215

Eashwar, M., Subramanian, G.; Chandrasekaran, P.; Manickam, S.T; Maruthamuthu S. and Balakrishnan K. (1995). The interrelation of cathodic protection and marine macrofouling. Biofouling, 8, 4, 303-312.

Elbourne, P. D., & Clare, A. S. (2010). Ecological relevance of a conspecific, waterborne settlement cue in *Balanus amphitrite* (Cirripedia). Journal of Experimental Marine Biology and Ecology, 392(1-2), 99–106.

Ellis, D.V.; Pattisina, L.A. (1990). Widespread neogastropods imposex: a biological indicator of global contamination? Marine Pollution Bulletin ,21, 248–253.

Ellis, D.V. (1991). New dangerous chemicals in the environment: lessons from TBT. Marine Pollution Bulletin, 22, 8–10

Evans, C.J.; Hill, R. (1983). Organotin-based antifouling systems. Reviews on Silicon, Germanium, Tin and Lead Compounds, 7, 57-125.

Evans, L.V.; Clarkson, N. (1993). Antifouling Strategies in the Marine Environment. Journal of Applied Bacteriology ,74 ,119-124

Fadnes, B.; Uhlin-Hansen, L.; Lindin, I.; Rekdal, Ø. (2011). Small lytic peptides escape the inhibitory effect of heparan sulfate on the surface of cancer cells. BMC cancer, 11 (1), 116.

Faimali, M.; Garaventa, F.; Terlizzi, A.; Chiantore, M.; Cattaneo-Vietti, R. (2004). The interplay of substrate nature and biofilm formation in regulating *Balanus amphitrite* Darwin, 1854 larval settlement. Journal of Experimental Marine Biology and Ecology, 306(1), 37–50.

Faulkner, D.J. (2002). Marine natural products. Natural Product Report, 19, 1–48.

Fautin, D.G.; Buddemeier, R.W. (2004) Adaptive bleaching: a general phenomenon. Hydrobiologia, 530, 459– 467

Fent, K. (2006) Worldwide occurrence of organotins from antifouling paints and effects in the aquatic environment, Handbook of Environmental Chemistry,5(5):71–100.

Fitridge, I. ; Dempster, T. ; Guenther, J. ; de Nys, R. (2012). The Impact and Control of Biofouling in Marine Aquaculture: A Review. Biofouling, 28, 7, 649–69.

Flaten, G. E.; Kottra, G.; Stensen, W.; Isaksen, G.; Karstad, R.; Svendsen, J. S.; Daniel, H.; Svenson, J. (2011). In Vitro Characterization of Human Peptide Transporter hPEPT1 Interactions and Passive Permeation Studies of Short Cationic Antimicrobial Peptides. Journal of Medicinal Chemistry, 54 (7), 2422-2432.

Fleming, H. C. (1996). Economical and technical overview in Microbially Influenced Corrosion of Materials, New York, Springer, 6–14.

Flemming, H.C.; Wingender, J. (2001) Relevance of microbial extracelular polymeric substances (EPSs)- Part I: Structural and ecological aspects. Water Science and Technology 43, 1-8.

Flemming, H. C. (2002). Biofouling in water systems - cases, causes and countermeasures. Applied Microbiology and Biotechnology 59, 6, 629-640.

Flemming, K.; Klingenberg, C.; Cavanagh, J. P.; Sletteng, M.; Stensen, W.; Svendsen, J. S.; Flægstad, T. (2009) High in vitro antimicrobial activity of synthetic antimicrobial peptidomimetics against staphylococcal biofilms. Journal of Antimicrobial Chemotherapy, 63 (1), 136-145.

Fletcher, R.L. and Baier, R.E. (1984). Influence of surface energy on the development of the green algal *Enteromorpha*. Marine Biology Letters, 5, 251–254.

Frey, M.; Therriault, T.W. (2014). Fouling around: vessel sea-chests as a vector for the introduction and spread of aquatic invasive species and spread of aquatic invasive species, Management of Biological Invasions, Volume 5, Issue 1, 21–30.

Fusetani N. (2004). Biofouling and antifouling. Natural Product Reports, 21, 94-104.

Fusetani, N. (2011). Antifouling marine natural products. Natural Product Reports 2011, 28 (2), 400-410.

Fux, C.A; Shirtliff, M.; Stoodley, P. *et al*. (2005) Can laboratory reference strains mirror “real-world” pathogenesis? Trends in Microbiology 13, 58–63.

Gardiola, F. A.; Cuesta, A.; Meseguer, J.; Esteban, M. A. (2012) Risks of Using Antifouling Biocides in Aquaculture. International Journal of Molecular Sciences, 13 (2), 1541-1560.

Gardner, T. A. (2003). Long-Term Region-Wide Declines in Caribbean Corals. Science, 301,5635, 958–960.

Gebeshuber I.C.; Stachelberger H. and Drack M. (2005) Diatom bionanotribology - Biological surfaces in relative motion: their design, friction, adhesion, lubrication and wear, Journal of. Nanoscience and. Nanotechnology. 5(1), 79-87.

Geesey, G.G.; Wigglesworth Cooksey, B.; Cooksey, K. (2000). Influence of calcium and othercations on surface adhesion of bacteria and diatoms: a review. Biofouling 15, 195–205.

Gerwick, W. H.; Moore, B. S (2012). Lessons from the past and charting the future of marine natural products drug discovery and chemical biology. Chemical Biology, 19, 85−98.

Gibbs, P.E., Bryan, G.W., (1986). Reproductive failure in populations of the dogwhelk, *Nucella lapillus*, caused by imposex induced by the tributyltin from antifouling paints. Journal of Marine Biology Association,UK,66, 767–777.

Gibbs, P.E; Bryan, G.W; Pascoe,P.L; Burt, G.R.(1987). The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. Journal of Marine Biology Association,UK, 67,507–524.

Gifford, J.L.; Hunter, H.N.; Vogel, H.J. (2005). Lactoferricin. Cellular and Molecular Life Science,. 62,2588–2598.

Gilbert, P.; Collier,P.J.; Brown,M.R. (1990). Influence of Growth Rate on Susceptibility to Antimicrobial Agents: Biofilms, Cell Cycle, Dormancy, and Stringent Response. Antimicrobial Agents and Chemotherapy, 34 ,10, 1865–68.

Gittens, J. E., Smith, T. J., Suleiman, R., & Akid, R. (2013). Current and emerging environmentally-friendly systems for fouling control in the marine environment. Biotechnology Advances, 31(8), 1738–53

Glynn PW., (1993). Coral reef bleaching-ecological perspectives. Coral Reefs, 1 (2),1-17.

Glynn, P.W, Maté,J.L; Baker,A.C.; Calderón,M.O. (2001). Coral bleaching and mortality in Palama and Ecuador during the 1997–1998 EL Nino–southern oscillation event: spatial

/temporal patterns and comparisons with the 1982–1983 event. Bulletin of Marine Science, 69 (1), 79–109.

Gohad, N. V.; Aldred,N.; Orihuela,B.; Clare,A.S.; Rittschof, D. and Mount,A.S. (2012). Observations on the settlement and cementation of barnacle (*Balanus amphitrite*) cyprid larvae after artificial exposure to noradrenaline and the locations of adrenergic-like receptors. Journal of Experimental Marine Biology and Ecology, 416–417,153-161

Gomez-Gil, B.; Soto-Rodriguez, S.; Garcia-Gasca, A.; Roque, A.; Vazquez-Juarez, R.; Thompson, F.L.; Swings, J. (2004). Molecular identification of *Vibrio harveyi* related isolates associated with diseased aquatic organisms. Microbiology, 150, 1769–1777.

González-Chávez, S. A.; Arévalo-Gallegos, S.; Rascón-Cruz, Q., (2009). Lactoferrin: structure, function and applications. International Journal of Antimicrobial Agents, 33 (4).

Goulet, T.L. (2006) Most corals may not change their symbionts. Marine Ecology Progress Serie,321,1–7.

Guillard, R.R.L. and Ryther, J.H. (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gram. Canadian Journal of Microbiology, 8, 229–239.

Hall, J.R.; Giddings, L.W.; Solomon, J.M.; Balcomb, R. (1999). An Ecological Risk Assessment for the Use of Irgarol 1051 as an Algaecide for Antifoulant Paints. Critical Reviews in Toxicology, 29, 367-437.

Hallers-Tjabbes, T.; Cato, C.; Walmsley, S. (2009). Consequences of Antifouling Systems,An Environmental Perspective. In *Biofouling*, 243-51.

Hanssen, K.; Cervin, G.; Trepos, R.; Petitbois, J.; Haug, T.; Hansen, E.; Andersen, J.; Pavia, H.; Hellio, C.; Svenson, J. (2014). The Bromotyrosine Derivative Ianthelline Isolated from

the Arctic Marine Sponge *Stryphnus fortis* Inhibits Marine Micro- and Macrobiofouling. Marine Biotechnology, 16 (6), 684-694.

Harder, T., & Yee, L. H. (2009). Bacterial adhesion and marine fouling. In Advance Marine Antifouling Coating Technology. ,1, 113–131.

Haug, B. E.; Stensen, W.; Stiberg, T.; Svendsen, J. S., Bulky. (2004). Non proteinogenic Amino Acids Permit the Design of Very Small and Effective Cationic Antibacterial Peptides. Journal of Medicinal Chemistry, 47 (17), 4159-4162.

Haug, B. E.; Stensen, W.; Kalaaji, M.; Rekdal, Ø.; Svendsen, J. S. (2008). Synthetic Antimicrobial Peptidomimetics with Therapeutic Potential. Journal of Medicinal Chemistry, 51 (14), 4306-4314.

Hellio, C.; Berge, J.P.; Beaupoil, C.; Le Gal, Y.; Bourgougnon, N. (2002) Screening of marine algal extracts for anti-settlement activities against microalgae and macroalgae, Biofouling ,18, 205-215.

Hellio, C., Simon-Colin, C., Clare, A. S., & Deslandes, E. (2004). Isethionic acid and floridoside isolated from the red alga, *Grateloupia turuturu*, inhibit settlement of *Balanus amphitrite* cyprid larvae. Biofouling, 20, 139–145.

Hellio, C., Bado-Nilles, A., Gagnaire, B., Renault, T., & Thomas-Guyon, H. (2007). Demonstration of a true phenoloxidase activity and activation of a ProPO cascade in Pacific oyster, *Crassostrea gigas* (Thunberg) in vitro. Fish and Shellfish Immunology, 22, 433–440.

Hellio C. and Yebra D. M., (2009). Advances in Marine Antifouling Coatings and Technologies. Woodhead Publishing Cambridge, UK.

Hellio, C. (2010). The potential of marine biotechnology for the development of new antifouling solutions. Journal Des Sciences Halieutique et Aquatique, 35–41.

Hennige, S. J., Suggett, D. J., Warner, M. E., McDougall, K. E., & Smith, D. J. (2009). Photobiology of Symbiodinium revisited: Bio-physical and bio-optical signatures. Coral Reefs, 28(1), 179–195.

Hill M, Wilcox T., (1998). Unusual mode of symbiont population after bleaching in *Anthosigmella varians*: acquisition of different zooxanthellae strains. Symbiosis 25 :279-89.

Hoegh-Guldberg, O. (1999). Climate change coral bleaching and the future of the world's coral reefs. Marine Freshwater Reserve, 50, 839-66.

Hoegh-Guldberg, O.; Jones, R.J.; Ward, S. and Loh, W.K. (2002) Ecology – Is coral bleaching really adaptive? Nature, 415 6872, 601-602.

Hoegh-Gulberg, O.; Mumby, P. J.; Hooten, A. J.; Steneck, R. S.; Greenfield, P.; Gomez, E.; Harvell, C.D.; Sale, P. F.; Edwards, A.J.; Caldeira, K.; Knowltonn, N.; Eakin, C. M.; Iglesias -Prieto, R.; Muthiga, N.; Bradbury, Y. R. H.; Dubi, A. and Hatziolos, M. E. (2007) Coral reefs under rapid climate change and ocean acidification. Science, 318, 1737–1742.

Hofmann, G.E.; Todgham, A.E. (2010). Living in the Now: Physiological Mechanisms to Tolerate a Rapidly Changing Environment. Annual Review of Physiology, 72, 127–45.

Horn, J. and Jones, D. (2002) Microbiological influenced corrosion: Perspectives and Approaches, Manual of Environmental Microbiology, 1072-1083.

Houssen, W.E.; Bent, A.F.; McEwan, A.R.; Pieiller, N.; Tabudravu, J.; Koehnke, J.; Mann, G.; Adaba, RI.; Thomas, L.; Hawas, U.W.; Liu, H.; Schwarz-Linek, U.; Smith, M.C.M.; Naismith, J.H.; Jaspars, M. (2014). 'An Efficient Method for the *In Vitro* Production of Azol(in)e-Based Cyclic Peptides'. Angewandte Chemie International Edition, 53, 51, 14171-14174.

Huggett, M.; Williamson, J.; de Nys, R.; Kjelleberg, S.; Steinberg, P. (2006). Larval settlement of the common Australian sea urchin *Heliocidaris erythrogramma* in response to bacteria from the surface of coralline algae. Oecologia, 149 (4), 604-619.

Hughes, T P T.; Baird, A.H.; Bellwood, D.R.; Card, M.; Connolly, S.R.; Folke, C.; Grosberg, R.; Hoegh-Guldberg, O.; Jackson, J.B.C.; Kleypas, J.; Lough, J.M.; Marshall, J; Nystro, M.; Palumbi, S.R.; Pandolfi, J.M.; Rosen, B.; Roughgarden, J. (2003). Climate Change, Human Impacts, and the Resilience of Coral Reefs. Science, 301, 929–933.

Hung, O.S.; Thiyagarajan, V.; Wu, R.S.S.; Qian, P.Y. (2005) Effects of ultraviolet radiation on films and subsequent settlement of *Hydroides elegans*. Marine Ecology Progress Series, 304,155–66

Hunter, H. N.; Vogel, H. J. (2005). Lactoferricin. Cellular and. Molecular. Life Science, 62 (22), 2588-2598.

Imhoff, J. F.; Labes, A.; Wiese, J. (2011). Bio-mining the microbial treasures of the ocean: new natural products. Biotechnology Advances, 29(5), 468–82.

Isaksson, J.; Brandsdal, B. O.; Engqvist, M.; Flaten, G. E.; Svendsen, J. S. M.; Stensen, W. (2011). A Synthetic Antimicrobial Peptidomimetic (LTX 109): Stereochemical Impact on Membrane Disruption. Journal of Medicinal Chemistry, 54 (16), 5786-5795.

Ivanisevic, J.; Thomas, O.P.; Lejeusne, C.; Chevaldonne, P.; Perez T. (2011) Metabolic fingerprinting as an indicator of biodiversity: towards understanding inter-specific relation- ships among Homoscleromorpha sponges. Metabolomics, 7, 289–304.

Jackson, J B. (2001). What Was Natural in the Coastal Oceans? Proceedings of the National Academy of Sciences ,98, 10, 5411–5418.

Jayaraman, M.; Seetharaman, J., (2003). Physiochemical analysis of the exopolysaccharides produced by a marine biofouling bacterium *Vibrio alginolyticus*. Process biochemistry., 38 (6), 841-847 (7)

Jellali, R.; Campistron, I.; Pasetto, P.; Laguerre, A.; Gohier, F.; Hellio, C.; Mouget, J. L. (2013). Antifouling activity of novel polyisoprene-based coatings made from photocurable natural rubber derived oligomers. Progress in Organic Coatings, 76, 1203–1214.

Jones, R. J.; Kerswell, A. P. (2003). Phytotoxicity of Photosystem II (PSII) herbicides to coral. Marine Ecology Progress Series, 261(February), 149–159.

Jones, A.M.; Berkelmans, R.; Van Oppen, M.J.H.; Mieog, J.C.; Sinclair, W.; Apprill, A.M; Gates, R.M. (2008). A Community Change in the Algal Endosymbionts of a Scleractinian Coral Following a Natural Bleaching Event: Field Evidence of Acclimatization. Proceedings of the Royal Society of Biological Sciences, 275 (1641), 1359–65.

Jorge, P.; Lourenço, A.; Pereira, M. O. (2012). New trends in peptide-based anti-biofilm strategies: a review of recent achievements and bioinformatic approaches. Biofouling, 28 (10), 1033-1061.

Kamino, K.; Odo, S.;Maruyama, T. (1996). Cement proteins of the acorn barnacle, *Megabalanus rosa*. Biological Bulletin, 190(3), 403–409.

Karlsson,J.; Eklund,B. (2004). New Biocide Antifouling Paints Are Toxic New Biocide-Free Anti-Fouling Paints Are Toxic. Marine Pollution Bulletin ,49 ,456–464

Karlsson, J., Ytreberg, E., & Eklund, B. (2010). Toxicity of anti-fouling paints for use on ships and leisure boats to non-target organisms representing three trophic levels. Environmental Pollution, 158(3), 681–7.

Karstad, R.; Isaksen, G.; Brandsdal, B.-O.; Svendsen, J. S.; Svenson, J. (2010). Unnatural Amino Acid Side Chains as S1, S1′, and S2′ Probes Yield Cationic Antimicrobial Peptides with Stability toward Chymotryptic Degradation. Journal of Medicinal Chemistry, 53 (15), 5558-5566.

Karstad, R.; Isaksen, G.; Wynendaele, E.; Guttormsen, Y.; De Spiegeleer, B.; Brandsdal, B.-O.; Svendsen, J. S.; Svenson, J. (2012). Targeting the S1 and S3 Subsite of Trypsin with Unnatural Cationic Amino Acids Generates Antimicrobial Peptides with Potential for Oral Administration. Journal of Medicinal Chemistry, 55 (14), 6294-6305.

Kelly, S.R.; Garo,E.; Jensen, P.R.; Fenical, W.; Pawlik,J.R.(2005). Effects of Caribbean Sponge Secondary Metabolites on Bacterial Surface Colonization. Aquatic Microbial Ecology,40,2, 191–203.

Kientz, B., Thabard, M., Cragg, S. M., Pope, J., & Hellio, C. (2011). A new method for removing microflora from macroalgal surfaces: an important step for natural product discovery. Botanica Marina, 54(5), 457-469.

Kleypas, J. A.; Feely, R. A.; Fabry, V. J, Langdon, C., Sabine C. L. and Robbins L. L. (2006). Impacts of Ocean Acidification on Coral Reefs and Other Marine Calcifiers. A Guide for Future Research, Report of a workshop sponsored by NSF, NOAA and the US Geological Survey. 88pp.

Knutson, S., Downs, C. A., & Richmond, R. H. (2012). Concentrations of Irgarol in selected marinas of Oahu, Hawaii and effects on settlement of coral larval. Ecotoxicology, 21(1), 1–8.

Konstantinou, I.K., Albanis, T.A., (2004). Worldwide occurrence and effects of antifouling paint booster biocides in the aquatic environment: a review. Environment International, 30, 235–248.

Kotake Y. (2012). Molecular mechanisms of environmental organotin toxicity in mammals. Biological and Pharmaceutical Bulletin, 35, 1876-1880.

Kottuparambil, S., Lee, S., & Han, T. (2013). Single and interactive effects of the antifouling booster herbicides Diuron and Irgarol 1051 on photosynthesis in the marine cyanobacterium, Arthrospira maxima. Toxicology and Environmental Health Sciences, 5(2), 71–81.

Kristensen, J. B., Meyer, R. L., Laursen, B. S., Shipovskov, S., Besenbacher, F., & Poulsen, C. H. (2008). Antifouling enzymes and the biochemistry of marine settlement. Biotechnology Advances, 26(5), 471–81

Kristensen, J. B., Meyer, R. L., Laursen, B. S., Shipovskov, S., Besenbacher, F., Poulsen, C. H., Negri, A. P. (2016). Validation of Reef-Scale Thermal Stress Satellite Products for Coral Bleaching Monitoring. Marine Drugs, 8(1), 59.

Krueger, T.; Hawkins, T.D.; Becker, S.; Pontasch, S.; Dove, S.; Hoegh-Guldberg, O.; Leggat, W.; Fisher, P.L.; Davy, S.K. (2015). Differential coral bleaching contrasting the activity and response of enzymatic antioxidants in symbiotic partners under thermal stress. Comparative Biochemistry and Physiology, part A, Molecular and Integrative Physiology; 190:15-25.

Krug, P.J (2006) Defense of benthic invertebrates against surface colonization by larvae: a chemical arms race. Progress in Molecular and Subcellar Biology 42:1-53

Kubo, I., Muroi, H., & Himejima, M., (1992). Antibacterial activity of Totarol and its potentiation. Journal of natural products, 55 (10), 1436-1440.

Küster, A.; Altenburger, R. (2007). Development and validation of a new fluorescence-based bioassay for aquatic macrophyte species. Chemosphere, 67, 194-201.

Kuwata, H.; Yip, T.-T.; Tomita, M.; Hutchens, T. W. (1998). Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1429 (1), 129-141.

LaJeunesse, T. C.; Loh, W. K.; Van Woesik, R.; Hoegh- Guldberg, O.; Schmidt, G. W.; Fitt, W. K. (2003) Low symbiont diversity in southern Great Barrier Reef corals, relative to those of the Caribbean, Limnology Oceanography, 48, 2046–2054.

LaJeunesse, T.C.; Smith, R.; Walther, M.; Pinzón, J.; Pettay, D.T.; McGinley, M.; Aschaffenburg, M. (2010). Host-Symbiont Recombination versus Natural Selection in the Response of Coral-Dinoflagellate Symbioses to Environmental Disturbance. Proceedings. Of the Royal Society of Biological Sciences, 277 ,1696, 2925–34.

Langhamer, O.; Haikonen, K.; Sundberg, J. (2010). Wave power—Sustainable energy or environmentally costly? A review with special emphasis on linear wave energy converters. Renewable and Sustainable Energy Reviews**,** 14 (4), 1329-1335.

Lasa, I. (2006) Towards the identification of the common features of bacterial biofilm development. International Microbiology ,9:21–8

Larsen, P; Nielsen, J.L; Dueholm, M.S; Wetzel, R; Otzen, D; Nielsen, P.H. (2007) Amyloid adhesins are abundant in natural biofilms. Environmental Microbiology ,9(12):3077–90

Latasa, C.; Solano, C.; Penadés, J.R. and Lasa, I. (2006) Biofilm-associated proteins. Comptes. Rendus. Biologie, 329:849–57

Lee, R.F.; Valkirs, A.O. and Seligman,P.F. (1989) Importance of microalgae in the biodegradation of tributyltin in estuarine waters, Environmental Science and Technology, Vol. 23, No 12, 1515–1518.

Lee, J.; Marsden.; Glover, C.N. (2010). The Influence of Salinity on Copper Accumulation and Its Toxic Effects in Estuarine Animals with Differing Osmoregulatory Strategies. Aquatic Toxicology, 99 (1), 65–72.

Lejars, M.; Margaillan, A.; Bressy, C. (2012). Fouling Release Coatings: A Nontoxic Alternative to Biocidal Antifouling Coatings. Chemical Reviews, 112 (8), 4347-4390.

Leroy, C.; Delbarre-Ladrat, C.; Ghillebaert, F.; Compere, C.; Combes, D. (2008). Effects of commercial enzymes on the adhesion of a marine biofilm-forming bacterium. Biofouling, 24,11–22.

Lesser MP, Farrell JH, (2004). Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. Coral Reefs; 23(3): 367-377.

Lessios, H A.; Robertson, D.R.; Cubit, J.D. (1984). Spread of Diadema Mass Mortality through the Caribbean. Science, 226, 4672, 335–337.

Lewis, M. R.; Horne, W; Cullen, J.J; Oakey, N.S and Platt, M. (1984). Turbulent motions may control phytoplankton photosynthesis in the upper ocean. Nature 311: 49–50.

Lewis, J.A., (1998). Marine biofouling and its prevention on underwater surfaces. Mater. Forum 22, 41–61.

Lewis, J.A.; Baran, I.J; Carey, J.M; Fletcher, L.E. (2010). A Contaminant in Decline: Long-Term Tbt Monitoring at a Naval Base in Western Australia. Australasian Journal of Ecotoxicology 16 (1): 17–34.

Li, C.J. and Trost B.M. (2008). Green chemistry for chemical synthesis. Proceeding of National Academy Science, 105, 36, 13197–13202.

Li, X. J.; Hopmann, K. H.; Hudecova, J.; Isaksson, J.; Novotna, J.; Stensen, W.; Andrushchenko, V.; Urbanova, M.; Svendsen, J. S.; Bour, P.; Ruud, K. (2013) Determination of Absolute Configuration and Conformation of a Cyclic Dipeptide by NMR and Chiral Spectroscopic Methods Journal of Physical Chemistry, vol. A, 117, 1721−1736.

Lind, J. L.; Heimann, K.; Miller, E. A.; Van Vliet, C.; Hoogenraad, N. J.; & Wetherbee, R. (1997). Substratum adhesion and gliding in a diatom are mediated by extracellular proteoglycans. Planta, 203(2), 213–221.

Lind, U.; Rosenblad, M. A.; Frank, L. H.; Falkbring, S.; Brive, L.; Laurila, J. M.; Pohjanoksa, K.; Vuorenpaa, A.; Kukkonen, J. P.; Gunnarsson, L.; Scheinin, M.; Lindblad, L.; Blomberg, A. (2010). Octopamine Receptors from the Barnacle *Balanus improvisus* Are Activated by the alpha (2)-Adrenoceptor Agonist Medetomidine. Molecular Pharmacology, 78 (2), 237-248.

Litaudon, M. and Guyot, M. (1986) Ianthelline, un nouveau derive de dibromo-3,5 tyrosine, isole de l'eponge *Ianthella ardis*(Bahamas). Tetrahedron Letter,27(37),4455-4456.

Liu, M., Hansen, P. E., & Lin, X. (2011). Bromophenols in marine algae and their bioactivities. Marine Drugs, 9(7), 1273–92.

Lough, J. (2007). Climate and climate change on the Great Barrier Reef. Climate Change and the Great Barrier Reef: A Vulnerability Assessment. Great Barrier Reef Marine Park Authority, Townsville, 15-50

Macdonald, A.H.H.; Sampayo, E.M.; Ridgway, T.; Schleyer, M.H. (2008). Latitudinal Symbiont Zonation in *Stylophora Pistillata* from Southeast Africa. Marine Biology, 154, 2, 209–217.

Macherla, V.R.; Liu, J.; Sunga, M.; White, D.J.; Grodberg, J.; Teisan, S.; Lam, K.S.; Potts, B.C.M. (2007). Lipoxazolidinones A, B, and C: Antibacterial 4-Oxazolidinones from a Marine Actinomycete Isolated from a Guam Marine Sediment. Journal of Natural Products 70 (9), 1454–1457.

Maggs,C.A. and Callow, M. E. (2003). Algal Spores. In Encyclopedia of Life Sciences. Chichester, UK: John Wiley & Sons

Magin, C. M.; Cooper, S. P.; & Brennan, A. B. (2010). Non-toxic antifouling strategies. Materials Today, 13(4), 36–44.

Magnusson, M.; Heimann, K.; Negri, A.P. (2008). Comparative effects of herbicides on photosynthesis and growth of tropical estuarine microalgae. Marine Pollution Bulletin, 56, 1545-1552.

Majumdar, P.; Lee, E.; Patel, N.; Ward, K.; Stafslien, S. J.; Daniels, J.; Chisholm, B. J.; Boudjouk, P.; Callow, M. E.; Callow, J. A.; Thompson. (2008a). S. E. M., Combinatorial

materials research applied to the development of new surface coatings IX: An investigation of novel antifouling/fouling-release coatings containing quaternary ammonium salt groups. Biofouling, 24 (3), 185-200.

Majumdar, P.; Lee, E.; Patel, N.; Stafslien, S.; Daniels, J.; Chisholm, B. (2008b). Development of environmentally friendly, antifouling coatings based on tethered quaternary ammonium salts in a crosslinked polydimethylsiloxane matrix. J Coat Technol Res, 5 (4), 405-417.

Maki, J.S.; Rittschof, D.; Costlow, J.D.; Mitchell, R. (1988). Inhibition of attachment of larval barnacles, *Balanus amphitrite*, by bacterial surface films. Marine Biology,97, 199–206.

Manivasagan, P., and Kim, S.-K. (2014). Extracellular polysaccharides produced by marine bacteria. Advances in Food and Nutrition Research, 72: 79–94

Manzello, D.P.; Brandt, M.; Smith, T.B.; Lirman, D.; Hendee, J.C.; Nemeth, R.S. (2007). Hurricanes Benefit Bleached Corals. Proceedings of the National Academy of Sciences, 104 (29), 12035–12039.

Marabotti, I. ; Morelli, A. ; Orsini, L.M. ; Martinelli, E. ; Galli, G. ; Chiellini, E. ; Lien, E.M. ; Pettit, M.E. ; Callow, M.E. ; Callow, J.A. (2009). Fluorinated/siloxane copolymer blends for fouling release: chemical characterisation and biological evaluation with algae and barnacles. Biofouling, 25, 481-493.

Maréchal, J-P.; Culioli, G.; Hellio, C.; Thomas-Guyon, H.; Callow, M.E.; Clare, A.S.; Ortalo-Magné, A. (2004). Seasonal variation in antifouling activity of crude extracts of the brown alga *Bifurcaria bifurcata* (Cystoseiraceae) against cyprids of *Balanus amphitrite* and the marine bacteria *Cobetia marina* and *Pseudoalteromonas haloplanktis.* Journal of Experimental Marine Biology and Ecology, 313, 1, 47-62.

Maréchal, J.-P., & Hellio, C. (2009). Challenges for the development of new non-toxic antifouling solutions. International Journal of Molecular Sciences, 10(11), 4623–37.

Maréchal, J. P., & Hellio, C. (2011). Antifouling activity against barnacle cypris larvae: Do target species matter (*Amphibalanus amphitrite* versus *Semibalanus balanoides*)? International Biodeterioration and Biodegradation, 65, 92–101.

Marshall,P.A. and Schuttenberg, H.Z. (2006). A Reef Manager's Guide to Coral Bleaching. Great Barrier Reef Marine Park Authority, Australia

Martinelli, E.; Suffredini, M.; Galli, G.; Glisenti, A.; Pettitt, M. E.; Callow, M. E.; Callow, J. A.; Williams, D.; Lyall, G. (2011). Amphiphilic block copolymer/poly(dimethylsiloxane) (PDMS) blends and nanocomposites for improved fouling-release. Biofouling ,27 (5), 529-541.

Martinelli, E.; Guazzelli, E.; Bartoli, C.; Gazzarri, M.; Chiellini, F.; Galli, G.; Hill, S. (2015). Amphiphilic pentablock copolymers and their blends with PDMS for antibiofouling coatings. Journal of Polymer Science Part A: Polymer Chemistry, 53(10), 1213–1225.

McClintock, J.B. and Baker, B.J. (2001). Marine chemical ecology.CRC Marine Science Series, CRC Press, Boca Raton, 609 pp.

Melander, C.; Moeller, P.D.R; Ballard, T.E; Richards, J.J; Huigens, R.W.; Cavanagh, J. (2009). Evaluation of dihydroOroidin as an antifouling additive in marine paint. International Biodeterioration and Biodegradation, 63, 529-532

Menna, M.; Fattorusso, E.; Imperatore, C. (2011). Alkaloids from Marine Ascidians. Molecules 16 (12), 8694–8732.

Mielczarski, J.A. ; Mielczarski, E. ; Galli, G. ; Morelli, A. ; Martinelli, E. and Chiellini, E. (2010). The Surface-Segregated Nanostructure of Fluorinated Copolymer−Poly(dimethylsiloxane) Blend Films. 26 (4), American Chemical Society, 2871–2876.

Miller, M.B. and Bassler, B.L. (2001) Quorum sensing in bacteria, Annual Reviews of Microbiology 55 (1) 165–199.

Miyao, M.; Ikeuchi, M.; Yamamoto, N.; Ono, N. (1995). Specific Degradation of the D1 Protein of photosystem II by treatment with hydrogen peroxide in darkness: Implications for the mechanism of degradation of the D1 protein under illumination. Biochemistry, 34, 10019-10026.

Miyata, Y.; Diyabalanage, T.; Amsler, C.D; McClintock, J.B; Valeriote, F.A.; Baker, B.J. (2007). Ecdysteroids from the Antarctic Tunicate *Synoicum Adareanum*. Journal of Natural Products ,70, 12, 1859–1864.

Molino, P. J.; Wetherbee, R. (2008). The biology of biofouling diatoms and their role in the development of microbial slimes. Biofouling, 24 (5), 365-379.

Müller, W.E.G; Wang, X.; Proksch, P.; Perry, C.C; Osinga, R.; Gardères, J.; Schröder, H.C. (2013). Principles of Biofouling Protection in Marine Sponges: A Model for the Design of Novel Biomimetic and Bio-Inspired Coatings in the Marine Environment? Marine Biotechnology, 15, 375−398.

Murguía, J. S.; Rosu, H. C.; Jimenez, A.; Gutiérrez-Medina, B.; García-Meza, J. V. (2015). The Hurst exponents of *Nitzschia sp*. diatom trajectories observed by light microscopy. Physica A: Statistical Mechanics and Its Applications, 417: 176–184.

Myan, F.W.Y.; Walker, J. and Paramor, O. (2013). The interaction of marine fouling organisms with topography of varied scale and geometry: a review. Biointerphases, 8, 30.

Negri, A., Vollhardt, C., Humphrey, C., Heyward, A., Jones, R., Eaglesham, G., & Fabricius, K. (2005). Effects of the herbicide Diuron on the early life history stages of coral. Marine Pollution Bulletin, 51(1-4), 370–383.

Negri, A.; Marshall, P. (2009). TBT contamination of remote marine environments: Ship groundings and ice-breakers as sources of organotins in the Great Barrier Reef and Antarctica. Journal of Environmental Management, 90(SUPPL. 1).

Negri, A. P.; Flores, F.; Röthig, T.; Uthicke, S. (2011). Herbicides increase the vulnerability of corals to rising sea surface temperature. Limnology and Oceanography, 56(2), 471–485.

Nicolaus, M.E.E; Barry, J. (2015). Imposex in the Dogwhelk (Nucella *Lapillus*): 22-Year Monitoring around England and Wales. Environmental Monitoring Assessment, 187:736

Núñez-Pons, L.; Carbone, M.; Vázquez, J.; Rodríguez, J.; Nieto, R.M.; Varela, M.M.; Gavagnin, M.; Avila, C. (2012). Natural Products from Antarctic Colonial Ascidians of the Genera Aplidium and Synoicum: Variability and Defensive Role. Marine Drugs ,10 ,12, 1741–64.

Ödling, K; Albertsson, C.; Russell, J; Mårtensson, L. (2007) An in vivo study of cement proteins from barnacle *Balanus improvisus* cyprid larva. Journal of Experimental Biology ,209: 956–64

Okamura, H.; Aoyama, I.; Liu, D.; Maguire, J.; Pacepavicius, G. J.; Lau, Y. L., (1999). Photodegradation of Irgarol 1051 in water. *Journal of Environmental Science & Health Part B*, *34*(2), 225-238.

Okamura, H.; Aoyama, I.; Liu, D.; Maguire, R.J.; Pacepavicius, G.J.; Lau, Y.L. (2000). Fate and ecotoxicity of the new antifouling compound Irgarol 1051 in the aquatic environment. Water Research 34, 3523–3530.

Okoro, H.K.; Fatoki, O.S.; Adekola, F.A.; Ximba, B.J.; Synman, R.G. (2011): Sources Environmental Levels and Toxicity of Organotin in Marine Environment- A Review, Asian Journal of Chemistry. 23(2), 473 – 482.

Olsen, S.M.; Pedersen, L.T.; Laursen, M.H.; Kiil, S.; Dam-Johansen, K. (2007). Enzyme-based antifouling coatings: a review. Biofouling 23:369–383.

Omae, I., (2003). General aspects of tin-free antifouling paints. Chemical reviews, 103(9), 3431-3448.

Ortlepp, S.; Sjogren, M.; Dahlstrom, M.; Weber, H.; Ebel, R.; Edrada, R.; Thoms, C.; Schupp, P.; Bohlin, L.; Proksch, P. (2007). Antifouling activity of bromotyrosine-derived sponge metabolites and synthetic analogues. Marine Biotechnology**,** 9 (6), 776-785.

Orme, J.A.C; Masters, I; Griffiths, R.T. (2001). Investigation of the effect of biofouling on the efficiency of marine current turbines. MAREC, Institute of Marine Engineers; 91-99.

Pandolfi, J. M.; Bradbury, R. H.; Sala, E.; Hughes, T. P.; Bjorndal, K. A.; Cooke, R. G.; McArdle, D.; McClenachan, L.; Newman, M. J. H.; Paredes, G. (2003). Global trajectories of the long- term decline of coral reef ecosystems, Science, 301, 955–958.

Parks, R., Donnier-Marechal, M., Frickers, P. E., Turner, A., & Readman, J. W. (2010). Antifouling biocides in discarded marine paint particles. Marine Pollution Bulletin, 60(8), 1226–30

Penesyan, A., Kjelleberg, S., & Egan, S. (2010). Development of novel drugs from marine surface associated microorganisms. Marine Drugs, 8(3), 438–59.

Perez, M; Sanchez, M; Stupak, M; Garcia, M; de Almeida, MTR; Oberti, JC ;Palermo, JA ; Blustein, G. (2014). Antifouling Activity of Celastroids Isolated from *Maytenus* species. Natural and Sustainable Alternatives for Marine Coatings. Industrial and engineering chemistry research, 53, 18,7655-7659.

Peters, E.C, Gassman, N.J, Firman, J.C, Richmond, R.H, Power, E.A. (1997). Ecotoxicology of tropical marine ecosystems. Environmental Toxicology Chemistry; 16(1): 12-40.

Piola, R. F., Dafforn, K. a, & Johnston, E. L. (2009). The influence of antifouling practices on marine invasions. Biofouling, 25(7), 633–44.

Pinori, E; Elwing, H.; Berglin, M. (2013). The impact of coating hardness on the anti-barnacle efficacy of an embedded antifouling biocide, Biofouling, Vol. 29, 7, 763-773.

Plass-Johnson, J.G; Cardini,U. ; Van Hoytema, N.; Bayraktarov, E.; Wilde, C. (2015) Coral Bleaching, in book: Environmental Indicators, Edition: 1st, Chapter: 9, Springer,117-146.

Plouguerné, E., Ioannou, E., Georgantea, P., Vagias, C., Roussis, V., Hellio, C., Stiger-Pouvreau, V. (2010). Anti-microfouling activity of lipidic metabolites from the invasive brown alga *Sargassum muticum* (Yendo) Fensholt. Marine Biotechnology, 12, 52–61.

Pochon, X.; Gates, R.D.G. (2010). A New Symbiodinium Clade (Dinophyceae) from Soritid Foraminifera in Hawai'i. Molecular Phylogenetics and Evolution, 56 (1), 492–97.

Poulsen, N. C., Spector, I., Spurck, T. P., Schultz, T. F., & Wetherbee, R. (1999). Diatom gliding is the result of an actin-myosin motility system. Cell Motility and the Cytoskeleton, 44(1), 23–33.

Proksch, P. (1994). Defensive Roles for Secondary Metabolites from Marine Sponges and Sponge-Feeding Nudibranchs Toxicon, Official Journal of the International Society on Toxinology, 32 (6), 639–55.

Qian, P. Y. ; Lau, S. C. K. ; Dahms, H. U. ; Dobretsov, S. ; Harder, T. (2007). Marine biofilms as mediators of colonization by marine macroorganisms: Implications for antifouling and aquaculture. Mar Biotechnol, 9 (4), 399-410.

Qian, P. Y.; Xu, Y.; Fusetani, N. (2010). Natural products as antifouling compounds: recent progress and future perspectives. Biofouling**,** 26 (2), 223-234.

Qian, P.-Y. ; Li, Z. ; Xu, Y. ; Li, Y. ; Fusetani, N. (2015). Mini-review: Marine natural products and their synthetic analogs as antifouling compounds: 2009–2014. Biofouling**,** 31 (1), 101-122.

Qiu, J.-W., Thiyagarajan, V., Cheung, S., & Qian, P.-Y. (2005). Toxic effects of copper on larval development of the barnacle Balanus amphitrite. Marine Pollution Bulletin, 51(8-12), 688–93.

Raimbault, P. ; Lantoine, F. ; Neveux, J. (2004). Dosage rapide de la Chlorophylle *a* et des phéopigments *a* par fluorimétrie après extraction au methanol. Comparaison avec la method classique d'extraction à l'acétone. Océanis, 30, 189-205.

Railkin, A.I. (2004) Marine biofouling : colonization processes and defences. Boca Raton: CRC Press LLC

Ralph, P.J.; Schreiber, U.; Gademann, R.; Kühl,M.; and Larkum, A.W.D. (2005). Coral photobiology studied with a new imaging pulse amplitude modulated fluorometer. Journal of Phycology 41 (2), 335–42.

Ralston, E. and Swain, G. (2011). Can biomimicry and bioinspiration provide solutions for fouling control? Marine Techology Society Journal, 45, 4, 216-227.

Rands, M.L.; Loughman, B.C.; Douglas, A.E.(1993) The Symbiotic Interface in an Alga Invertebrate Symbiosis. Process Royal Society.of London Biological. Science., 253, 161–165.

Rascio, V. J., Giudice, C. A., & del Amo, B., (1988). Research and development of soluble matrix antifouling paints for ships, offshore platforms and power stations. A review. *Corrosion Reviews*, *8*(1-2), 87-154.

Rascio, V. J., (2000). Antifouling coatings: where do we go from here. *Corrosion Reviews*, *18*(2-3), 133-154.

Readman, J.W., Kwong, L.L.W., Grondin, D., Bartocci, J., Villeneuve, J.P., Mee, L.D., (1993). Coastal water contamination from a triazine herbicide used in antifouling paints. *Environ. Sci. Technol*. 27, 1940–1942.

Reuter, K., Steinbach, A., & Helms, V. (2016). Interfering with Bacterial Quorum sensing. Perspectives in Medicinal Chemistry, 8: 1–15

Rittschof, D.; Clare, A. S.; Gerhart, D. J.; Mary, S. A. and Bonaventura, J. (1992). Barnacle *in vitro* assays for biologically active substances: Toxicity and Settlement inhibition assays using mass cultured *Balanus amphitrite amphitrite* Darwin. *Biofouling*, *6*(2), 115–122.

Rittschof, D. (2001) Natural Product Antifoulants and Coatings Development. In Marine Chemical Ecology, McClintock, J. B.; Baker, B. J., Eds. CRC Press: New York, 543-566.

Roberts, D.; Rittschof, D.; Holm, E.; Schmidt, A. R. (1991). Factors influencing initial larval settlement: temporal, spatial and surface molecular components. Journal of Experimental Marine Biology and Ecology, 150 (2), 203-221.

Roberts, J.; Tsamenyi, M. (2008). International legal options for the control of biofouling on international vessels. Marine Policy, 32(4), 559–569.

Robison, J.D.; Warner, M.E. (2006). Differential impacts of photoacclimation and thermal stress on the photobiology of four different phylotypes of symbiodinium. Journal of Phycology, 42, 3, 568-579.

Rodriguez-Lanetty, M.; Loh, W.; Carter, D.; Hoegh-Guldberg, O. (2001) Latitudinal variability in symbiont specificity within the widespread scleractinian coral *Plesiastrea versipora*. Marine Biology, 138,1175–1181

Rodriguez-Lanetty, M.; Krupp, D.A. and Weis, V.M. (2004). Distinct ITS Types of *Symbiodinium* in Clade C Correlate with Cnidarian/dinoflagellate Specificity during Onset of Symbiosis. Marine Ecology Progress Series, 275, 97–102.

Roth, M.S.; Goericke, R.; Deheyn, D.D. (2012). Cold Induces Acute Stress but Heat Is Ultimately More Deleterious for the Reef-Building Coral *Acropora yongei*. Scientific Reports, 2, 240, 1-5.

Roth, M.S. (2014). The Engine of the Reef: Photobiology of the Coral-Algal Symbiosis Frontiers in Microbiology, 5, 422;

Rowan, R. and Knowlton, N. (1995). Intraspecific diversity and ecological zonation in coral-algal symbiosis. Proceed of National Academy of Science, 92, 2850–2853.

Rowan, R.; Knowlton, N.; Baker, A. C.; Jara, J. (1997). Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. Nature, 388, 265-269.

Rowan, R. (1998). Diversity and ecology of zooxanthellae on coral reefs. J. Phycol. 34:407-17

Rowan, R. (2004) Coral bleaching - Thermal adaptation in reef coral symbionts. Nature, 430, 742–742.

Salta, M.; Wharton, J.A; Stoodley, P.; Dennington, S.P.; Goodes, L.R.; Werwinski, S.; Mart, U.; Wood, R.J.K. and Stokes K.R. (2010). Designing biomimetic antifouling surfaces. Phil Trans R Soc A, 368, 4729-4754.

Sampayo, E.M.; Ridgway, T.; Bongaerts, P.; Hoegh-Guldberg, O. (2008). Bleaching suscepti- bility and mortality of corals are determined by fine-scale differences in symbiont type. Proceeding of National Academy of Science, 105, 10444–10449

Sangeetha, R.; Kumar; Doble, M. and Venkatesan R. (2010). Barnacle cement: An etchant for stainless steel 316L? Colloids and Surfaces B-Biointerfaces, 79, 2, 524-530.

Sapozhnikova, Y., Wirth, E., Schiff, K., Brown, J., Fulton, M., (2007). Antifouling pesticides in the coastal waters of Southern California. Marine Pollution Bulletin, 54, 1972–1978.

Saravolatz, L. D.; Pawlak, J.; Johnson, L.; Bonilla, H.; Saravolatz, L. D.; Fakih, M. G.; Fugelli, A.; Olsen, W. M. (2012). In Vitro Activities of LTX-109, a Synthetic Antimicrobial Peptide, against Methicillin-Resistant, Vancomycin-Intermediate, Vancomycin-Resistant, Daptomycin-Nonsusceptible, and Linezolid-Nonsusceptible Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 56 (8), 4478-4482.

Scardino, J.; Hudleston, D.; Peng Z.; Paul N.A and de Nys R. (2009). Biomimetic characterisation of key surface parameters for the development of fouling resistant materials Biofouling: The Journal of Bioadhesion and Biofilm Research, Volume 25, Issue 1, pages 83-93.

Shai, Y. (1999). Mechanism of the Binding, Insertion and Destabilization of Phospholipid Bilayer Membranes by α-Helical Antimicrobial and Cell Non-Selective Membrane-Lytic Peptides. Biochimica et Biophysica Acta (BBA) – Biomembranes, 1462 (1), 55–70.

Schoepf, V.; Grottoli, A.G.; Levas, S.L.; Aschaffenburg, M.D.; Baumann, J.H.; Matsui, Y.; Warner, M.E. (2015) Annual coral bleaching and the long-term recovery capacity of coral. Proceed of the Royal Society of Biology,282.

Schultz, M. P. (2007). Effects of coating roughness and biofouling on ship resistance and powering. Biofouling**,** 23 (5), 331-341.

Schultz, M. P.; Bendick, J. A.; Holm, E. R.; Hertel, W. M. (2010). Economic impact of biofouling on a naval surface ship. Biofouling**,** 27 (1), 87-98.

Sekar, R.; Venugopalan, V. P.; Satpathy, K. K.; Nair, K. V. K.; Rao, V. N. R. (2004). Laboratory studies on adhesion of microalgae to hard substrates. Hydrobiologia, 512, 109–116.

Sjogren, M.; Goransson, U.; Johnson, A. L.; Dahlstrom, M.; Andersson, R.; Bergman, J.; Jonsson, P. R.; Bohlin, L. (2004). Antifouling activity of brominated cyclopeptides from the marine sponge *Geodia barretti*. Journal of Natural Products, 67 (3), 368-372.

Sjögren, M.; Jonsson, P.R.; Dahlström, M.; Lundälv, T.; Burman, R.; Göransson, U. and Bohlin L. (2011). Two brominated cyclic dipeptides released by the coldwater marine sponge *Geodia barretti* act in synergy as chemical defense. Journal of Natural products, 74, 449–454.

Slavov, C.; Schrameyer, V.; Reus, M.; Ralph, P.J.; Hill, R.; Büchel, C.; Larkum, A.W.D and Holzwarth, R.A. (2016). Super-Quenching State Protects *Symbiodinium* from Thermal Stress — Implications for Coral Bleaching. Biochimica et Biophysica Acta – Bioenergetics, 1857 (6), 840–47.

Sohm, J. A., Edwards, B. R., Wilson, B. G., & Webb, E. A. (2011). Constitutive Extracellular Polysaccharide (EPS) Production by Specific Isolates of *Crocosphaera watsonii*. Frontiers in Microbiology,2 (229).

Sonak, S.; Bhosle, N. B., A simple method to assess bacterial attachment to surfaces. (1995). Biofouling, 9 (1), 31-38.

Stat, M., Morris, E., & Gates, R. D., (2008). Functional diversity in coral-dinoflagellate symbiosis. Proceedings of the National Academy of Sciences of the United States of America, 105(27).

Stat, M.; Gates, R.D. (2011). Clade D *Symbiodinium* in scleractinian corals: a “nugget” of hope, a selfish opportunist, an ominous sign, or all of the above? Journal of Marine Biology, 2011, 1-9.

Statz, A., Finlay, J., Dalsin, J., Callow, M., Callow, J. A., & Messersmith, P. B. (2006). Algal antifouling and fouling-release properties of metal surfaces coated with a polymer inspired by marine mussels. *Biofouling*, *22*, 391–399.

Stewart, J.; Katsuyama, I.; Fahmy, H.; Fronczek, F.R.; Zjawiony, J.K. (2004). A Direct Synthesis of 3,5-Dibromo- *O* -methyl- L -tyrosine. Synthetic Communications 34 ,3, 547–55.

Strøm, M. B.; Haug, B. E.; Rekdal, Ø.; Skar, M. L.; Stensen, W.; Svendsen, J. S. (2002). Important structural features of 15-residue lactoferricin derivatives and methods for improvement of antimicrobial activity. Biochemistry and Cell Biology, 80 (1), 65-74.

Strøm, M. B.; Haug, B. E.; Skar, M. L.; Stensen, W.; Stiberg, T.; Svendsen, J. S. (2003). The Pharmacophore of Short Cationic Antibacterial Peptides. Journal of Medicinal Chemistry,46 (9), 1567-1570.

Sudaryanto, A.; Takahashi, S.; Monirith, I.; Ismail,A.; Muchtar,M.; Zheng,J.; Richardson,B.J.; Subramanian,A.; Prudente,M.; Hue, N.D.;Tanabe,S. (2002). Asia-Pacific Mussel Watch: monitoring of butyltin contamination in coastal waters of Asian developing countries. Environmental Toxicology and Chemistry 21, 2119-2130.

Svenson, J.; Stensen, W.; Brandsdal, B.-O.; Haug, B. E.; Monrad, J.; Svendsen, J. S. (2008). Antimicrobial Peptides with Stability Toward Tryptic Degradation. Biochemistry, 47 (12), 3777-3788.

Svenson, J.; Karstad, R.; Flaten, G. E.; Brandsdal, B.-O.; Brandl, M.; Svendsen, J. S. (2009). Altered Activity and Physicochemical Properties of Short Cationic Antimicrobial Peptides by Incorporation of Arginine Analogues. Molecular Pharmaceutics**,** 6 (3), 996-1005.

Svenson, J. (2013). MabCent: Arctic Marine Bioprospecting in Norway. Phytochemistry Reviews 12 ,3, 567–78.

Tadesse, M.; Gulliksen, B.; Strøm, M.B; Styrvold, O.B; Haug, T. (2008). Screening for Antibacterial and Antifungal Activities in Marine Benthic Invertebrates from Northern Norway. Journal of Invertebrate Pathology 99 (3), 286–93.

Tadesse, M.; Stroem, M.B.; Svenson, J.; Jaspars, M.; Milne, B.F.; Toerfoss, V.; Andersen, J.H.; Hansen, E.; Stensvaeg, K.; Haug, T. (2010). Synoxazolidinones A and B: Novel Bioactive Alkaloids from the Ascidian *Synoicum Pulmonaria*. Organic Letter, 12, 4752−4755.

Tadesse, M.; Svenson, J.; Jaspars, M.; Strøm, M.B.; Abdelrahman, M.H.; Andersen, J.H.; Hansen, E.; Kristiansen, P.E.; Stensvåg, K.; Haug, T. (2011). Synoxazolidinone C; a Bicyclic Member of the Synoxazolidinone Family with Antibacterial and Anticancer Activities. Tetrahedron Letters 52 ,15, 1804–1806.

Tadesse, M. ; Svenson, J. ; Sepčić, K. ; Trembleau, L. ; Engqvist, M. ; Andersen, J.H. ; Jaspars, M. ; Stensvåg, K. ; Haug, T. (2014). Isolation and Synthesis of Pulmonarins A and B, Acetylcholinesterase Inhibitors from the Colonial Ascidian *Synoicum Pulmonaria*. Journal of Natural Products, 77,2, 364–369.

Takahashi, S.; Lee,L.T.H.; Saeki,H.; Nakatani,N.; Tanabe,S.; Miyazaki,N.; Fujise,Y. (2000). Accumulation of butyltin compounds and total tin in marine mammals. Water Science and Technology,42, 97-108.

Tanabe, S. (1999). Butyltin contamination in marine mammals – a review. Marine Pollution Bulletin 39, 62-72.

Terlizzi, A.; Geraci, S.; Minganti, V. (1998). Tributyltin (TBT) Pollution in the Coastal Waters of Italy as Indicated by Imposex in *Hexaplex trunculus* (Gastropoda, Muricidae), Marine Pollution Bulletin, Vol. 36, No. 9, 749-752.

Thabard, M.; Gros, O.; Hellio, C.; Marechal, J. P. (2011). *Sargassum polyceratium* (Phaeophyceae, Fucaceae) surface molecule activity towards fouling organisms and embryonic development of benthic species. Botanica Marina, 54 (2), 147-157.

Thomas, K. V., (2001). The environmental fate and behaviour of antifouling paint booster biocides: a review. Biofouling, 17(1), 73-86.

Thomas, K., (2009). The use of broad-spectrum organic biocides in marine antifouling paints. In: Hellio, C., Yebra, D. (Eds.), Advances in Marine Antifouling Coatings and Technologies. Woodhead Publishing Limited, Cambridge, 522–553.

Thornhill, D.J.; Kemp, D.W.; Bruns, B.W.; Fitt, W.K.; Schmidt, G.W. (2008). Correspondance between cold tolerance and temperate biogeography in a western atlantic Symbiodinium (Dinophyta) lineage. Journal of Phycology, 44, (5), 1126–35.

Tomita, M.; Bellamy, W.; Takase, M.; Yamauchi, K.; Wakabayashi, H.; Kawase, K. (1991). Potent Antibacterial Peptides Generated by Pepsin Digestion of Bovine Lactoferrin. Journal of Dairy Science**,** 74 (12), 4137-4142.

Townsin, R. L., The Ship Hull Fouling Penalty. (2003). Biofouling**,** 19 (sup1), 9-15.

Trench RK., (1993). Microalgal-invertebrate symbiosis-a review. Endocytobiosis Cell Res. 9:135-75.

Trepos, R.; Cervin, G.; Hellio, C.; Pavia, H.; Stensen, W.; Stensvåg, K.; Svendsen, J.-S.; Haug, T.; Svenson, J. (2014). Antifouling Compounds from the Sub-Arctic Ascidian *Synoicum pulmonaria*: Synoxazolidinones A and C, Pulmonarins A and B, and Synthetic Analogues. Journal of Natural Products**,** *77* (9), 2105-2113.

Tsoukatou, M., Maréchal, J. P., Hellio, C., Novaković, I., Tufegdzic, S., Sladić, D., Roussis, V. (2007). Evaluation of the activity of the sponge metabolites avarol and avarone and their synthetic derivatives against fouling micro- and macroorganisms. Molecules, 12, 1022–1034.

Tsukamoto, S.; Kato, H.; Hirota, H.; Fusetani, N. (1996a). Mauritiamine, a new antifouling Oroidin dimer from the marine sponge *Agelas mauritiana*. Journal of Natural Products**,** 59 (5), 501-503.

Tsukamoto S, Kato H, Hirota H, Fusetani N. (1996b). Ceratinamides a and b: new antifouling dibromotyrosine derivatives from the marine sponge *Pseudoceratina purpurea*. Tetrahedron, 52:8181-8186.

Van Dam, J. W., Negri, A. P., Uthicke, S., & Mueller, J. F., (2011). Chemical pollution on coral reefs: exposure and ecological effects. *Ecological impact of toxic chemicals*, 187-211.

Van Dam, J.W.; Negri, A.P.; Mueller, J.F.; Altenburger, R.; Uthicke, S. (2012). Additive pressures of elevated sea surface temperatures and herbicides on symbiont- bearing foraminifera. PLoS One 7, e33900.

Van Dam, J. W., Uthicke, S., Beltran, V. H., Mueller, J. F., & Negri, A. P. (2015). Combined thermal and herbicide stress in functionally diverse coral symbionts. Environmental Pollution, 204, 271–279.

Van Zoelen, W.; Buss, H. G.; Ellebracht, N. C.; Lynd, N. A.; Fischer, D. A.; Finlay, J.; Hill, S.; Callow, M. E.; Callow, J. A.; Kramer, E. J.; Zuckermann, R. N.; Segalman, R. A. (2014). Sequence of Hydrophobic and Hydrophilic Residues in Amphiphilic Polymer Coatings Affects Surface Structure and Marine Antifouling/Fouling Release Properties. ACS Macro Letters, 3 (4), 364-368.

Vieira, E. A., Duarte, L. F. L., & Dias, G. M. (2012). How the timing of predation affects composition and diversity of species in a marine sessile community? Journal of Experimental Marine Biology and Ecology, 412, 126–133.

Vogel, H. J.; Schibli, D. J.; Jing, W.; Lohmeier-Vogel, E. M.; Epand, R. F.; Epand, R. M. (2002). Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides. Biochemistry and Cell Biology**,** 80 (1), 49-63.

Walt, D. R., Smulow, J. B., Turesky, S. S., Hill, R. G. (1985). The effect of gravity on initial microbial adhesion. J. Col- loid Interface Sci. 107 (2): 334-336

Warner, M. E.; Fitt,W.K. and Schmidt, G.W. (1996). The Effects of Elevated Temperature on the Photosynthetic Efficiency of Zooxanthellae in Hospite from Four Different Species of Reef Coral: A Novel Approach. Plant, Cell and Environment ,19 (3), 291–299.

Watanabe, J.; Utsunomiya,Y.; Yuyama,I. (2007) Comparison of the antivascular and cytotoxic activities of TZT-1027 (Soblidotin) with those of other anticancer agents. Anticancer Drugs 18, 905–911.

Waters,C.M; Bassler,B.L.(2005) Quorum sensing: cell-to-cell communication in bacteria, Annual Review of Cell and Developmental Biology 21 319–346.

Webster, N. S., Negri, A. P., Botté, E. S., Laffy, P. W., Flores, F., Noonan, S., … Uthicke, S. (2016). Host-associated coral reef microbes respond to the cumulative pressures of ocean warming and ocean acidification. Nature Publishing Group, (August 2015), 1–9.

Weis, V.M. (2008). Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis. Journal of Experimental Biology; 19:3059-66.

Wetherbee, R.; Lind, J. L.; Burke, J.; & Quatrano, R. S. (1998). Minireview-the first kiss: establishment and control of initial adhesion by raphid diatom. Journal of Phycology, 34(1), 9–15.

Wetzel, R.; Shivaprasad, S.; Williams,A.D.(2007) Plasticity of amyloid fibrils. Biochemistry 46(1):1–10.

Wikström, S.A and Pavia H. (2004). Chemical settlement inhibition versus post-settlement mortality as an explanation for differential fouling of two congeneric seaweeds. Oecologia. , 138, 2, 223-30.

Wilhelmsson, D.; Malm, T. (2008). Fouling assemblages on offshore wind power plants and adjacent substrata. Estuarine, Coastal and Shelf Science, 79 (3), 459-466.

Wilkinson, A. D., Collier, C. J., Flores, F., & Negri, A. P. (2015). Acute and additive toxicity of ten photosystem-II herbicides to seagrass. Scientific Reports, 5, 17443.

Wood, R. J. K., A. S. Bahaj, S. R. Turnock, L. Wang and M. Evans (2010). Tribological design constraints of marine renewable energy systems. Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences 368, 1929, 4807-4827.

Wooldridge, S.A. (2009). Water Quality and Coral Bleaching Thresholds: Formalising the Linkage for the Inshore Reefs of the Great Barrier Reef, Australia. Marine Pollution Bulletin, 58 (5), 745–751.

Wustman, B.A; Gretz, M.R.; Hoagland, K.D. (1997). Extracellular matrix assembly in diatoms (Bacillariophyceae), a model of adhesives based on chemical characterization and localization of polysaccharides from the marine diatom *Achnanthes longipes* and other diatoms. Plant Physiology ,113:1059–69

Yamada A, Kitamura H, Yamaguchi K, Fukuzawa S, Kamijima C, Yazawa K, Kuramoto M, Wang GYS, Fujitani Y, Uemura D. (2000). Development of chemical substances regulating biofilm formation. Biology and Chemistry Society of Japan,70:3061-3069.

Yamaoka, N.; Suetomo, Y.; Yoshihisa, T.; Sonobe, S. (2016). Motion analysis and ultrastructural study of a colonial diatom, *Bacillaria paxillifer*. Microscopy, Oxford, England, 65(3), 211–21.

Yebra, D. M.; Kiil, S.; Dam-Johansen, K. (2004). Antifouling technology - past, present and future steps towards efficient and environmentally friendly antifouling coatings. Progress in Organic Coatings, 50 (2), 75-104.

Yebra, D. M.; Kiil, S.; Weinell, C. E.; Dam-Johansen, K. (2006). Presence and effects of marine microbial biofilms on biocide-based antifouling paints. Biofouling, 22(1-2), 33–41.

Yebra, D.M.; Weinell, C.E. (2009). Key Issues in the Formulation of Marine Antifouling Paints. In Advances in Marine Antifouling Coatings and Technologies Woodhead Publishing Cambridge, UK, 308–33.

Yellowlees, D.; Rees, T.A.W; Leggat, W. (2008). Metabolic interactions between algal symbionts and invertebrate hosts. Plant, Cell Environment. 31:679–694.

Yorisue, T.; Chan, B. K. K.; Kado, R.; Watanabe, H.; Inoue, K.; Kojima, S.; Høeg, J. T. (2016). On the morphology of antennular sensory and attachment organs in cypris larvae of the deep-sea vent/seep barnacles, Journal of Morphology, 277(5), 594–602.

Ytreberg, E.; Karlsson, J.; Eklund, B., (2010). Comparison of toxicity and release rates of Cu and Zn from anti-fouling paints leached in natural and artificial brackish seawater. Science of the Total Environment, (408), 2459-2466.

Yule, A.B. and Walker, G. (1984). The adhesion of the barnacle*, Balanus balanoides*, to slate surfaces. Journal of Marine Biology Association, 64, 146–156.

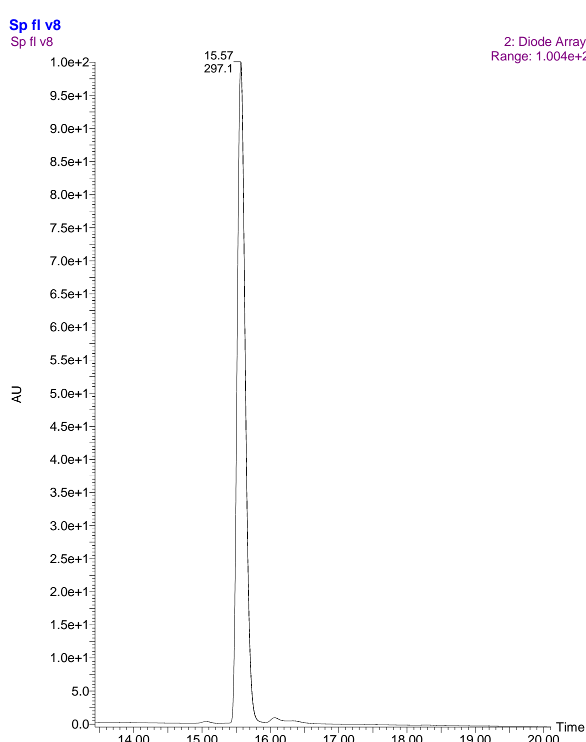
Zhang, H.; Wang, D.-Z.; Xie, Z.-X.; Zhang, S.-F.; Wang, M.-H.; & Lin, L. (2015). Comparative proteomics reveals highly and differentially expressed proteins in field-collected and laboratory-cultured blooming cells of the diatom *Skeletonema costatum*. Environmental Microbiology, 17(10), 3976–91.

Appendix

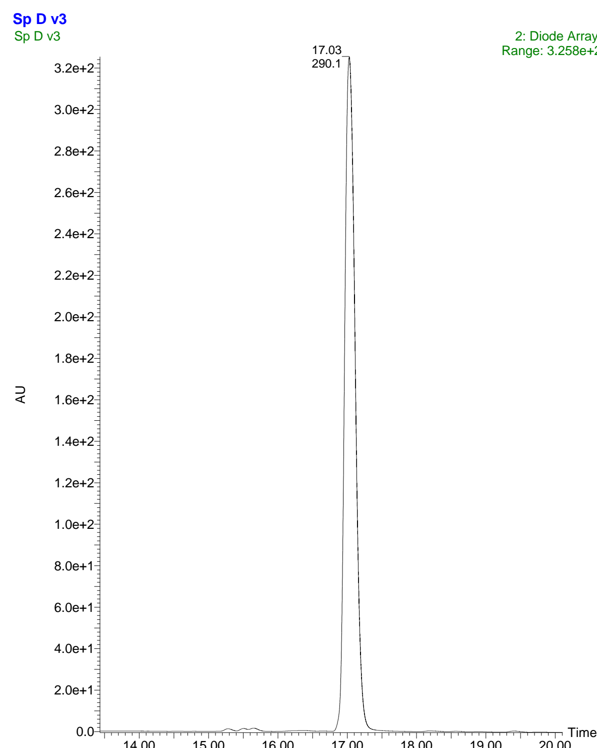
Appendix I

Supporting Information: Antifouling compounds from the sub-Arctic ascidian *Synoicum pulmonaria*: Pulmonarin A & B, Synoxazolidinone A & C and synthetic analogs

Figure S1. HPLC chromatograms of synoxazolidinone A & C (**I** & **II**)

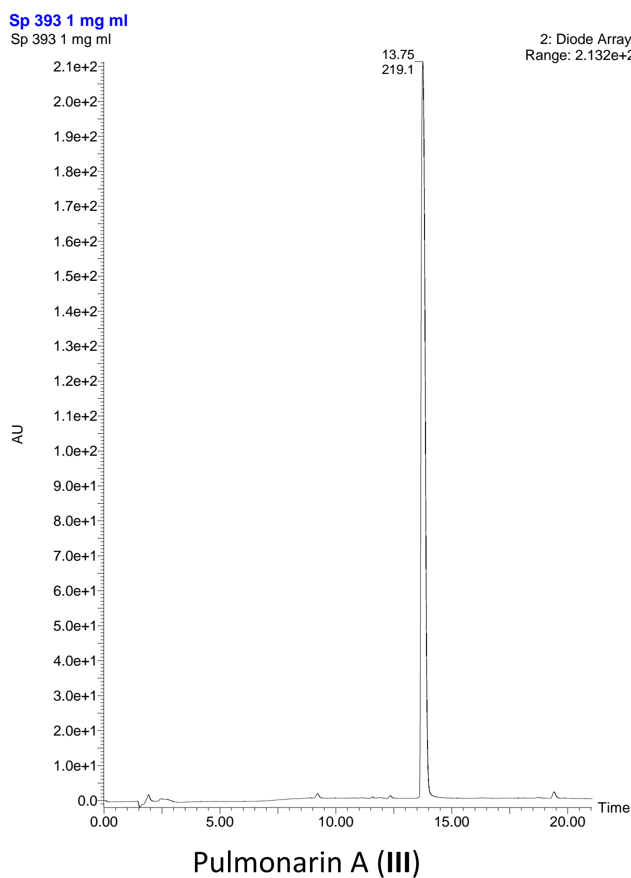


Synoxazolidinone A (**I**)

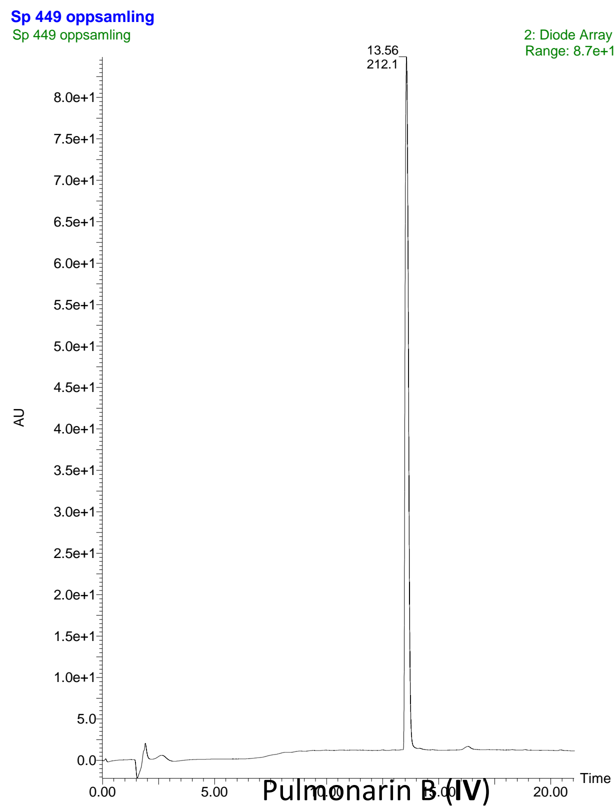


Synoxazolidinone C

Figure S2. HPLC chromatograms of Pulmonarin A & B (**III** & **IV**).

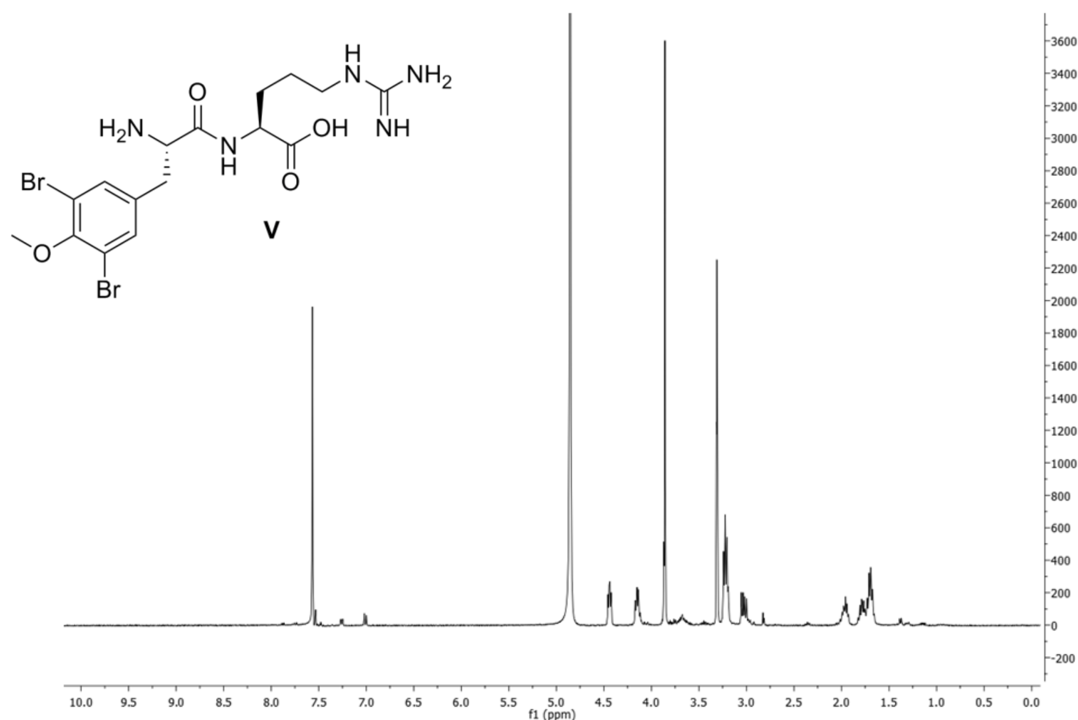


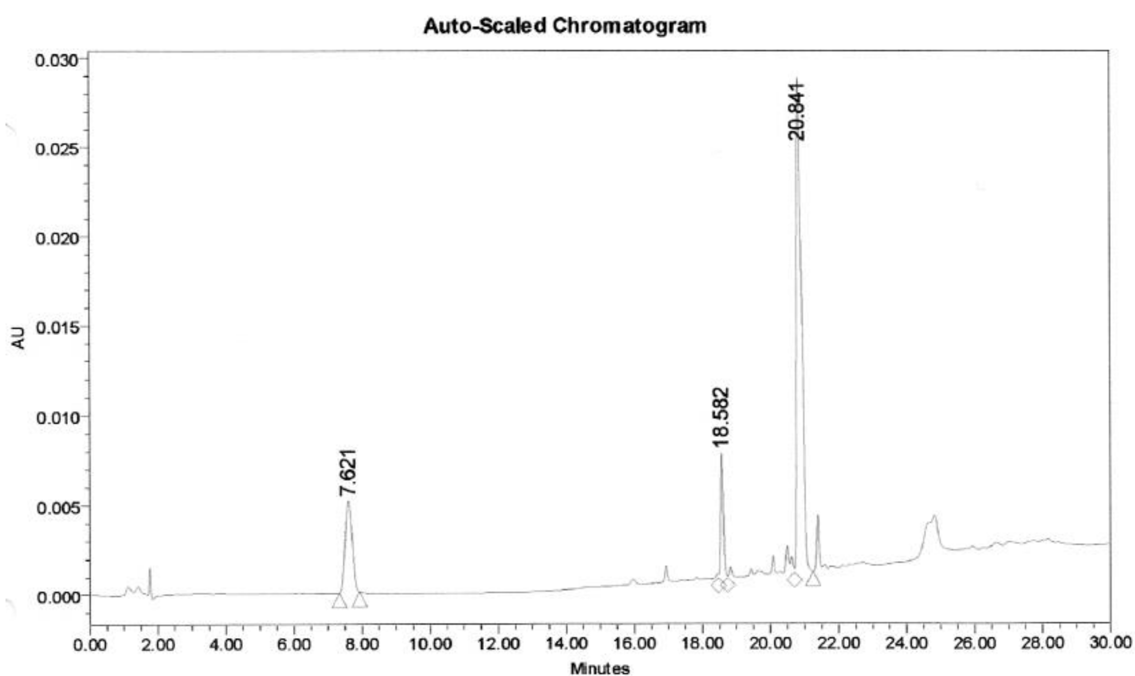
Pulmonarin A (**III**)



Pulmonarin B (**IV**)

Figure S3. ^{1}H-NMR spectrum and LC-trace of synthetic (**V**) in methanol-d_4.

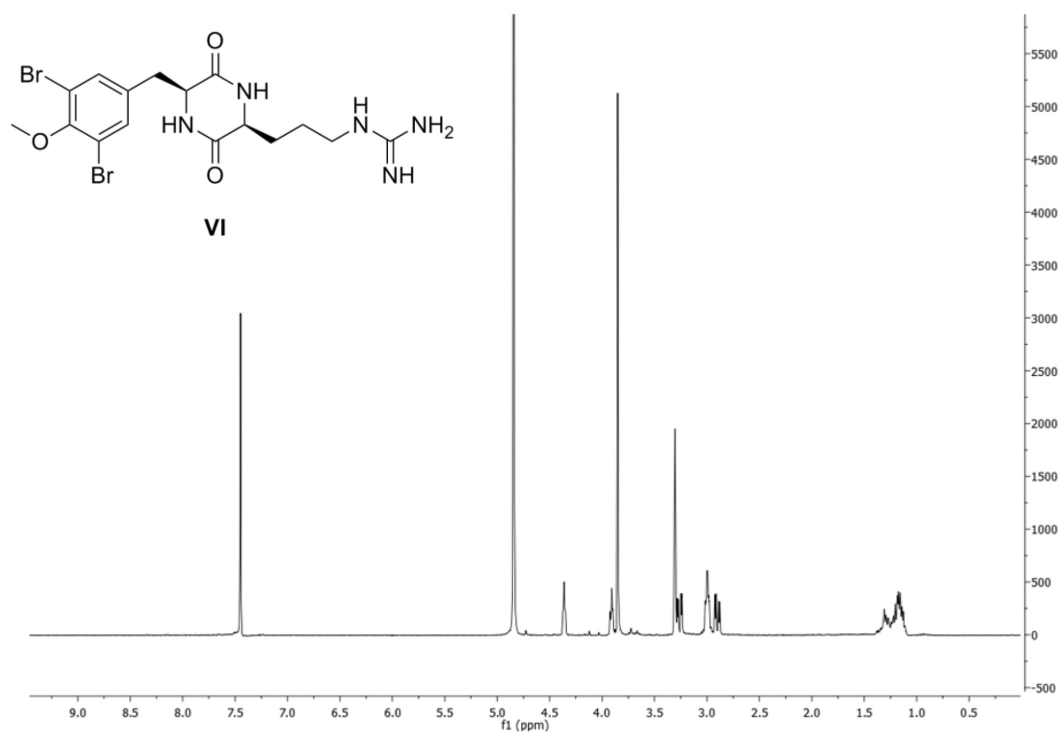




Note to analytical LC-trace and ^{1}H-NMR spectrum of **V**. Sample contains a small amount of residual HOBt giving rise to the minor upfield peaks at 7.02 and 7.26 ppm in the NMR

spectrum. The UV-absorbance of the compound is however strong and results in a significant peak at 7.62 min in the LC-trace (checked via co-injections). The peak at 18.58 min is not seen in the NMR.

Figure S7. ^{1}H-NMR spectrum of synthetic (**VI**) in DMSO-d_6.



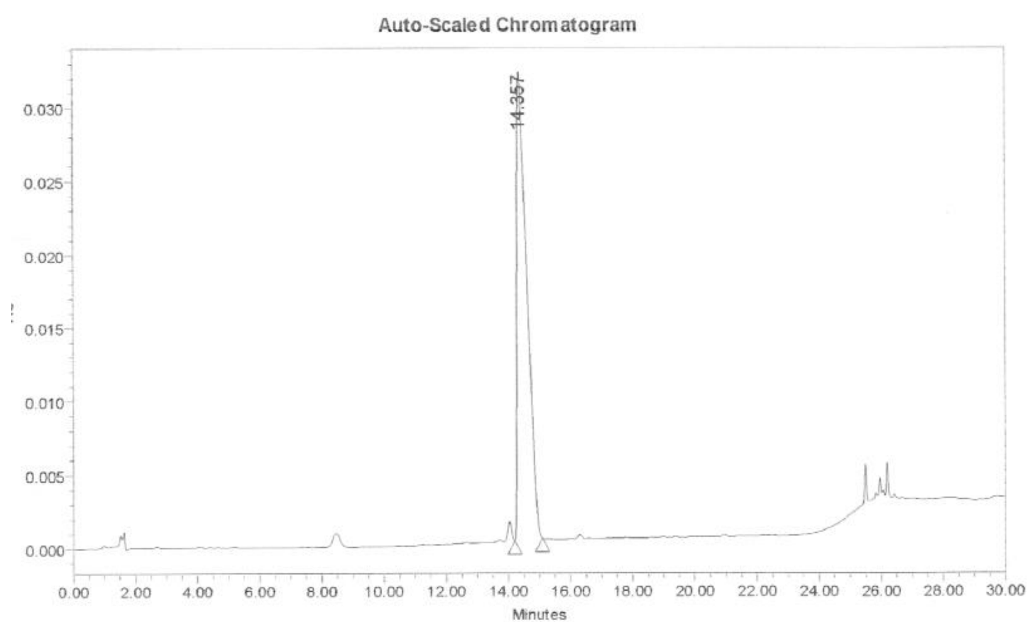
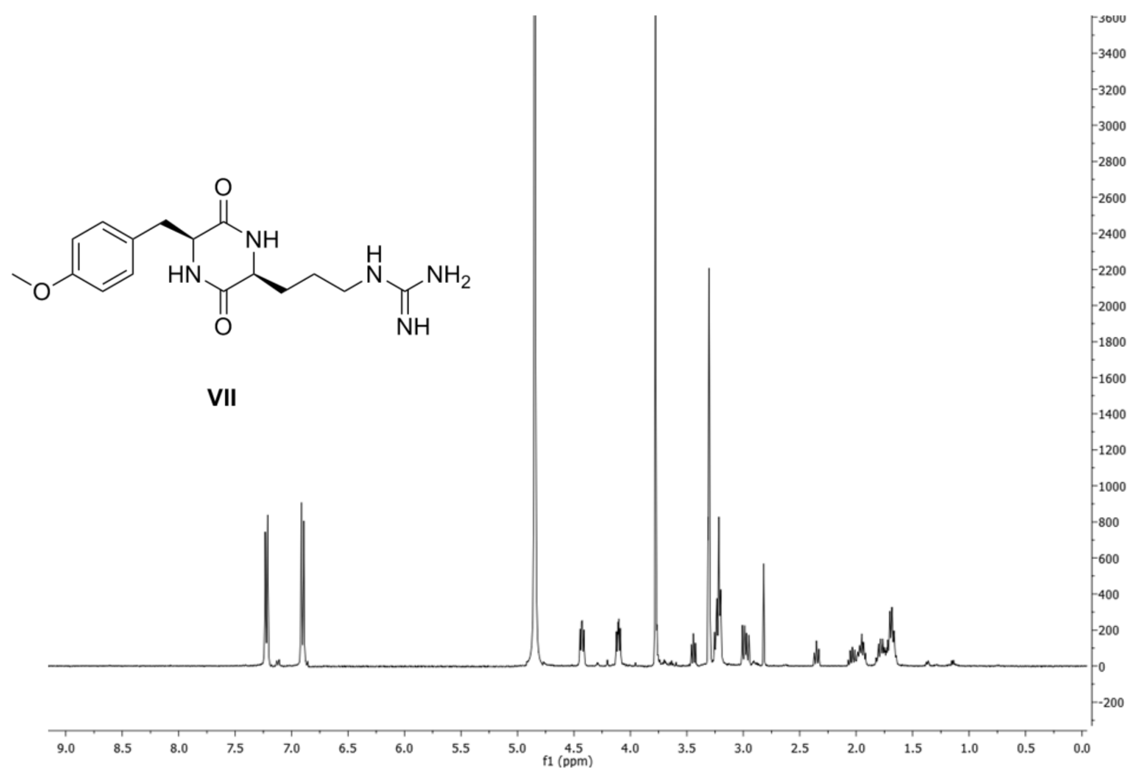
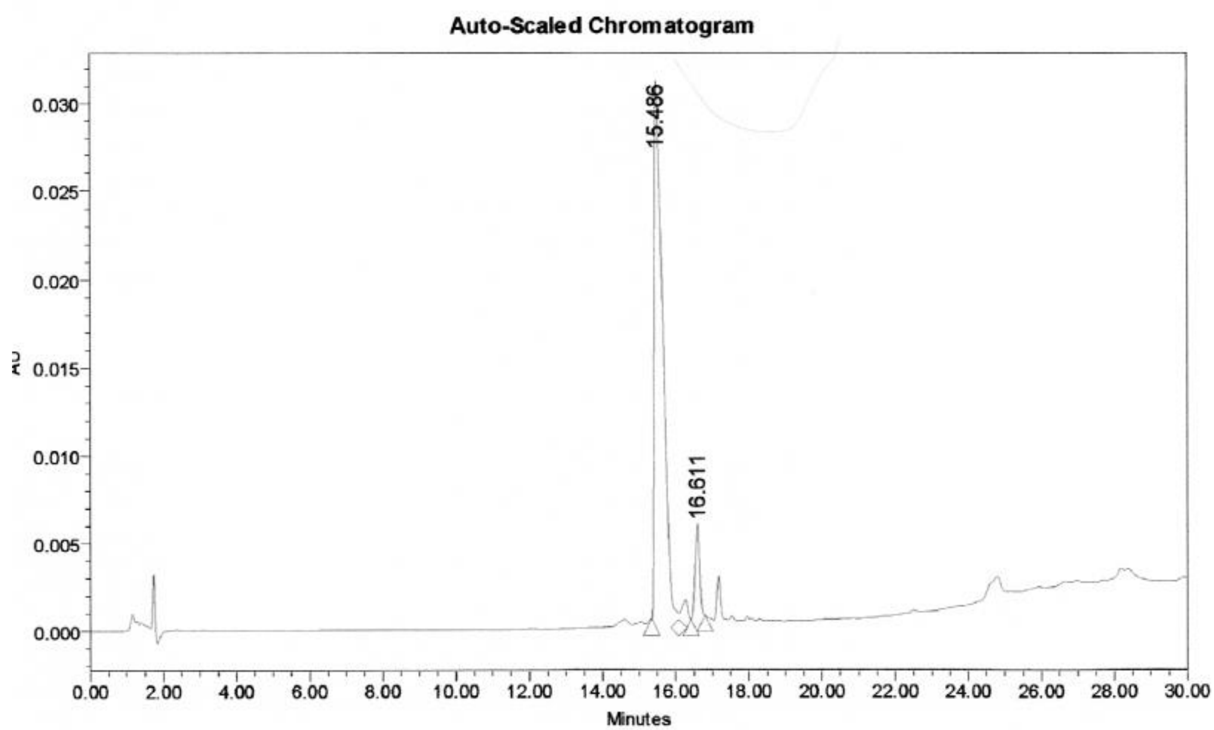


Figure S7. ^{1}H-NMR spectrum of synthetic (**VII**) in DMSO-d_6.





Note to analytical LC-trace and ^{1}H-NMR spectrum of **VII**. Sample contains a small amount of residual NMM giving rise to the peaks at 3.44, 2.82 and 2.35 ppm and the peak at 16.61 min. The NMM eluted too near the product peak to allow complete separation and isolation.