UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

CENTRE FOR BIOLOGICAL SCIENCES

Biofouling and its control for *in situ* lab-on-a-chip marine environmental sensors

by

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ABSTRACT

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES
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BIOFOULING AND ITS CONTROL FOR *IN SITU* LAB-ON-A-CHIP MARINE ENVIRONMENTAL SENSORS

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Biofouling is the process by which biological organisms attach to surfaces in an aqueous environment. This occurs on nearly all surfaces in all natural aquatic environments, and can cause problems with the functioning of scientific equipment exposed to the marine environment for extended periods. At the National Oceanographic Centre in Southampton (NOCS), the Centre for Marine Microsystems (CMM) is developing lab-on-chip micro-sensors to monitor the chemical and biological environment *in situ* in the oceans. Due to the long periods (up to several months) that these sensors will be deployed, biofouling by microbial biofilms is an important concern for the efficient running of these sensors. The aim of this project was therefore to determine the potential level of fouling within the sensors and to investigate the potential use of low-concentration diffusible molecules (LCDMs) to remediate biofouling.

Many of the sensors in development by CMM are designed to sense specific chemical species and they use various chemical reagents to achieve this. The effects of some of these reagents on the formation of biofilms by mixed marine communities were investigated. It was shown that Griess reagent and ortho-phthadialdehyde (OPA), used to sense nitrites and ammonium respectively, effectively stop biofilm formation by killing microorganisms before they can attach to surfaces.

Biofouling on two different polymers, cyclic olefin copolymer (COC) and poly (methyl methacrylate) (PMMA), used in the construction of micro-sensors, was compared with biofouling on glass. No differences were observed between COC and PMMA, however a small but significant difference in surface coverage was observed between glass and COC at the early stages of exposure to the marine environment. The lack of differences between the two polymers suggests that biofouling is not an important consideration when deciding whether to construct sensors from COC or PMMA. However, the larger degree of fouling on hydrophobic COC compared with hydrophilic glass indicates a potential use of surface modifications as an antifouling strategy.

The effects on biofouling of the LCDMs nitric oxide (NO), *cis*-2-decenoic acid (CDA) and patulin, were investigated to evaluate their potential for anti-fouling in marine micro sensors. All three molecules were shown to reduce the formation of biofilms by mixed marine communities, but colony counts suggested that the effect of patulin was due to toxicity as opposed to a physiological effect. Investigation of biofilm growth in the light and the dark revealed that there was less biofilm formation in the light that the dark and this effect was determined to be due to an interaction with the polystyrene growth substratum.

Analysis of the biofilm communities grown in the presence of LCDMs by denaturing gradient gel electrophoresis (DGGE), showed no clear differences in community profiles depending on the LCDMs. However those biofilms grown in the light appeared to have a greater proportion of Alphaproteobacteria than those grown in the dark.

Further study is needed to determine the level of fouling and the applicability of LCDMs in real micro-sensor systems. However, this study has shown that LCDMs have the potential to remediate, at least in part, the biofouling of marine micro-sensors.

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Declaration of authorship

I, David Ian Walker declare that this thesis entitled 'Biofouling and its control for *in situ* lab-on-a-chip marine environmental sensors' and the work presented in the thesis are both my own, and have been generated by me as the result of my own research. I confirm that:

- This work was done wholly while in candidature for a research degree at the university;
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution, this has been clearly stated;
- Where I have consulted the published work of other, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- None of this work has been published before submission.

Signed: …….

Date: ……

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And above all my profound thanks to my patient and understanding partner Mari Haughton.

List of abbreviations

AHL	-	Acyl homoserine lactones
AI-2	-	Autoinducer-2
ANOVA	-	Analysis of variance
ARDRA	-	Amplified rDNA restriction analysis
BDSF	-	*Burkholderia* diffusible signal factor
BLAST	-	Basic local alignment search tool
BSA	-	Bovine serum albumin
CDA	-	*cis*-2-decenoic acid
cdGMP	-	Cyclic di-GMP
CFU	-	Colony forming unit
CLSM	-	Confocal laser scanning microscopy
CMM	-	Centre for Marine Microsystems
COC	-	Cyclic olefin copolymer
CTAB	-	Cetyl trimethylammonium bromide
DAPI	-	4',6-diamidino-2-phenylindole
DDBJ	-	DNA databank of Japan
DGGE	-	Denaturing gradient gel electrophoresis
DIC	-	Differential interference contrast
DLVO	-	Derjaguin-Landau-Verwey-Overbeek
dsDNA	-	Double stranded DNA
DSF	-	Diffusible signal factor
EDIC	-	Episcopic differential interference contrast
EMBL-NSD	-	European Molecular Biology Laboratory nucleotide sequence database
EPS	-	Extracellular polymeric substances
FISH	-	Fluorescence *in situ* hybridisation
LCDM	-	Low-concentration diffusible molecule
MET	-	Marine exposure tube
MIC	-	Minimum inhibitory concentration
NED	-	N-(1-napthyl)ethylenediamine dihydrochloride
NOCS	-	National Oceanography Centre, Southampton
OPA	-	Orthophthadialdehyde
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
LCDM	-	Low-concentration diffusible molecule
PEG	-	Poly (ethylene glycol)
PET	-	Poly (ethylene terephthalate)
PFA	-	Paraformaldehyde
PMMA	-	Poly (methylmethacrylate)
PMP	-	Poly (methylpentene)
RDP	-	Ribosomal database project
SDS	-	Sodium dodecyl sulphate
SNP	-	Sodium nitroprusside
SSW	-	Sterile seawater
TAE	-	Tris base, acetic acid and EDTA
TBT	-	Tributyl tin
TGGE	-	Temperature gradient gel electrophoresis
T-RFLP	-	Terminal restriction fragment length polymorphism
UV	-	Ultraviolet
XDSF	-	*Xanthomonas* diffusible signal factor

Chapter 1 Introduction

1.1. Background

The oceans play a key role in the regulation of the Earth's climate (Bigg *et al.*, 2003, Field *et al.*, 2002). However, much of the oceans' biogeochemical processes remain under-sampled, making future predictions on the Earth's climate unreliable. Oceanographic surveys are limited to a relatively small scale due to the high costs of ship based water sampling (~£15k per ship, per day) or the opacity of deep seawater to electromagnetic radiation used for remote sensing. For this reason *in-situ* sensors have been identified as a solution to providing large scale data on biogeochemical processes in the world's oceans (Gallager and Whelan, 2003).

In situ sensors are used to characterise the physical (depth, salinity, temperature), chemical (pH, nutrient and other chemical concentrations) and biological (plankton density and species) environment and achieved this using several methodologies (Mills and Fones, 2012).

Conductivity, temperature and depth (CTD) are commonly detected simultaneously in a single device, which not only provides useful data in itself about the condition of the oceans, but also provides an invaluable reference for the physical environment in which other measurements are made. Oxygen saturation is also commonly measured using a Clark polarographic electrode, or more recently developed luminescent technology (Reimers, 2007).

Many other *in situ* devices have been created which are essentially miniaturisations of existing laboratory based methodologies. For example, Floch and colleagues (1998) developed a device to detect dissolved silicic acid in sea water by adapting the existing colorimetric methodology developed by Jolles and Neurath (1898). In this method the formation of yellow beta silicomolybdic acid when silicic acid is reduced in molybdenum blue is detected and quantified to determine silicic acid concentrations.

Plant and colleagues (2009) developed an in situ sensor to detect levels of ammonium by adapting the approach used by Hall and Aller (1992) in which the water the be analysed is adjusted to pH 10.5 to convert ammonium ions to ammonia gas. The gas diffuses across a gas permeable membrane into 50 μM hydrochloric acid where a reduction in conductivity is interpreted as the amount of ammonia which has diffused across the membrane. By using this method, Plant and colleagues were able to develop a sensor with a dection limt of 0.014 μM ammonium.

Many existing sensors are large "macro-sensors". These existing sensors are limited in the amount of data that can be collected, because their large size and cost make them both expensive and inconvenient to use (Schmidt *et al.*, 1991). A potential solution to this problem is to miniaturise sensors, reducing production costs and energy consumption. This will allow for many more sensors to be deployed simultaneously, thereby increasing the volume of data on the biogeographical processes which occur in the sea. Some commercially available miniaturised sensors already exist, such as the Star-Oddi (Star-Oddi, 2013) and Cefas G5 (CTL,

2011) fish tags and At the National Oceanography Centre in Southampton (NOCS), the Centre for Marine Microsystems (CMM) is developing such "micro-sensors".

Several sensors are in development, including those which detect specific chemical species such as nitrates, iron, ammonium and others, as well as sensors to identify the diversity of phytoplankton based on morphological and molecular markers.

	Argo CTD (large)	Star-Oddi fish tag	Cefas (G5 fish tag)	Goal for CMM project (fish tag size)
Conductivity (accuracy)	0.005 psu	0.75 psu	none	<0.01 psu
T (accuracy)	0.002°C	0.1°C	0.1°C	0.002°C

Table 1.1: Comparisons of some current sensor technologies shows that while it is possible to achieve high accuracy with existing technology, this comes at the cost of increased size. Conversely, existing small devices have low accuracy. Devices being developed by CMM aim to have high accuracy and small size.

The miniaturisation of these sensors will be achieved by creating microfluidic devices, through which seawater can be passed and analysed. Such devices commonly have fluid channels with diameters of less than 10 μm, and so it is important to reduce any possible source of blockage. One potentially major issue in the use of these sensors is biofouling, which could result in the blockage of the microchannels, as well as causing other potentially serious issues with sensor operation.

The aim of this project was therefore to first identify the potential extent of the biofouling problem for these sensors, then to test methods of biofouling remediation and investigate the effects that antifouling methods have on biofouling communities. The following chapters first discuss the importance of biofouling, how it occurs, how it can be quantified and analysed in more detail, and existing methods for biofouling remediation. It will then go on to discuss experimental work to address the problems of biofouling in marine microsensors.

1.2. Introduction to biofouling

Biofouling is the process in which biological organisms attach to surfaces in an aqueous environment (Kerr *et al.*, 1998). This occurs on nearly all surfaces in all natural aquatic environments. Biofouling can be broadly broken into two categories, macro-biofouling and micro-biofouling. Macro-biofouling occurs when macroscopic organisms such as algae and invertebrates attach to a surface. This is often preceded by micro-biofouling, where microorganisms attach to a surface (O'Connor and Richardson, 1998). Once attached, these microorganisms form an extracellular support matrix. The combination of the cells and the

matrix is known as a biofilm, and is believed to be the most common form in which bacteria exist in the environment (Lappin-Scott and Costerton, 1995).

Macro-foulers settle on biofilms for many reasons. For example, biofilms may alter the physical properties of a surface, making it easier to colonise (Gray *et al.*, 2002). The presence of a biofilm can increase the stability of attachment for macro-foulers in comparison to a clean surface (Zardus *et al.*, 2008). Additionally, it has been speculated that the presence of a biofilm indicates a surface that is has been immersed for a long period and is therefore in a non-toxic environment (Johnson *et al.*, 1997).

The settlement of invertebrate larvae and algal spores is meditated in part by the production of settlement signals by microbial biofilms. Harder et al. (2002) showed that extracts of metabolites from marine bacterial species increased the settlement of the tubeworm *Hydroides elegans* larvae and a study by Joint et al. (2002) showed that bacterial quorum sensing molecules act as a cue for the settlement of the spores of the green alga *Enteromorpha*.

1.3. Importance of marine biofouling

Biofouling occurs on most surfaces that are immersed in a non-sterile aqueous environment. This makes biofouling an important consideration for medicine, where bacterial biofilms that form both on biological surfaces and non-biological implants such as catheters, dental implants and ventricular assist devices can be very difficult to remove, and so cause severe reoccurring infections (Costerton *et al.*, 2005, Costerton *et al.*, 1999).

Another major area of research is that of environmental biofilms. It has been suggested that the default state of many bacterial species is in a biofilm as opposed to in a free-swimming form as previously thought (Costerton *et al.*, 1995, Ellwood *et al.*, 1982, Jefferson, 2004). Therefore any attempt to understand the ecology of an ecosystem is not complete unless biofilms are considered.

The marine environment is a particularly important environment for biofilms. With around two thirds of the Earth's surface covered with oceans, the seafloor represents a vast area over which biofilms can form.

New habitats are created for marine biofilms when artificial structures such as ships, drilling rigs and sensing equipment are put into the sea. The resulting biofilm formation can have considerable impact on the functioning of marine equipment. Biofilms alone can increase fuel consumption of naval vessels by around 10% (Haslbeck and Bohlander, 1992, Schultz *et al.*, 2011) due to increased water resistance. If macrofouling is allowed to develop, the additional fuel consumption can rise to around 20% (Schultz *et al.*, 2011).

This project is concerned with biofouling inside the thin channels of lab-on-chip oceanographic sensors, through which chemical reagents and seawater flow and mix (Figure 1.1). The constant presence of seawater means that the build-up of biofilms in these channels is inevitable.

Biofilms range in thickness from around one or two microns up to many millimetres (Wimpenny *et al.*, 2000), and in the thin channels of the sensors (often as thin as 10 μm) biofilms may drastically reduce the width of the channels, reducing flow rates. Additionally, this biofilm growth has the potential to stop all flow through sensor channels thus rendering sensors inoperable (Drescher *et al.*, 2013).

A biofilm's mass and metabolism makes it difficult to maintain a constant set of experimental conditions. This means that data recorded at different times are not reliably comparable. Biofilms are known to alter the composition of metal compounds in water systems by immobilising ions in the EPS or cellular component, as reviewed by van Hullebusch and colleagues (2003). The composition of biofilm communities has the potential to affect the chemical environment. Iron reducing bacteria such as *Shewanella* or *Geobacter* species (Caccavo *et al.*, 1992, Coates *et al.*, 1996, Straub and Buchholz-Cleven, 2001) may change the proportion of ferric iron (Fe^{3+}) and ferrous iron (Fe^{2+}); while bacteria involved in nitrogen cycling such as the nitrogen fixer *Vibrio diazotrophicus* or the nitrifying *Nitrospira* and *Nitrococcus* genera may change the relative proportion of nitrogen species (Guerinot *et al.*, 1982, Watson and Waterbury, 1971) leading to false data from the sensors.

Another problem caused by biofilms in the sensors is that of reduced or otherwise altered light transmission. Most of the CMM sensors rely on the detection of light for their measurements. As biofilm accumulates on a surface, it absorbs more light (Bakke *et al.*, 2001). In a study by Marrs and colleagues (1999) where marine biofilms were grown on glass coupons, up to 65% of the light shone on the samples was absorbed by the biofilm. Therefore biofilms growing at the points where light is detected or transmitted into the sensors may alter the results obtained.

One of the sensors in development by the CMM is a micro-fluidic flow-cytometer which will be used for *in situ* sampling and identification of phytoplankton using a combination of optical and electrical measurements. Biofilm accumulation effectively concentrates microorganisms, which may be a major concern for the design of the cytometer. Biofilms are dynamic systems, where cells are constantly multiplying and detaching (Stoodley *et al.*, 2001b). Thus, presence of a biofilm in the cytometer will potentially alter the detected community drastically, with a bias towards the biofilm community.

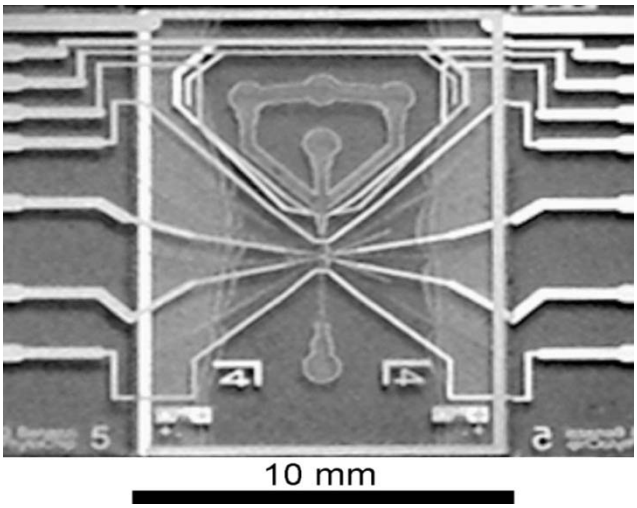


Figure 1.1: Prototype of a cytometer, one of the lab-on-a-chip sensors being developed by the CMM group at NOCS. The light areas represent channels through which water will pass during its operation. These channels are thin and may become blocked with biofilm.

1.4. Colonisation and development of biofilms on surfaces

The formation of biofilms on a surface is a highly regulated process which tends to follow a series of stages (Characklis, 1990). This lifecycle is outlined in Figure 1.2 and is discussed in more detail in the following sections

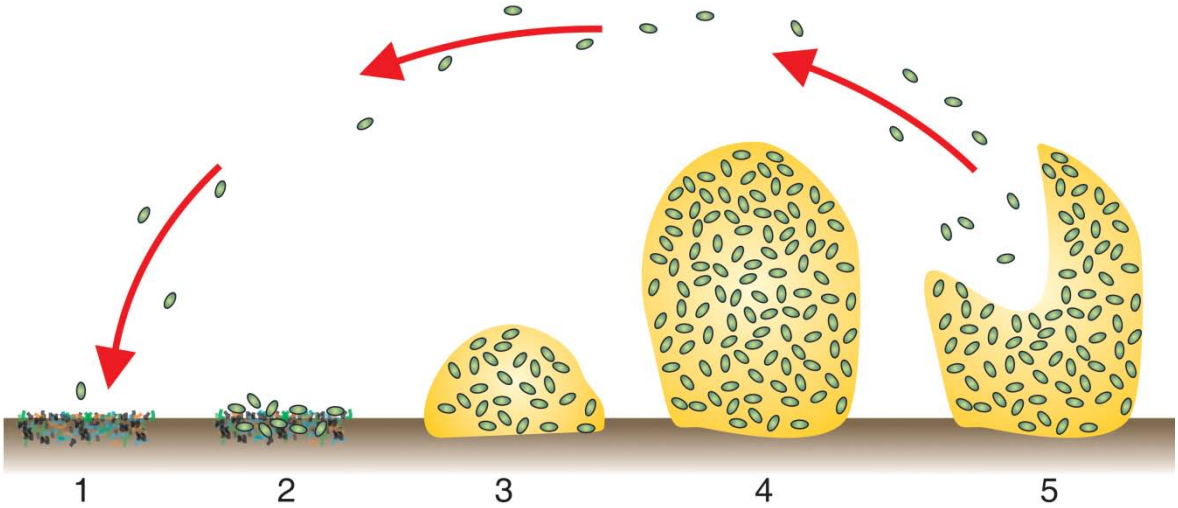


Figure 1.2: Biofilm lifecycle, adapted from Monroe (2007). (1) A conditioning film first forms on the surface. (2) Cells then attach to the surface. These cells then (3) start to form structured micro-colonies which (4) mature and (5) disperse cells back into the planktonic phase.

1.4.1. Conditioning film

When a clean surface is immersed in water, it is quickly coated in a thin film of organic molecules. This is known as the conditioning film and is usually made up of substances such as polysaccharides, proteins, lipids, humic acids, nucleic acids and amino acids (Siboni *et al.*, 2007). A study by Jain and Bhosle (2009) showed that without the conditioning film, the number of bacterial cells that attach to a surface is significantly reduced. They also found that depending on the composition of the conditioning film, different species were more successful at attachment. For example it was found that the concentration of carbohydrates in a marine conditioning film was positively correlated to *Pseudomonas* species attachment, but negatively correlated to *Bacillus* species attachment. This may have been a result of the differences in cell hydrophobicity between the different species (Jain and Bhosle, 2009) or due to chemical attraction or repulsion (Chet *et al.*, 1975, Gubner and Beech, 2000). Other research suggests that proteins forming part of the conditioning film can competitively inhibit attachment of bacterial cells by occupying suitable binding sites on the attachment surface (Helke *et al.*, 1993). However many of the molecules (such as proteins) present in a conditioning film are made up of long chains of monomers which themselves may provide additional binding sites for microorganisms (Lappin-Scott and Costerton, 1995).

As well as providing attachment sites for microorganisms, the conditioning film may increase attachment by altering the physical properties of the attachment surface. Such properties include surface tension, surface free energy and surface roughness (Schneider, 1996).

1.4.2. Initial attachment

The ability of a cell to come in to contact with a surface in order to attach to it depends in part on a combination of repulsive electrostatic forces and attractive van der Waals forces between the cell and the attachment surface as described by the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloid stability (Derjaguin and Landau, 1941, Verwey and Overbeek, 1948). This theory was first applied to the study of bacterial attachment to surfaces by Marshall and colleagues (1971) and continues to be applied to explain bacterial adhesion to surfaces (Hwang *et al.*, 2012, Lerner *et al.*, 2012). When a material is immersed, Coulomb interactions between charged molecules in the material and those in solution result in a region of transiently bound counter-ions at the surface of the material. This is known as the Stern layer. With increasing distance from the Stern layer, interactions between incompletely neutralised surface ions and ions in solution create a diffuse electric double layer. The repelling force which limits bacterial attachment is caused by repulsive osmotic pressure between the overlapping double layers of the attachment substratum and the bacterium surface.

When considering a cell's attachment to a surface, DLVO theory is complicated by changes in ion concentration and pH, caused by biogenic substances from the cell. In addition, the DLVO theory describes the particle as a sphere, where an increased radius results in a higher degree of repulsion. The shape of a cell is often not spherical, depending on species and nutrient depletion (Slonczewski and Foster, 2009), and surface appendages complicate this even further. However, the theory is still useful for explaining general trends in attachment.

DLVO theory can also help to determine why microorganisms have evolved certain characteristics. A study by Feldner and colleagues (1983) showed that *Mycoplasma pneumoniae*, a bacterium with no rigid cell wall, treated with glucose attached more readily to a glass surface than those not treated with glucose. It was found that as *M. pneumoniae* approached the glass surface they became elongated and thin (Figure 1.3) and so were able to penetrate the repulsive forces of the two surfaces. Those that had not been treated with glucose did not change shape so readily, indicating that this process requires a large amount of energy. It is hypothesised that these bacteria have evolved to elongate in this way to reduce the radius of part of the cell and so be able to penetrate the repulsive electrostatic layer (Feldner *et al.*, 1983).

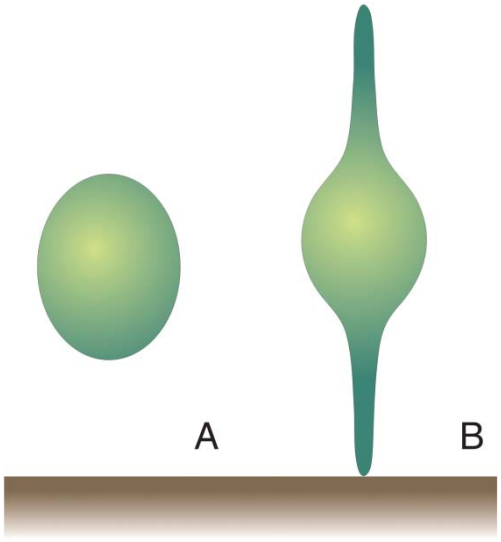


Figure 1.3: As *Mycoplasma pneumoniae* approaches a surface it does not change shape in the absence of glucose (a) but does when glucose is present (b) This is probably an adaption to penetrate the electrostatic boundary. Adapted from Feldner and colleagues (1983).

Initial attachment of microorganisms to surfaces is also affected by the system's free energy (Palmer *et al.*, 2007). In order to measure the free energy of a surface the contact angle between a droplet of liquid (or a gas bubble) and the surface is measured (Figure 1.4). When the free energy is low, the contact angle will be greater (Genzer and Efimenko, 2006). In general, a surface with a contact angle of greater than sixty degrees is said to be hydrophobic (Vogler, 1998). Inversely, a surface with a contact angle of less than ninety degrees is said to

be hydrophilic. If the system's free energy is reduced when a cell contacts a surface then it will remain attached (Genzer and Efimenko, 2006). This means that a surface whose free energy is already low, and is therefore hydrophobic, will in theory have a greater potential to adsorb microorganisms than one with high surface free energy.

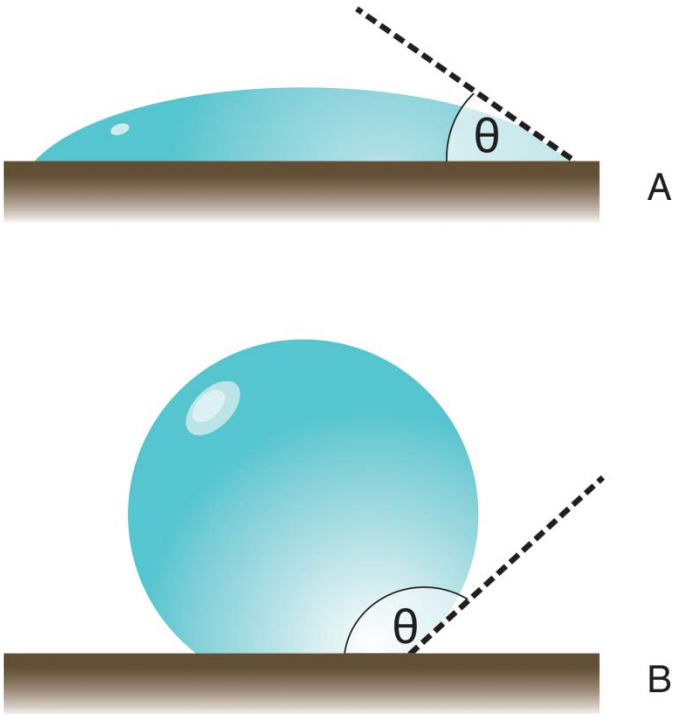


Figure 1.4: The contact angle (θ) of a water droplet on a surface gives a measure of its free energy, with those with a high free energy and a contact angle lower than 90° being referred to as hydrophilic (A) and those with a low free energy and a contact angle of greater than 90° being referred to as hydrophobic (B).

Baier (1970) showed that surfaces with a free energy level of between 20 and 30 $mJ.m^{-2}$ resulted in low biofilm formation, whereas those surfaces with a surface free energy of below 20 $mJ.m^{-2}$ and above 30 $mJ.m^{-2}$ demonstrated greater biofilm formation. However, there is some doubt over the validity of these findings, as the chemistry of the surfaces studied varied greatly. This introduces other chemical factors which may be directly responsible for the differences seen by Baier.

Wienck and Fletcher (1997) looked at the adhesion of *Pseudomonas* sp. to two similar long-chain alkanethiols with differing hydrophobicities. They found that bacteria attached much more readily to surfaces composed of the hydrophobic undecanethiol than to the hydrophilic mercaptoundecanol. This indicated that where materials have similar chemistries, an increase in hydrophobicity will increase the fouling potential of a surface.

1.4.3. Biofilm Development

Within a few minutes of a cell's attachment to a surface, genes that produce extracellular polymeric materials are often upregulated (Nobile and Mitchell, 2005) increasing the production of materials which cement the cell to the surface, thereby increasing the strength of attachment. These adhesins often take the form of thin, thread-like structures known as fimbriae in bacteria, or hyphae in fungi. In the fungal species *Candida albicans*, attachment to a surface triggers the up-regulation of the *tec1* gene (Blankenship and Mitchell, 2006). *Tec1* mutants form poor biofilms which cannot adhere well to a surface. This is because *Tec1* is part of the pathway for forming hyphae, which help the fungus adhere to the surface (Nobile and Mitchell, 2005).

Once cells have adhered to the surface, they often proliferate by binary division to form cells clusters which spread over the surface (Lappin-Scott and Costerton, 1995). As the initial colonisers grow and divide, they produce more polymeric substances. This extracellular polymer layer traps additional organic and inorganic substances (Lappin-Scott and Costerton, 1995). Other microorganisms as well as daughter cells from the primary colonisers join these substances to form a matrix, referred to as extracellular polymeric substances (EPS). The EPS is usually composed of fibrous polysaccharides, proteins, extracellular DNA, phosphorous, monosaccharides, hexosamines and other substances (Baillie and Douglas, 2000). This composition can vary however depending on nutrient availability (Myszka and Czaczyk, 2009). It is possible that EPS serve several purposes including defence against predation; support for the biofilm structure; and limitation of the diffusion of toxic compounds(Baillie and Douglas, 2000, Costerton *et al.*, 1995).

Some monospecies biofilms may grow as heterogeneous, mushroom shaped micro-colonies over a surface as opposed to a flat, homogenous layer (Lawrence *et al.*, 1991), while other monospecies and polymicrobial biofilm communities grow as stacks of aggregated micro-colonies (Rogers *et al.*, 1991). Between these micro-colonies channels exist, through which fluids can flow. This increases the amount of oxygen and nutrients that can be accessed by the biofilm (de Beer *et al.*, 1994), although oxygen concentration remains low inside the microcolonies relative to the outside (Robinson *et al.*, 1995). It is within these micro-colonies that different species will be found at different levels. Research by Davey and colleagues (2003) suggests that the channels between the colonies are maintained by rhamnolipid surfactants in *Pseudomonas aeruginosa* biofilms.

Many studies have been conducted to look at the role of flagella on the development of bacterial biofilms (Delpin *et al.*, 2000, Hossain and Tsuyumu, 2006, Kim *et al.*, 2008, Lemon *et al.*, 2007, O'Toole and Kolter, 1998a, Watnick *et al.*, 2001). In several mutant strains that either have no flagella or non-moving flagella, development of biofilms is severely altered. For

example, Delphin and colleagues (2000) compared the development of biofilms by wild type marine bacteria *Vibrio* sp. S141 and the M8.2 (flagellar mutant) strain of the same species. When grown under the same conditions (on glass in flowing media) it was found that while the wild type bacteria produced mushroom shaped microcolonies, the M8.2 strain did not and instead formed a homogenous layer of cells across the substratum. This indicates that flagella play an important role in biofilm development after initial attachment of cells.

1.4.4. Biofilm dispersal

Part of a biofilm's lifecycle is the dispersal of cells in order for them to colonise new surfaces. These dispersal events are triggered by physical processes such as shearing that result in sloughing or erosion of clusters of cells (Stoodley *et al.*, 2001a, Stoodley *et al.*, 2001b) or genetically controlled processes that result in the release of individual cells (Webb, 2006).

Genetically controlled dispersal (herein referred to as dispersal) has been observed by biofilms formed by the marine bacterium, *Pseudoalteromonas tunicata* (Mai-Prochnow *et al.*, 2004), as well as species that are found both in marine and freshwater environments (McDougald *et al.*, 2007, Thormann *et al.*, 2006). Such dispersal events may be triggered by several environmental cues. For example it has been shown that nutrient starvation can induce dispersal in *P. aeruginosa* biofilms (Gjermansen *et al.*, 2005, Hunt *et al.*, 2004). Conversely, Sauer and colleagues (2004) showed that rapidly increasing nutrient availability can also induce dispersal in *P. aeruginosa*, likely a consequence of the planktonic life mode becoming more beneficial.

The regulation of such dispersal events is regulated by several inter- or intra-cellular signalling mechanisms including quorum sensing systems (Davies and Marques, 2009, Rice *et al.*, 2005) intracellular cyclic-di-GMP (cdGMP) (Simm *et al.*, 2004) and production of free radicals (Barraud *et al.*, 2006, Barraud *et al.*, 2009b). These signals may ultimately give rise to dispersal by triggering cells to produce enzymes such as polysaccharide lyases that breakdown components of the EPS (Ott *et al.*, 2001) and surfactants that loosen cells from the biofilm (Davey *et al.*, 2003).

Populations of dispersing biofilm also demonstrate high levels of peroxynitrite ($ONOO^-$), a chemical which is produced when nitric oxide (NO) and superoxide (O_2^-) are combined (Beckman and Koppenol, 1996). In addition, Barraud and colleagues (2006) have shown that introducing low concentrations of NO to a biofilm can trigger a dispersal event. The levels of NO required to trigger dispersal are in the non-toxic, nano-molar range, indicating that NO acts to modify the physiological functioning of the biofilm as opposed to inducing cell death. This hypothesis was supported by evidence that Δ*nirS* mutants of *P. aeruginosa* (which lack the ability to produce metabolic NO) were unable to disperse from biofilms.

The role of NO in biofilm dispersal has been linked to the cyclic-di-GMP (cdGMP) intracellular signalling system (Barraud *et al.*, 2009a). It was shown that NO stimulates the activity of phosphodiesterases and decreases the activity of diguanylate cyclases which are involved in the degradation and synthesis of cdGMP respectively. Therefore the introduction of NO decreased the levels of cdGMP and in turn increased dispersal. Using microarray analyses, Barraud and colleagues (Barraud *et al.*, 2009a) found that NO altered the expression of several genes involved in biofilm formation and dispersal of biofilms.

1.4.5. Quorum sensing

The development of a biofilm is a highly regulated process, for which communication between cells is essential. Bacteria and other microorganisms use diffusible molecules to communicate with members of the same or different species in a process known as quorum sensing (Fuqua *et al.*, 1994). This was first seen in the Gram-negative bacterium *Vibrio fischeri* (Nealson and Hastings, 1979) which is associated with the light producing organ of *Euprymna scolopes*, a small sepiolid squid found around the Hawaiian Islands. *V. fischeri* produces acyl homoserine lactones (AHLs), which once at a threshold concentration (achieved by having a high density population) are bound by the LuxR protein. This complex activates luciferase production, which oxidises luciferin molecules to produce light (Stevens *et al.*, 1994). In this situation, it would be inefficient for *V. fischeri* to produce luciferin at a low population density, as the light emitted would be so dim. It is only at high population densities that the production of luciferase becomes beneficial and so they have evolved a quorum sensing system able to detect population density.

It has since been found that quorum sensing is common in microorganisms (Brown and Johnstone, 2001). There are several biological processes that are regulated by quorum sensing including biofilm formation (Davies *et al.*, 1998), and its nature may vary between species. Some processes are regulated by chemicals that are only detectable by organisms of the same species. Due to the high specificity of these chemicals, there are several types of compound used by different groups. Gram-positive bacteria commonly use oligopeptides as intraspecific signalling molecules (Greenberg, 2003). Oligopeptides regulate metabolic processes by initiating a phosphorylation cascade that changes the activity of transcription factors (Waters and Bassler, 2005). Gram-negative bacteria mostly use AHLs for intraspecies communication (Manefield and Turner, 2002), however some have also been shown to use autoinducer-2 (AI-2), which is usually associated with interspecies communication (Hooshangi and Bentley, 2008).

While some species can form biofilms even with a reduced capacity for quorum sensing, the biofilms that are produced are less stable (Greenberg, 2003). A study by Sakuragi and Kolter (2007) showed that *las* mutants of *P. aeruginosa*, which do not produce the quorum

sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone, produce smaller biofilm colonies than do wild type *P. aeruginosa*. This has been linked with a reduction in the expression of the *pel* gene cluster, which encodes proteins responsible for the production of EPS (Friedman and Kolter, 2004).

C. albicans produces a quorum sensing molecule known as farnesol (Ramage *et al.*, 2002). When farnesol is present in high concentrations, the expression of genes important in hyphal production is reduced (Cao *et al.*, 2005), resulting in less biofilm formation. This allows *C. albicans* to regulate biofilm formation and aid its dispersal (Greenberg, 2003).

The majority of quorum sensing studies that have been conducted have looked at medically important species. However, studies have also shown that quorum sensing is important in marine biofilms. Huang and colleagues (2007) reported the presence of AHLs in marine biofilm communities grown in tropical subtidal waters. This was done using a *Chromobacterium violaceum* reporter strain which produced a purple pigment in the presence of short chain AHLs, and an *Agrobacterium tumefaciens* reporter which produced a blue pigment in the presence of long chain AHLs. It was found that only short chain AHLs were present in 2-day old biofilms.

1.4.6. Environmental influences on biofilms

The sensors in development by CMM will be deployed in various locations throughout the oceans and will therefore encounter many different environmental conditions. The variations in environmental conditions will affect not only the species of fouling organisms that are present in the water column, but also the physical characteristics of any biofilms that form.

1.4.6.1. pH

The average pH of the open ocean is just over pH 8 (Hofmann *et al.*, 2011). However, due to ocean acidification, oceanic pH is likely to decline in the coming decades (Doney *et al.*, 2009). According to Hofmann and colleagues (2011), the pH of the oceans varies depending on local environmental conditions. For example while in the open ocean pH remains relatively constant over time, at upwellings, estuaries and CO2 vents, pH may vary between pH 8.2 and pH 7.0. These changes in pH are likely to affect the biofilms that grow in any sensors deployed in these types of environments. While no studies could be found that linked a change in ocean pH with marine biofilm formation, Lianou and Koutsoumanis (2012) showed that *Salmonella enterica* biofilm formation had a general decline with decreasing pH (pH 7.0 to pH 3.8). There was also a large amount of variation in biofilm formation across different strains of *S. enterica*. It is likely that a shift in pH will alter the community composition of

marine biofilms as conditions become more favourable for different species. However, the effect of pH on the biomass of marine biofilms remains to be determined.

1.4.6.2. Salinity

Salinity has been shown to alter the formation of biofilms in several studies (Choi *et al.*, 2013, Lianou and Koutsoumanis, 2012, O'Toole and Kolter, 1998b, Qurashi and Sabri, 2012). In general, biofilm formation appears to be reduced with increasing salinity. However, increased salinity often increases the production of EPS (Mishra and Jha, 2009, Qurashi and Sabri, 2012), presumably to act as a buffer to the increased osmolarity.

Those species adapted to live in relatively low salt concentrations may not be able to survive in high salt environments, halophilic species such as *Halomonas meridiana* show high levels of biofilm development at salt concentrations as high as 58 ppt, compared with the average oceanic salinities of around 37 ppt (Qurashi and Sabri, 2012).

deFran (de França *et al.*, 2000) and colleagues showed that the relative numbers of different groups of bacteria grown on steel surfaces changed with increasing salinity. While aerobic and anaerobic bacteria CFU counts decreased with increased salinity, counts of iron-reducers increased. This is likely due to iron-reducing bacteria gaining a completive advantage over other types of bacteria in increased salinity.

1.4.6.3. Temperature

Several studies have shown that an increase in temperature will increase the formation of biofilms (Lianou and Koutsoumanis, 2012, Rao, 2010, Santos Mendonça *et al.*, 2012, Stratil *et al.*, 2013) up to a certain point where further temperature increases are inhibitory. However, Villanueva and colleagues (2010) showed that while riverine biofilms formed at higher temperatures (average 12.2°C vs average 15.4°C) accumulated faster and were thicker than those formed at low temperatures, temperature did not affect the final biomass. This indicated that higher temperature biofilms were less dense than low temperature biofilms.

The species present within a biofilm are also affected by temperature. Rao (2010) investigated biofilms formed near the waste water outlet for a nuclear reactor. Here the water temperature varied between 28.5°C and 35.7°C. Those biofilms formed at higher temperatures had higher levels of bacteria and lower levels of diatoms than at lower temperatures. In contrast, Villanueva (2010) showed that there was an increased level of micro algae relative to bacteria in higher temperatures (15.4°C) than at low temperatures (12.2°C). The differences between these studies is likely due to the increased range of environmental conditions in which bacteria as a group can flourish, and Villanueva's study was conducted closer to the ideal growth conditions for eukaryotic microorganisms.

Stratil and colleagues (2013) studied the effects of temperature on epiphytic biofilm communities forming on brown algae using next generation sequencing. They found that with an increase in temperature, around 50% of the species present in biofilms increased in relative abundance, 40% of species decreased and around 10% maintained the same relative abundance. In addition, the species richness was lowest at low temperatures (5°C), increased at moderate temperatures (15°C) and decreased slightly at higher temperatures (25°C). This indicates that temperature has important effects on biofilm communities, which may have implications on the application of antifouling strategies.

1.4.6.4. Hydrodynamics

The flow of water through a system affects the formation and development of biofilms broadly in three ways: transport of colonising microorganisms to a surface, transport of solutes to and from biofilms and frictional forces (causing changes in shape and shearing).

The Reynolds number is used in the study of hydrodynamics to characterise the relationship between viscous and inertial forces in a system. A system with a small flow diameter and high flow velocity has a high Reynolds number and conversely, a system with a large flow diameter and low velocity has a low Reynolds number. In lab-on-chip sensors the Reynolds number is an important parameter, as it affects the way in which reagents are distributed as well as determining whether flow is turbulent or laminar (which is particularly important in flow-cytometry). Additionally, the Reynolds number will have a significant impact on the formation of biofilms on the surface of sensor channels. In a low velocity flowing environment, there will be fewer potentially biofilm forming bacteria flowing through the system. However, those that come into contact with a surface may be more likely to be able to permanently attach without being swept away.

With an increase in flow velocity, solutes such as oxygen and nutrients are replenished and waste products are removed at a higher rate, allowing micro-organisms within a biofilm to grow and multiply at a faster rate. Additionally, at higher Reynolds numbers the mass transfer coefficient between the bulk fluid and the biofilm is increased, allowing increased transport of solutes into and out from the biofilm (Mašić *et al.*, 2010, Rasmussen and Lewandowski, 1998, Stoodley *et al.*, 1997). The increase in the level of available nutrients and oxygen through increased flow not only increases the overall biomass of the biofilm, but Zhang and colleagues (2013) have also shown that it can alter the relative abundances of different species within a mixed species biofilm.

The morphology of the biofilm is also influenced by the Reynolds number of the system. In an environment where mass transfer is low, biofilms respond by growing in towers or stacks (Vaughan *et al.*, 2010). This increases the surface area to volume ratio and therefore mass transfer between the biofilm and the surrounding fluid. It has also been reported in that

biofilms formed in fast flowing water are thicker and have a higher biomass than those formed at lower velocities (Zhang *et al.*, 2013). Conversely, Chang and colleagues (1991) showed that in systems where the flow exerts high shear forces, biofilms become thinner, but have denser biomass.

In lab-on-chip sensors, it is unlikely that Reynolds numbers will be high enough to significantly reduce biofilm formation by shear forces. However, the biomass and shape of fouling biofilms is likely to change from sensor to sensor and perhaps within individual sensors depending on flow rates and channel thickness.

Given the large range of environmental conditions to which the sensors will be deployed and within the sensors themselves, any attempts at remediating the formation of biofilms by individual species or in a specific environment will unlikely produce feasible antifouling solutions. Therefore any antifouling strategies used in the marine environment should be broad ranging to take account of this variability.

1.5. Analysis of Biofilms

1.5.1. Physical characterisation

Studies of biofilms need to address the differences in biofilm phenotype under variable growth conditions. These differences may be in the form of the quantity of biomass in a biofilm or other physical characteristics such as the morphology of cells present or the amount of EPS.

1.5.1.1. Surface coverage

One aspect of the biofilm that may be studied is the total coverage over the attachment surface. This can be measured by viewing the biofilm under a microscope and measuring the proportion of the surface that is covered with biofilm.

Under bright-field transmission microscopy, biological specimens often appear transparent and so are difficult to visualise. To improve the contrast and ease visualisation, stains are often used which bind to the sample. One such stain that is commonly used is crystal violet (CV), which when in aqueous solution dissociates into a CV^+ ion and a Cl^- ion. The CV^+ diffuses into cells and binds with negatively charge molecules such as proteins and DNA (Bartholomew and Finkelstein, 1954, Santhanalakshmi and Balaji, 2001, Wistreich and Bartholomew, 1969). This makes cells appear pink or purple and much clearer under bright-field microscopy. A drawback associated with this technique however is that inorganic particles that may also be present on the surface may be visible, which would make it difficult to quantify only the biological components. An additional problem with transmission

microscopy is that it can only be used effectively on transparent surfaces. An alternative is to use epifluorescence microscopy techniques.

Fluorescence microscopy works by exciting a fluorescent dye with a specific wavelength of light and then detecting the emitted light. When a photon of a specific wavelength is absorbed by a fluorescent molecule, an electron is excited to a higher energy state. The electron then returns to its ground state, emitting a photon. Some energy is lost in this process, so the emitted photon has a longer wavelength than the absorbed photon. Due to this difference in wavelength, the emitted light can be distinguished from the excitation light and so used to form an image of the fluorescing specimen. The difference in excitation and emission wavelengths is called the Stokes shift. A large Stokes shift means that there is little overlap in the excitation and emission light making the two wavelengths easier to isolate using interference filters (Murphy, 2001).

Several filters can be used with a fluorescence microscope, depending on the dyes which are used. Each of these filters is designed to expose the stained specimen with the relevant excitation wavelength, while allowing the transmission of the emission wavelength (Murphy, 2001). This is achieved using a dichroic mirror, which reflects the excitation light onto the specimen, but allows transmission of the emitted wavelength.

The use of a dichroic mirror allows the light source to be on the same side of the specimen as the objective lens, a technique called episcopic microscopy. This layout is depicted in Figure 1.5. By using episcopic microscopy instead of transmission microscopy more light can be detected, without interference from transmission through an object (Murphy, 2001).

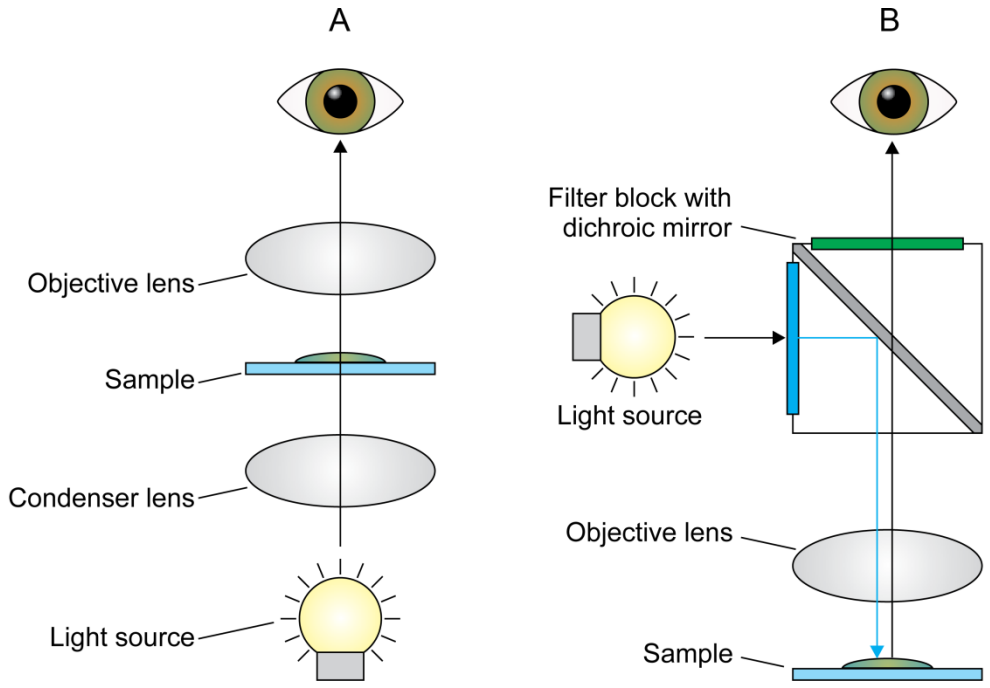


Figure 1.5: Unlike transmission microscopy (A), in episcopic microscopy (B) the light source is above the sample. White light travels through a filter and the filtered light excites the sample at a specific wavelength. The emitted light is then detected.

Fluorescence microscopy allows the user to detect specific molecules under a microscope, by using dye molecules that bind specifically to a target molecule in the specimen being examined. When studying biofilms, the application of the SYTO (Invitrogen) stains has been successful (Giao *et al.*, 2009). SYTO stains have a low level of fluorescence when in solution, but when bound to nucleotides they fluoresce strongly (Haughland, 2002). For this reason, they can be used to stain biofilms and allow high contrast images to be taken which only include biological materials containing polynucleotides. This reduces the amount of interference from inorganic materials and so allows more accurate quantification of biofilms. However, if the inorganic materials present (including the substratum on which the biofilm is growing) fluoresce in the wavelength that is detected by the microscope, this technique can prove difficult to use. It is possible to reduce this problem by first removing the biofilm from a surface and quantifying it *ex-situ*. For example, Giao and colleagues (2009) quantified *Legionella pneumophila* biofilms by removing them from the growth substratum through the use of bead beating followed by cell counts of samples stained with SYTO 9.

1.5.1.2. Thickness

A measurement of the surface coverage only gives a two dimensional representation of the biofilm. Biofilms are three-dimensional structures, which extend out from the surface. Therefore to accurately quantify a biofilm, its thickness must also be measured. There are several ways to measure biofilm thickness, some more accurate and practical than others. Mauricio and colleagues (2006) used a micrometre against the edge of a biofilm to measure its thickness. While this is a simple method, it requires the edge of the biofilm to be accessible, which is not always possible. Mauricio and colleagues (2006) also developed a technique whereby the change in electrical capacitance between a clean substratum and one with a biofilm can be interpreted as a thickness measurement. However, not only were the electrical capacitance measurements inconsistent, but they also did not give an absolute thickness measurement (only a measurement proportional to other samples).

Paramonova (2007) developed a technique which measured the heights of a plate when at the top of a biofilm and when in contact with the attachment surface. The difference between these heights could then be interpreted as the biofilm thickness. This technique, although useful, measures the maximum biofilm thickness and not the average biofilm thickness. Biofilms are not homogenous structures and so their thickness will change across their surface. For this reason it is desirable to take thickness measurements at several points over the surface instead of just one, which can be achieved through the use of microscopy.

To utilise microscopy in this manner, measurements are made by first focusing on the top of the biofilm and then the substratum and measuring the focal distance between the two.

In order to make accurate measurements this way, it is desirable to use an episcopic technique. This allows the top of the biofilm to be viewed reliably, while also allowing focus on the substratum. In this setup, if the biofilm was stained to increase its visibility, it would be very difficult to determine where the top of the biofilm was, due to interference from lower focal planes. Episcopic differential interference contrast (EDIC) microscopy produces high contrast images in an episcopic setup without the need for staining (Keevil, 2003). This makes it ideally suited for measuring biofilm thickness.

EDIC is a modified form of the commonly used differential interference contrast microscopy (DIC). This technique gives high resolution images of low contrasting biological materials without the need for staining. DIC requires a complex setup for the microscope as outlined in Figure 1.6. In a DIC microscope light is first passed through a light polariser and then through a Normarski-modified Wollaston prism. The prism splits the polarised light into two beams which travel closely together through a condenser lens and then through the sample. The waves of the beams are altered as they pass through the sample depending on its properties (such as thickness, slope and refractive index). The beams are focused by the objective lens and they pass through another prism and an analyser which recombines the beams. When the beams recombine the outputted light will differ depending on how the beams were altered when they traversed the sample. If the sample altered the beams so that they recombined productively, then that section of the sample will appear brighter, while destructive combinations will make the samples appear darker. Variation in properties across a sample will result in variations in destructive and productive combinations, resulting in a high contrast image.

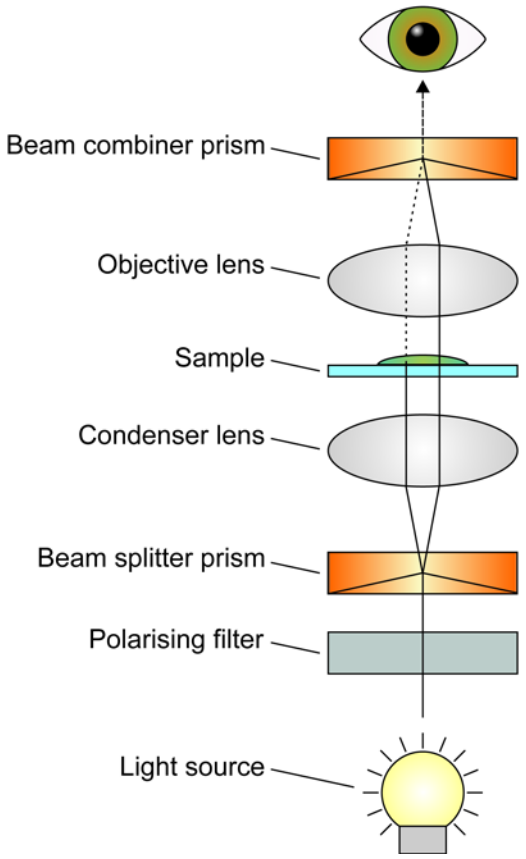


Figure 1.6: In differential interference contrast microscopy light is split by a beam splitter before passing through the sample. Differences within the sample cause the recombined beams to interact destructively, neutrally or constructively, increasing the contrast of the resulting image.

Another form of microscopy that has been used successfully to measure biofilm thickness is confocal laser scanning microscope (CLSM) (Costerton, 1999, Moller *et al.*, 1996, Murga *et al.*, 1995). One of the drawbacks of conventional EF microscopy is that not only areas of the sample that are in focus that fluoresce. This means that out of focus light from many focal planes is detected, resulting in an unsharp image. To counter this problem, CLSM works by introducing a pinhole to the microscope just before the camera. The pinhole eliminates light from the out of focus planes, so that only light from the focus planes is detected. The use of a pinhole means that only very small areas of the sample can be seen at one time, and so a scanning mirror is used which redirects light to different points on the sample. The brightness of light emitted from each point is measured and translated to and an image is constructed (rasterised) from this information. Lasers are used in confocal microscopy because they are high powered and easy to focus, making them compatible with the illumination of small points of the sample. CLSM produces high quality images of three-dimensional structures by introducing a stepper motor so that several focal planes can be imaged sequentially. CLSM can produce powerful data about the nature of biofilms.

1.5.1.3. Optical density

An alternative approach to quantifying biofilms by microscopy is to measure optical density. The build-up of biofilm on a surface can reduce the transmission of light waves. This can be detected using a spectrophotometer, which measures the difference between light intensity at a light source and the light intensity after transmission through a sample. This is reported as light absorbance, which is the inverse of transmission and relates to the amount of light absorbed by a sample. Christensen and colleagues (1985) used absorbance as a technique for measuring biofilm formation in microplates. Biofilms were stained with crystal violet, to increase their optical density relative to biomass, allowing for more accurate readings. The optical densities were then measured using a spectrophotometer. This gave a significant correlation with the mass of the biofilm, indicating that its optical density suitably represented biomass.

This technique was later optimised by Stepanovic and colleagues (2000) by redissolving the bound crystal violet in acetic acid, and then measuring the optical density of the resulting solution. This allowed the biofilm on the side of the microplate wells to be accounted for as well as that on the base of the well.

Optical density gives a quick, accurate measurement of relative biomass on a surface, which makes it ideal for high throughput measurements. However, without other data to compare, such as mass, surface coverage and thickness, a biofilm can only be semi-quantified.

1.5.2. Community analysis

Many studies of biofilms have been conducted using a mono-species culture. This is often the case where a single, pathogenic species is being considered, and so interactions with other microorganisms may not be very important. However, due to the mixed nature of the planktonic microbial community found in natural water systems, the biofilms that form inevitably contain mixed communities also. Within these communities, each species fills a niche which allows the community to function as a whole (Costerton *et al.*, 1995). Thus, in order to be able to understand biofilm ecology and so develop the most effective methods of remediation, it is important to study the entire community, and not just a single species within it.

The first step towards understanding a community's ecology is to identify which species occur within it. Traditionally, microbiological community analysis has relied on culture-based analyses. Such analyses involve placing a microbial community sample in a medium containing the nutrients necessary for microbial growth. Inoculated medium is then left in a suitable environment to allow the microorganisms within it to proliferate. The resulting colonies of species that appear in the medium can then be studied and identified. However, the majority

of microbial species cannot yet be cultured in this way (Rappe and Giovannoni, 2003) and so this method cannot give an accurate picture of the community.

1.5.2.1. Molecular tools for studying species richness

Due to the problems involved in culture dependent community analysis, molecular techniques have become popular in microbial community studies (Brummer *et al.*, 2000, Casamayor *et al.*, 2000, Herrera and Cockell, 2007, Jones *et al.*, 2007). By far the most common marker used in molecular microbial ecology is the 16S rRNA gene a region of the genome which is found in all bacterial species. Some regions of the 16S are highly conserved while others are variable. This makes it ideal for use in taxonomy because the conserved regions can be targeted by molecular probes, while the variable regions help to infer phylogeny.

The sequences of 16S rRNA have been elucidated for thousands of species of bacteria, and many are available on Internet-based databases such as the European Molecular Biology Laboratory Nucleotide Sequence Database (EMBL-NSD) (Stoesser *et al.*, 1999), GenBank (Benson *et al.*, 2005), the DNA database of Japan (DDBJ) (Tateno *et al.*, 2002) or the Ribosomal Database Project (RDP) (Maidak *et al.*, 1997). Using a basic local alignment search tool (BLAST) (Altschul *et al.*, 1990), new sequences can be compared to those already within a database to find sequences from closely related organisms. If they are at least 97% similar, it is probable that the sequences came from the same species (Gevers *et al.*, 2005). On this principle, 16S rRNA sequences from an unknown species can be compared with sequences from known species to allow identification.

This is a very powerful tool in microbial ecology. It allows researchers to make species identifications from natural environments in a culture independent way. This does of course depend on a record for each species already existing in the database. However, even where species identifications cannot be made, it can still give an indication of species richness in a community or flag species that are new to science.

In practice 16S rRNA is not a perfect solution. Several copies of the gene exist in most cells (Case *et al.*, 2007), and their DNA sequences may differ in a single organism, potentially resulting in a single species being represented by more than one band in an electrophoretic gel (see below), therefore resulting in an over estimation of a community's species richness.

A potential solution to this problem is to use another gene, such as *rpoB*, of which there is only one copy in each cell (Case *et al.*, 2007). This gene has been used successfully to distinguish species of bacteria (Case *et al.*, 2007, Dahllof *et al.*, 2000) and has been shown to be more sensitive than 16S rRNA in many cases (Dahllof *et al.*, 2000). Therefore as a measure of species richness, *rpoB* is probably a much more powerful tool than 16S rRNA. However, many more studies have been conducted using 16S rRNA, and so databases have more sequence

data for 16S rRNA than for *rpoB*. As a result, 16S rRNA is currently a much more powerful tool for species identification.

1.5.2.2. DNA extraction and amplification

Before DNA can be used to elucidate community structure, it must first be extracted and purified from an organism and then amplified. Amplification of specific DNA strands is carried out using the polymerase chain reaction (PCR), a standard technique used in many laboratories. On the other hand, the process of extracting and purifying DNA (herein referred to as DNA extraction) varies widely between laboratories, depending on the nature of the material from which DNA must be extracted.

DNA extraction usually follows a general two-step process. The first step is to lyse the cell, thus exposing its contents to the environment. Physical techniques of cell lysis use mechanical force to break down cell walls and release the cell contents. One such method involves simply grinding the cells with a mortar and pestle or small beads (Miller, 2001). The efficiency of this method can be increased by freezing the cells first to make them more brittle and easier to break (Lee *et al.*, 2003). Freezing can also be used without grinding, and relying on the expansion of ice crystals within the cell to rupture the cell wall and membrane (Tsai and Olson, 1991). Care must be taken when using physical methods, because the same forces that break open the cells could also shear the target DNA, rendering it useless. Because of the high potential for shearing DNA, physical techniques are often not the preferred method of lysis. However, they are useful where the extracellular substances are particularly resilient or where there is a lot of unavoidable mineral contamination in the sample (such as when extracting DNA from soil samples) (Robe *et al.*, 2003).

Chemical and enzymatic extraction techniques are probably the most commonly used in bacterial analyses. Enzymes such as proteinase K break down the structural components of the cell. Enzymes are always used in combination with a buffer which usually contains a surfactant and a chelating agent such as EDTA. Surfactants such as sodium dodecyl sulphate (SDS) or cetyl-trimethylammonium bromide (CTAB) interact with the lipids of the cell membrane to rupture the cell (Robe *et al.*, 2003). Chelating agents are used to remove metal ions from the system. This serves both to reduce the integrity of cell membranes as well as reducing the activity of any nucleases which may be present.

Following lysis, the second step in DNA extraction is purification of the DNA. There are two commonly used techniques for purification in bacterial community analyses. The first uses phenol-chloroform to separate proteins from the nucleotides in aqueous solution. Nucleotides can then be precipitated from the aqueous solution by adding ethanol (Sambrook *et al.*, 1989). The second technique uses spin columns, in which DNA binds to silica in the presence of a chaotropic agent such as guanidinium thiocyanate or guanidinium hydrochloride (Miller *et al.*,

1999). Once the contaminating substances have been washed away, the chaotropic agent is removed to allow the DNA to unbind from the silica and be released into an aqueous solution. While the phenol-chloroform technique usually gives purer extracts with a greater yield of DNA, spin columns are often preferred due to their high throughput and freedom from toxic phenol-chloroform.

1.5.2.3. DNA cloning

If one were to sequence PCR amplicons from a mixed species system straight after the PCR process, the sequence obtained would be unreadable. This is because all of the various 16S rRNA sequences from the different species within the community would be sampled simultaneously, and so their sequences would overlap. Traditional automated sequencers cannot distinguish between species and so cannot separate out individual sequences. In order to do this, an additional step between PCR and sequencing is needed.

One such method to achieve this, known as cloning, is to incorporate individual PCR amplicons in the form of plasmids into the cells of well characterised bacterial species such as *Escherichia coli* (Schmidt *et al.*, 1991). These transformed bacterial cells are then cultured. As the transformed cells divide, they replicate the extra plasmid alongside their own genome. This results in many copies of the PCR amplicons but in separate colonies, which can be isolated. Plasmids extracted from the isolated, transformed colonies can then be sequenced to enable the identification of the original species.

1.5.2.4. Gel-based species separation

Cloning of PCR amplicons is a time consuming process. Another approach to separating PCR amplicons from different species is to separate them on an electrophoresis gel. Two commonly used methods to do this are denaturing gradient gel electrophoresis (DGGE) (Diez *et al.*, 2001, Muyzer *et al.*, 1993, Muyzer and Smalla, 1998, Sahan *et al.*, 2007) and temperature gradient gel electrophoresis (TGGE) (Heuer *et al.*, 1999, Muyzer and Smalla, 1998). These work on the principle that the bonding between cytosine (C) and guanine (G) is stronger than between thymine (T) and adenine (A). PCR products that are to be used with DGGE or TGGE must be amplified using primers that have a long chain of C and G (known as a CG clamp) attached to one end. In a DGGE gel, there is a concentration gradient of urea and formamide. These chemicals denature double stranded DNA (dsDNA) to give an open DNA chain which is held together by the CG clamp (TGGE uses a temperature gradient to denature dsDNA instead of a chemical gradient). As dsDNA runs through the DGGE gel, it encounters an increasing concentration of urea/formamide. At low concentrations only fragments with a low CG content are denatured, but as the concentration increases, fragments with increasing higher CG contents begin to denature as well. An open DNA chain has almost

double the area in contact with the gel than a fully bound dsDNA chain. This decreases the rate at which it can move through the gel (Osborn, 1995), and so over the course of the electrophoresis those fragments that were denatured sooner will move a shorter distance. During the evolution of a species, the number and position of C and G in its 16S rRNA gene will change. This means that 16S rRNA from different species will denature differently and so move through the gel at different rates. A DGGE/TGGE run with several species will give a series of bands, each representing a separate putative species. These bands can then be excised from the gel and sequenced. The sequences obtained can be compared to an existing database of 16S rRNA sequences to give a set of species identifications.

The banding pattern seen on a DGGE/TGGE gel itself gives some information about community structure, even before the bands are sequenced (Osborn, 1995). The number of bands gives a snapshot of the diversity of a community which can be useful for high throughput research, where a large number of samples need to be analysed quickly. If these are the only data that are needed in a study, other profiling techniques are available, which may in fact be quicker and simpler than DGGE. These include terminal restriction fragment length polymorphism (T-RFLP) (Moeseneder *et al.*, 1999), and amplified rDNA restriction analysis (ARDRA) (Dang and Lovell, 2000). These techniques use restriction enzymes to cut PCR products at specific locations. Mutations at restriction sites result in different length DNA strands for different species, whose positions on an electrophoresis gel depend on their fragment lengths. T-RFLP is commonly used to quantify the diversity of bacterial communities (Berga *et al.*, 2012, Garland *et al.*, 2001, Lehours *et al.*, 2005, Li *et al.*, 2009, Moeseneder *et al.*, 1999, Schwartz *et al.*, 2007, Skrivanová *et al.*, 2010, Tiquia *et al.*, 2002). In T-RFLP, a fluorescently labelled PCR primer is used, so that each resulting PCR amplicon is fluorescently labelled. A restriction digest is then performed to create many DNA fragments of varying length. These DNA fragments are then separated by acrylamide gel or capillary electrophoresis based on their size. Those fragments that are fluorescently labelled (the terminal fragments) are then visualised. The number of discrete bands on a gel should theoretically represent individual species (Moeseneder *et al.*, 1999). Because these techniques rely on markers that do not have a continuous sequence, they cannot be used in the same way as DGGE/TGGE, to separate sequenceable DNA for comparison to an existing database.

The relative positions of a species' band in different DGGE/TGGE gels will always be constant assuming that the conditions of the gels are the same. This means that once the identity of a band is known, if the band reoccurs in other samples, it can be identified without needing to sequence its DNA fragment. Generally this is achieved by creating a standard solution with known DNA sequences to run in parallel to the samples being studied.

1.5.2.5. Next Generation sequencing

In contrast to the Sanger chemistry sequencing methodology (Sanger and Coulson, 1975), which is often used to acquire DNA sequences after cloning or DGGE, next generation sequencers allow PCR amplicons from a mixed community to be sequenced in parallel and still be distinguished from each other. There are several different systems used for next generation sequencing; however at present Roche's 454 sequencing technology is currently the most suitable method for microbial community analysis due to its relatively long sequence read capabilities.

The 454 system works by first denaturing the amplicons to single strands of DNA, which are then modified with short adapter DNA sequences. The adapted amplicons are then immobilised onto DNA capture beads, resulting in one amplicon bound to each bead in the reaction mixture. Each bead is captured in a micelle, which provides an isolated environment for further reactions (much like a biological cell). The DNA bound to the bead is then amplified to produce several million copies of the DNA on each bead. The beads are put into a picotitre plate, which contains wells which have a diameter only large enough to fit one bead. Nucleotides are then flowed over the picotitre plate in a known order. When a complementary nucleotide binds to a DNA strand, a fluorescent signal is detected. By comparing the timing of fluorescent signals with the timing of nucleotide flow, a sequence can be compiled for each well of the picotitre plate (Shendure and Ji, 2008).

Next generation sequencing (NGS) has been used successfully to study microbial community structure in several environments including soil (Campbell *et al.*, 2010, Roesch *et al.*, 2007, Rousk *et al.*, 2010), sinkholes (Sahl *et al.*, 2010), hot springs (Miller *et al.*, 2009) and the marine environment (Andersson *et al.*, 2009, Brown *et al.*, 2009, Gilbert *et al.*, 2009, Kirchman *et al.*, 2010, Sogin *et al.*, 2006). It is fast becoming the standard method for elucidating microbial community compositions.

NGS is a rapidly evolving technology and the reliability of the data obtained by this technique is increasing quickly, while the increasing availability of the necessary equipment is bringing the costs of the analysis of microbial communities by NGS into the budgets of most laboratories. However, as with any technology, there are limitations involved with NGS. Possibly the most prominent of these limitations come from the very high volume of data which is produced. In a single 454 run, around 1 million sequence reads are made. This volume of data provides a significant challenge in analysis, and several algorithms must be run on the data to exclude a large amount of artifact and allow accurate annotation of the sequences (Gilbert and Dupont, 2011). Such analysis requires a large amount of processing power, which may make this unfeasible for many laboratories. However, many NGS service providers now offer basic bioinformatics after service at an additional cost.

1.6. Anti-fouling strategies

Several techniques exist for reducing the amount of biofilm that forms on surfaces. These can be broadly split into power-consuming strategies, biocidal strategies, physiology effecting strategies and polymer surface modification. The efficacy of such methods can be assessed using the techniques previously described. Some of these strategies are more effective than others and not all those previously used can be feasibly applied to anti-fouling of micro-sensors. Existing anti-fouling techniques are discussed below.

1.6.1. Power-consuming antifouling strategies

Two methods that use mechanical force to shear the biofilm from a surface are high-pressure water jets and wipers (Whelan and Regan, 2006). Both of these techniques are unsuitable for use inside micro-sensors, as not only do the small dimensions of the sensor channels make their application impractical, but they could also destroy delicate sensor components.

By applying an electrical charge across the seawater within a sensor, chlorine can be generated by the electrolysis of chloride ions in the seawater (Manmaru and Shimono, 1997). The chlorine produced can kill fouling organisms. An alternative use of electrolysis involves transferring electrons directly into the biofilm-forming organisms, to kill or immobilise them.

These methods have the common problem of requiring large amounts of energy to function. This makes them particularly unsuitable where the power supply is limited. The sensors in development by CMM will need to operate remotely for several months at a time and so these power intensive antifouling methods are probably unsuitable for this application.

1.6.2. Biocidal strategies

Using biocides to kill microorganisms can be an effective strategy for reducing biofouling (Delauney *et al.*, 2010). As a biofilm is a highly complex structure requiring physiological processes for it to develop, if a bacterium is killed before it reaches a surface, it will not be able to form a biofilm. For this reason, using biocides from the initial immersion of a surface into water may reduce biofilm accumulation. However, it must also be noted that introducing biocidal chemicals to a system may trigger a stress response in bacteria and increase the rate at which they form biofilms (O'Toole and Stewart, 2005).

1.6.2.1. Chlorine

The application of chlorine as an antifouling strategy has been tested by dissolving chlorine into the water at a controlled rate. Davis and colleagues (1997) used slowly dissolving tablets releasing chlorine and bromine to control the rate at which the halogens entered the

sensor system. They found that this system worked well for their chlorophyll sensors, reducing biofouling for at least 3.5 months. While chlorine may be useful in a sensor that is only detecting physical properties of the environment, chemical or biological measurements may be seriously affected by the presence of chlorine. For example the production of hypochlorous acid by electrolysis of seawater will reduce the pH of the seawater, effecting any measurements of acidity. Also, due to its highly reactive nature, chlorine can degrade the surfaces of sensors, rendering them inoperable.

1.6.2.2. Tributyl tin

In the past, tributyl tin (TBT) was commonly used in paint and other coatings to control biofouling. The antifouling effect of TBT works in two ways. Firstly, the chemical itself is toxic to fouling organisms and so kills many of them before they have a chance to settle. Secondly, TBT based paints are self-polishing. When in the alkaline environment of seawater, ester bonds in the paint are hydrolysed, leading to layers of the paint sloughing off of the surface, taking any settled biofouling organisms with it (Whelan and Regan, 2006). Unfortunately the toxic nature of TBT is not exclusive to fouling organisms. It has been found to have deleterious effects on the environment, and has been referred to as "the most toxic chemical that has ever been deliberately placed in natural waters" (Manov *et al.*, 2004). This led to the International Convention on the Control of Harmful Anti-Fouling Systems on Ships to be adopted by the International Maritime Organisation and as such, as of 2008 the use of TBT on ship hulls is no longer allowed (IMO, 2002).

1.6.3. Physiological effectors

Another possible strategy for reducing biofouling is to exploit the physiology of biofilm systems. Biofilm lifecycles are highly regulated processes which utilise molecular signalling. By studying how the biofilm lifecycles are regulated it is possible to interfere with formation by recreating the chemical conditions present at different stages in the biofilm lifecycle.

1.6.3.1. Nitric oxide

Nitric oxide has been shown to trigger dispersal of cells from biofilms in many species. Barraud (2006) showed that the addition of the nitric oxide donor, sodium nitroprusside (SNP), could reduce the level of biofilm on a surface by around 80%. It was also shown that the addition of nitric oxide makes biofilms more susceptible to treatment by antibiotics and biocides, which show a lower efficacy toward bacteria in a biofilm at a given concentration than in the same bacteria in a planktonic state (Buckingham-Meyer *et al.*, 2007). Therefore, using nitric oxide in combination with other antifouling methods may result in a synergistic reduction in biofouling.

While it has been shown that nitric oxide increases dispersal of single species biofilms of Gram negative and Gram positive bacteria and fungi (Barraud *et al.*, 2009b) no research has been conducted that investigates the effectiveness of nitric oxide on the removal of natural mixed species marine biofilms.

1.6.3.2. Furanones

Biofouling is applicable in an ecological context, with aquatic organisms also at risk. Left uncontrolled, biofouling may cause serious adverse effects to an organism's health (Littler and Littler, 1995). For this reason, several anti-fouling strategies have evolved in nature, some of which have the potential to be exploited for industrial purposes.

It was observed that while some marine macro-algae do acquire a biofilm, the amount of biofilm was lower than would be expected if the algae had no fouling control measures. This prompted studies to look at the strategies that macro-algae use to reduce fouling (Steinberg *et al.*, 1997). It was found that *Delisea pulchra* produced heterocyclic compounds known as brominated furanones. Furanones are similar in structure to the AHL compounds used in quorum sensing (Rasmussen *et al.*, 2000). When present, they compete for binding sites with AHL and inhibit the quorum signal produced by bacteria. This means that population level processes that rely on quorum sensing for their control are reduced. Quorum sensing is very important in biofilm formation and so its disruption leads to a reduction in fouling. A study by Hentzer and colleagues (2002) found that in the presence of a synthetic furanone (furanone 56), biofilm formation by *P. aeruginosa* was reduced to negligible concentrations after six hours.

1.6.3.3. Diffusible signal factors

Another group of molecules that are involved in the regulation of biofilm formation and dispersal are diffusible signal factors (DSF). These are mono-unsaturated fatty acids possessing a similar chemical structure (Ryan and Dow, 2008, Wang *et al.*, 2004). In this group, the most deeply studied chemical is cis-11-methyl-2-dodecenoic acid, which is also known as diffusible signal factor and is the chemical after which the whole group is named (Barber *et al.*, 1997, Ryan and Dow, 2008). Diffusible signal factor was first isolated from *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Barber *et al.*, 1997), and so is herein referred to as *Xanthomonas* DSF (XDSF) to avoid confusion between the general name for the group of chemicals, and that chemical specifically produced by *Xcc*. XDSF has been found to be involved in the regulation of biofilm formation and dispersal (Dow *et al.*, 2003). This appears to be achieved by the regulation of the production of enzymes that are needed for the disaggregation of bacterial cells (Dow *et al.*, 2003). XDSF has also been shown to prevent the conversion of yeast to mycelium in *C. albicans* which indicates that XDSF is a cross-kingdom signalling molecule (Wang *et al.*, 2004). Indeed, *C. albicans* has been shown to produce farnesoic acid,

which is structurally similar to XDSF and has similar effects on mycelium production (Oh *et al.*, 2001). The minimum concentration of XDSF needed to affect *C. albicans* is only slightly higher than that of farnesoic acid. However, the concentration of farnesoic acid needed to affect *Xcc* is 2000 times higher than for *C. albicans* (Wang *et al.*, 2004). This indicates that while *Xcc* requires a very specific signal structure, *C. albicans* is less stringent.

Other similar signalling factors have been found to be produced by other Gram negative bacteria (Boon *et al.*, 2008, Davies and Marques, 2009), as well as Gram positive bacteria (Vílchez *et al.*, 2010). Some of these DSFs appear to be interchangeable to some extent. A DSF isolated from *Burkholderia cenocepacia*, known as BDSF, reactivates the formation of extracellular polysaccharides by *Xcc* deficient in XDSF (Boon *et al.*, 2008).

Another DSF, *cis*-2-decenoic acid, was isolated from *P. aeruginosa* and has been shown to induce the dispersal of biofilms formed by both Gram negative and Gram positive bacteria, as well as fungi (Davies and Marques, 2009).

As DSFs appear to be a ubiquitous signalling system for microorganisms, it is possible that they could have potential as marine antifouling agents, where mixed species biofilms would be expected. Their suitability would however depend on the nature of the sensor it was applied to. This is because by their nature, fatty acid molecules are not chemically compatible with some of the reagents used in sensors, and so could be broken down before they can act as antifoulants.

1.6.4. Anti-fouling polymer surfaces

The techniques discussed thus far either consume electrical power or rely on the controlled release of chemicals into the sensor environment. While many of these techniques have proven to be fairly successful, their application to long-term sensor deployments is somewhat limited by battery power or reagent supply respectively. To overcome this problem several studies have been conducted to look at the effect of modified polymer surfaces on biofilm accumulation.

Although glass has previously been shown to have a lower tendency to foul than some polymers (Fletcher and Loeb, 1979), several groups, especially those working with lab-on-a-chip applications, opt to use polymers as the bulk material to construct their sensors. This is usually because polymers are generally easier to machine than glass (Mowlem, personal communication). The fouling properties of polymers have been tested by several groups, and their fouling properties vary greatly. Kerr and colleagues (2001) carried out tests comparing the fouling properties of six different polymers. They found that some of the polymers were more susceptible to fouling than others. For example polyethylenerephthalate (PET) had an average fouling coverage of 3.62%, relative to 51.01% for polymethylpentene (PMP). These results were obtained after only a 1-hour immersion of the materials in water. Kerr and

colleagues also reported a positive correlation between the contact angle of each material and the level of fouling. However, their argument is unconvincing. No probability values supporting this claim were published, and the graphs within their paper do not appear to show a significant correlation (Figure 1.7).

An earlier study by Fletcher and colleagues (1979), also showed differences in the fouling properties of several polymers. This group also related increasing fouling to an increase in contact angle, but with a much stronger correlation than Kerr and colleagues (2001). This suggested that the fouling potential of a material increases with increasing hydrophobicity, as discussed previously.

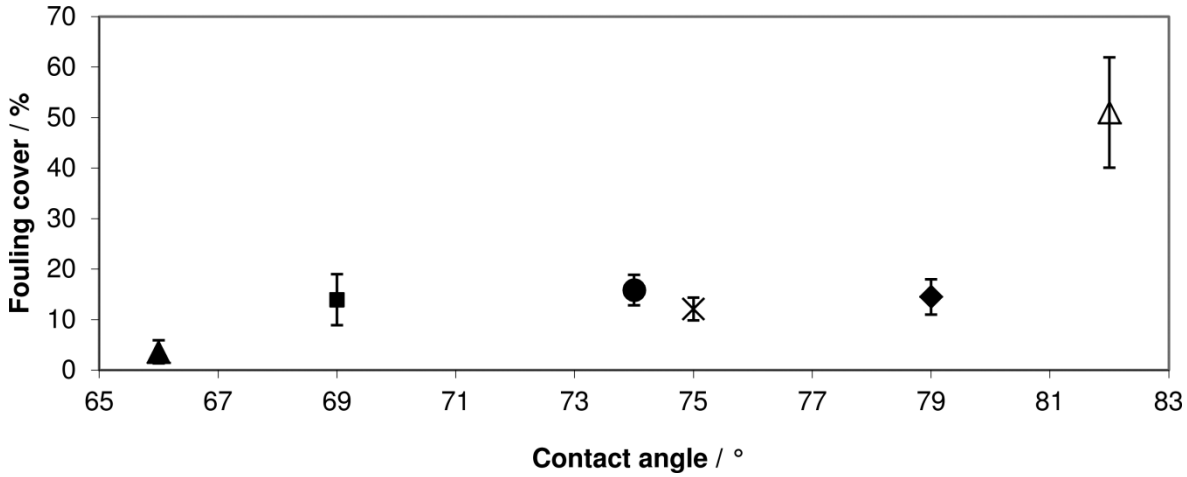


Figure 1.7: The relationship between contact angle and fouling cover for 6 different materials as measured by Kerr and colleagues (2001). While the authors reported a significant positive correlation, the removal of just the PMP (clear triangle) sample changes the relationship.

While studies of the antifouling properties of polymers reveal that some polymers such as PET reduce fouling, the reduction in fouling is generally not sufficient for extended immersion. For this reason, methods have been developed to increase antifouling effectiveness of polymer surfaces by modifying the physicochemical properties of materials.

Self-assembled mono-layers (SAM) are created with molecules that are terminated with a functional group that binds selectively to a surface at one end and a different functional group that will not bind to the surface at the other end (Whelan and Regan, 2006). This results in a carpet of molecular chains that extend perpendicular to the surface. Depending on the terminal groups used, SAMs can be constructed on different types of surfaces and endow the surface with new properties. Wiencek and Fletcher (Wiencek and Fletcher, 1995) used SAMs constructed with molecules with hydrophobic and hydrophilic terminal groups. The terminal groups used in this case were methyl (CH_2) and hydroxyl (OH), which are hydrophobic and hydrophilic respectively.

A material often used to produce sensors is SU-8, an epoxy based photoresist polymer. SU-8 is easily patterned using photolithography. It has a high chemical and thermal resistance,

and can easily produce a wide range of thicknesses (<1 to >200μm). However, it is fairly hydrophobic, with a contact angle of approximately 80° (Bennett, unpublished). This means that it is likely to be prone to fouling. To reduce this problem, Tao and colleagues (2008) attached chains of polyethylene glycol (PEG) to the surface of SU- 8. The addition of PEG decreased the contact angle to between 20° and 40° depending on the molecular weight of the PEG chains. The longer PEG chains gave a smaller contact angle and also showed less fouling after 16 hours than unmodified SU-8 and shorter chain PEG. However, the reduction was only by approximately 30% and so is by no means a perfect solution.

1.7. Project aims and rationale

Microfouling of artificial surfaces in the marine environment is a complex and dynamic process and tackling the problems that are associated with fouling has been on-going for centuries. Any surface that is immersed in the marine environment is likely to become rapidly fouled. This will potentially hinder the use of lab-on-chip devices in the sea, as is the proposed aim of the CMM project.

The use of toxic compounds such as TBT for anti-fouling is now banned, but even if their use were still allowed, then they would be poor choices for antifouling in a system that aims to measure environmental and biological conditions in the sensitive ecosystems of the world's oceans.

For this reason, the application of non-toxic, low-concentration diffusible molecules to a marine biofouling system were tested. The aim was to provide feasible antifouling solutions for marine lab-on-chip devices. However, in order to determine the best antifouling strategies, the extent to which lab-on-chip devices are likely to foul were investigated. The sensors in development by the CMM were still at the prototype stage and as such the ability to directly measure fouling in devices that have been deployed in the sea was beyond the scope of this project. For this reason, analyses of the fouling potential of the sensors were made to pre-empt any potential problems associated with biofouling that may be encountered.

1.7.1. Objectives

- Investigate the effects of the different reagents used by the chemical sensors on biofilm formation by mixed marine communities.
- Assess the relative levels of biofouling on different materials that are proposed for the construction of marine microsensors.
- Investigate the use of low-concentration diffusible molecules on mixed species marine biofilms to determine their effectiveness at reducing biofilm formation as well as determining what effect those molecules have on the biofilms at a community level.

Chapter 2 Effects of sensor reagents on the formation of marine biofilms

2.1. Introduction

Several of the sensors in development by the CMM are designed to measure the levels of different chemical species in the marine environment. To achieve this, different reagents are used depending on the sensor in question. When these reagents react specifically with the chemical species being measured, their optical properties change. The level of fluorescence or light absorbance during these reactions can be measured, and using these data the concentration of the chemical under investigation can be calculated.

The concentration of ammonium can be measured using a reagent based on that used by Kérouel and Aminot (1997) which contains ortho-phthadialdehyde (OPA), a known anti-bacterial that has been shown to be lethal to Gram negative and Gram positive bacteria and fungi (Akamatsu *et al.*, 2005, Walsh *et al.*, 1999). OPA is an aromatic compound containing two aldehyde groups that react with amines to form a chemical complex. This complex fluoresces with a peak emission of ~420 nm when excited at ~360 nm (Holmes *et al.*, 1999), and measurement of the level of fluorescence allows inference of ammonium concentration.

To detect nitrites (NO_2^-), Griess reagent is used. Griess reagent contains sulphanilamide, a bacteriostatic antibiotic that stops bacterial growth by competitively inhibiting the production of folic acid in bacterial cells (Slonczewski and Foster, 2009). In the Griess reaction, sulphanilamide reacts with nitrite to form a diazonium salt, which then reacts with N-(1-naphthyl)ethylenediamine dihydrochloride (NED) to form an azo compound which is pink in colour. The optical density of the solution can be measured, and interpreted to indicate the amount of azo compound present and therefore the concentration of nitrite (Dimitrios, 2007).

Iron is an important nutrient to nearly all forms of life, as reviewed by Hedich and colleagues (2011), and can be detected using ferrozine, which reacts with iron (II) and forms a stable, magenta coloured complex, with a maximum absorbance recorded at 562 nm (Stookey, 1970, Viollier *et al.*, 2000). It was developed for use in colorimetric *in-situ* analyses of dissolved iron by Chin and colleagues (1994). No information about ferrozine's toxicity to bacteria could be found in the literature but it is used as a reagent to determine the level of iron (III) reduction (Dailey and Lascelles, 1977, Dobbin *et al.*, 1995, Dobbin *et al.*, 1996), where cultures are grown in the presence of ferrozine.

In the microsensors in development by the CMM group, reagents will be flowed through microchannels at regular intervals. The presence of biofilm is likely to affect the flow rate, as well as the light used to measure chemical concentrations. In addition, the presence of biofilms may interfere with the chemical reactions in the sensors. For example if a sensor was fouled by iron reducing bacteria, such as *Shewanella* or *Geobacter* species (Caccavo *et al.*, 1992, Coates *et al.*, 1996, Straub and Buchholz-Cleven, 2001), the proportion of iron (III) and iron

(II) may be changed in such a way as to give false data in the iron sensor. The presence of nitrogen fixers such as *Vibrio diazotrophicus* (Guerinot *et al.*, 1982) or nitrifying (nitrite oxidising) bacteria such *as Nitrospira gracilis* or *Nitrococcus mobilis* (Watson and Waterbury, 1971) may also disrupt the functioning of ammonium and nitrite sensors respectively.

As some of the chemical reagents affect biological systems, it is likely that their presence will affect the level of fouling within chemical sensors. No literature was found that discusses the effects of these reagents on marine bacterial species. These experiments therefore investigated how the presence of the chemical reagents for ammonium, nitrites (Griess reagent), and iron affect marine biofilm formation, by studying planktonic and biofilm bacterial growth *in vitro* after treatment by the reagents.

In addition to investigating reagents directly used for sensing the chemical environment, the effect of hydrochloric acid (HCl) was investigated. HCl has been proposed as a cleaning fluid for use between chemical sensing operations, to remove residual chemicals that could affect chemical sensing assays. It is possible that the highly acidic nature of HCl would affect the growth of microorganisms either by inducing cell death, or by reducing their growth rate (Eifert *et al.*, 1997).

Measurement of biofilm formation was conducted using microscopy techniques as well as an optical density measurement based on the redissolved crystal violet assay developed by Stepanovic and colleagues (2000). As this investigation appears to be the first to use the crystal violet assay to quantify biofilms grown from a natural assemblage of marine bacteria, comparison of microscope and optical density methods was performed to confirm the applicability of this widely used method to mixed community marine biofilms.

2.2. Materials and methods

In the following experiments three chemical sensing reagents, ammonia reagent (with or without OPA), Griess reagent and ferrozine as well as HCl, were investigated for their effect on planktonic and biofilm organisms. Each chemical was used at the same concentration as it would be used in the sensor environment. By using these concentrations, it is possible to use the information gained from these experiments to predict how biofouling will be affected within the sensors.

2.2.1. Preparation of a standard marine inoculum

A standard inoculum was created to allow comparable growth conditions to be achieved in laboratory-based experiments. Forty litres of seawater were collected from the NOCS dock (50°53'28.36"N, 1°23'37.56"W (WGS-84)) on 29/03/2010. Using a high power peristaltic pump this seawater was serially pumped through two 100 μm pore-size, 47 mm diameter sterile filter membranes to remove large particulate matter. Microorganisms were collected on a sterile 0.2 μm pore-size, 120 mm diameter sterile filter membrane. Filter membranes were changed for every 15 litres of seawater filtered. The 0.2 μm membranes were cut into small pieces with sterile scissors and placed into sterile bottles containing 0.5 l of 0.2 μm filter sterilised seawater (SSW).

Approximately 2 grams of 2 mm diameter sterile glass beads were put into each bottle. The bottles were then shaken for 10 minutes to displace the microorganisms from the filter surfaces and resuspend them. This suspension was transferred into 50 ml centrifuge tubes and centrifuged for 30 minutes at 4800 RCF. After centrifugation the supernatant was removed to a sterile bottle, leaving a pellet in the bottom of the centrifuge tubes. The pellets were resuspended in 0.5 ml of SSW each and these suspensions were mixed in a separate centrifuge tube to make a master inoculum. Supernatants were re-centrifuged for 30 minutes at 4800 RCF. After centrifugation, supernatant was discarded and the pellets were resuspended in 0.5 ml of SSW. These suspensions were added to the master inoculum. This second centrifugation step was carried out to recover the maximum amount of cells from the original seawater.

Once collected, the master inoculum was further concentrated by centrifuging for 30 minutes at 4800 RCF. Enough supernatant was removed to leave 30 ml of supernatant. To this, 10 ml of sterile 60% glycerol (v/v) was added and mixed well by inversion. After these concentrating steps and the addition of glycerol, the resulting mixture had been concentrated 1000x. The 1000x mixture was separated into 300 μl aliquots in sterile cryo-tubes and stored at -80°C until needed.

2.2.2. Effect of chemical reagents on planktonic microbes

The effect of chemical reagents used in chemical sensors on planktonic marine microbes was investigated using agar plate based culture methods. A 300 μl aliquot of standard inoculum was diluted 100x in SSW, to give 30 ml of suspension. This suspension was then separated into fifteen 1 ml aliquots in sterile 1.5 ml tubes. Each tube was centrifuged for 15 minutes at 16000 RCF. The supernatant was removed and discarded, to leave a pellet in the base of the tube. Each pellet was then resuspended in 1 ml of reagent solutions (Table 2.1) giving three separate suspensions for each reagent and control. The tubes were then left for 5 minutes before being centrifuged for 15 minutes at 16000 RCF. The supernatant was removed, and the pellet was resuspended in 1 ml of SSW. The total amount of time that the microorganisms were in contact with the reagents was 20 minutes, during which time the tubes were kept in the dark as much as possible, to reduce ultraviolet oxidation of OPA. The tubes were again centrifuged for 15 minutes at 16000 RCF. The supernatant was removed and the pellet was resuspended in minimal marine medium with nutrients (3MN, Appendix 1).

Marine agar (Appendix 2) plates were inoculated with 100 μl of each suspension, and left for 4 days at 22°C followed by enumeration of the resultant bacterial colonies.

All statistical analyses were performed in the IBM SPSS statistic software package (version 17). An analysis of variance (ANOVA) test was used to compare differences in the numbers of CFU between reagent treatments, and a Tukey HSD post-hoc test was used for pair-wise comparisons for each reagent.

Table 2.1: Concentrations of reagents used in experiment to investigate their effects on marine microbial growth.

Reagent	Concentration (v/v with sterile seawater)	Final concentrations of components (M)	
Iron reagent (Appendix 3)	10%	Sodium acetate	0.08
		Acetic acid	4.0×10^{-3}
		Ferrozine	9.8×10^{-4}
Ammonium reagent with OPA (Appendix 4)	20%	Na_2SO_3	6.3×10^{-4}
		$Na_2B_4O_7$	0.02
		OPA[1]	5.0×10^{-3}
		Methanol	0.1
Ammonium reagent without OPA (Appendix 4)	20%	Na_2SO_3	6.3×10^{-4}
		$Na_2B_4O_7$	0.02
		Methanol	0.1
Griess reagent (Appendix 5)	50%	Sulfanilamide	2.9×10^{-3}
		HCl	0.06
		NED[2]	2.7×10^{-3}
HCl (Fisher Scientific,UK)	1.2 M (in pure water)	HCl	1.2

[1] ortho-phthadialdehyde
[2] N-(1-naphthyl)ethylenediamine

2.2.3. Effect of sensor reagents on early stage biofilm growth

To determine the effect of the sensor reagents on early stage biofilms, 6-well polystyrene microplates were inoculated with 2 ml of standard inoculum diluted 100x in SSW and left for 4 hours in static conditions in the dark to allow attachment of microorganisms to the surface of the plates.

After attachment, the inoculum was removed from the wells and replaced with 2 ml of the appropriate solution (Table 2.1), to give 3 wells per reagent. An additional set of wells was treated with SSW to act as a control. This was left for 20 minutes, after which time the liquid was removed from the plates and the wells were rinsed with 2 ml of SSW. This was then replaced and the plates were left in a 22°C static incubator for 5 days to allow the biofilms to grow. The growth media (SSW) was changed every 24 hours to replenish nutrients and removed waste products.

An additional plate, which was inoculated as before, was fixed with 4% (w/v) paraformaldehyde (PFA) for 1 hour. This served as a control for attached biomass before continued development. Those biofilms that were treated with the sensor reagents were also fixed in this way after 5 days growth.

2.2.4. Effect of sensor reagents on established biofilm growth

To test the effects of the sensor reagents on established biofilms, biofilms were grown in 6-well polystyrene microplates. Two sets of 18 wells (herein referred to as sample sets) were inoculated with 2 ml of standard inoculum diluted 100x in SSW and left in the dark in static conditions for 4 hours for attachment.

Following attachment, the inocula were removed and 2 ml of SSW was added to each well. The plates were left in a 22°C static incubator for 5 days with fresh media added every 24 hours.

After 5 days, each well was rinsed with 2 ml of sterile seawater. This liquid was then removed and discarded. To each well, 2 ml of the appropriate reagent solution (Table 2.1) was added, giving 3 wells per reagent per sample set. The reagents were left in the wells for 20 minutes, during which time the plates were kept in the dark to reduce ultraviolet oxidation of OPA. After this treatment, the wells were rinsed with 2 ml of SSW to remove residual reagents. SSW was added to one of the sample sets (Set 1) and growth was continued for a further 5 days before being fixed with 4% PFA. The other sample set (Set 2) was fixed immediately with 4% PFA and rinsed with sterile distilled water to remove residual PFA and salts.

2.2.5. Measurement and analysis of biofilm growth

2.2.5.1. Biofilm thickness, surface coverage and biovolume

After fixation the biofilm thicknesses were measured using EDIC microscopy at 1000x magnification. This was achieved by recording the difference in focal distance between the well surface and the top of the biofilm over 10 randomly selected fields of view.

To the each well, 0.5 ml of 5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in phosphate buffered saline (PBS) was added and left for 15 minutes in the dark. The DAPI stain was then pipetted off and the samples were rinsed with sterile distilled water. These were allowed to dry before being viewed under epifluorescence microscopy at 500x magnification. For each sample, 10 images in randomly selected locations were taken. Using ImageJ image analysis software (Abramoff *et al.*, 2004), the percentage surface coverage of biofilm in each image was calculated.

Biovolume of biofilms was calculated to give a 3 dimensional measure of the amount of biofilm growth. This was achieved by first calculating the means for both the thickness and surface coverage data for each sample and then calculating biovolume using Equation 1. Differences in biovolume between treatments were tested using one-way ANOVA tests and a Tukey HSD post-hoc test was used to test pair-wise differences between treatments.

$$V = T \times C \qquad \text{[Equation 1]}$$

where:

V = Biovolume ($\mu m^3 \cdot \mu m^{-2}$)

T = Thickness (μm)

C = Coverage ($\mu m^2 \cdot \mu m^{-2}$)

2.2.5.2. Measurement of biomass by optical density

To determine the relative amount of biomass in the microplates, a crystal violet assay similar to that used by Stepanovic and colleagues (2000) was used. To each well, 2 ml of crystal violet (2% w/v) was added. After 10 minutes, the crystal violet was removed and the plate was rinsed under slowly running water to remove excess crystal violet stain. The plate was then left to air dry before 2 ml of 33% (v/v) acetic acid in water was added to each well. This was left for 30 minutes on an orbital shaker to allow the bound crystal violet to be fully eluted into the acetic acid.

The optical densities of the solutions were recorded on a Tecan Sunrise microplate absorbance reader at 540 nm. Data were standardised against the negative control by first calculating a mean absorbance value for the negative control wells and subtracting this

number from the absorbance value for individual wells. Differences in optical densities between different treatments were tested using an ANOVA test and Tukey HSD post-hoc tests for pairwise comparisons.

To determine whether the optical density assay for measuring biomass gave an accurate indication of the amount of biomass in the microplates, biovolume and absorbance were compared using a Pearson's correlation.

2.2.5.3. Measurement of continued biofilm growth

To quantify the effect of the chemical reagents on the continued growth of established biofilms the absorbance values for sample Set 2 were subtracted from the absorbance data from the corresponding wells of sample Set 1. The resulting data were interpreted as biofilm growth after treatment with chemical reagents. The differences in growth were assessed using an ANOVA test, followed by a post-hoc Tukey HSD test for pair-wise comparisons between treatments.

2.3. Results

2.3.1.1. Effect of chemical reagents on planktonic microbes

CFU counts of bacteria exposed to different chemical sensor reagents revealed that HCl, Griess reagent and ammonium reagent (with OPA) completely stopped the growth of marine inoculum CFU. An ANOVA test to compare the control, ammonium reagent (without OPA) and iron reagent, indicated that there was a significant difference between CFU counts for these treatments ($p = 0.026$). Tukey post-hoc tests revealed that iron reagent had significantly less CFU than the control ($p = 0.02$).

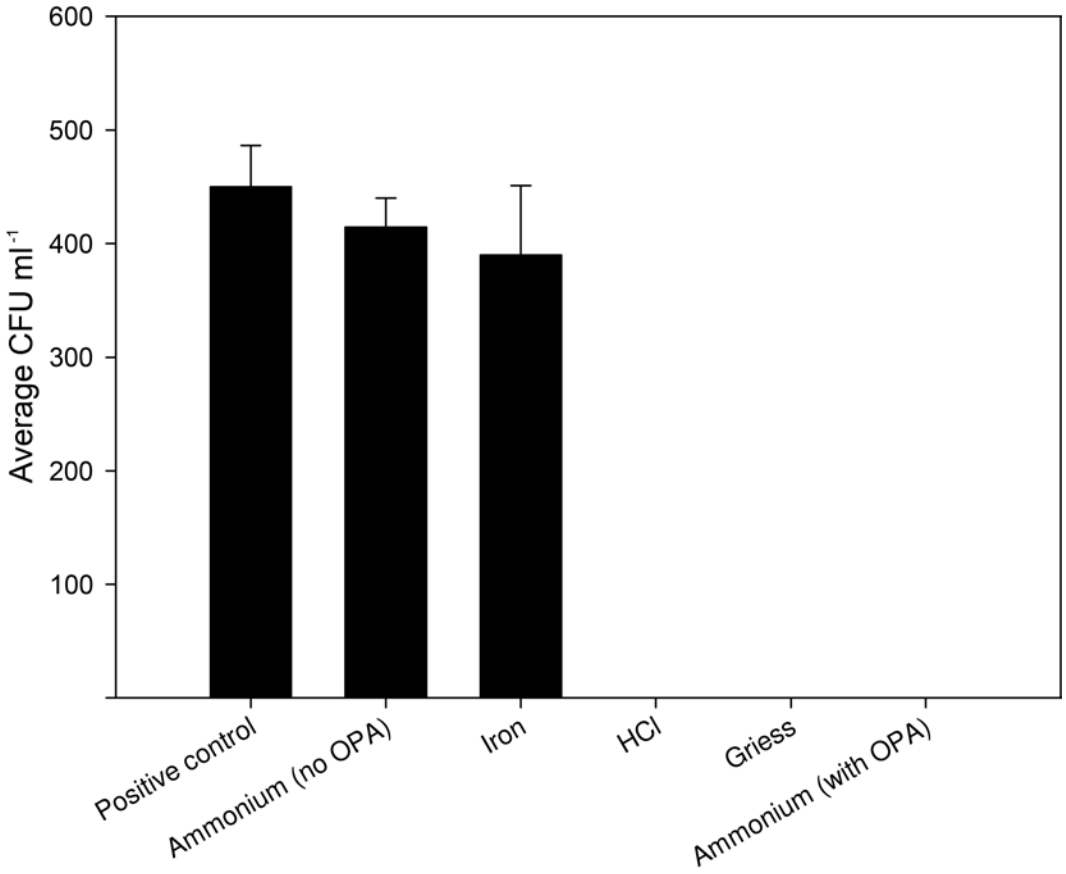


Figure 2.1: The average number of CFU ml^{-1} on marine agar after a standard marine inoculum was treated with different marine sensor reagents. Those samples treated with Griess reagent, ammonium reagent (with OPA) and HCl yielded no CFU, while ammonium reagent (without OPA) had no effect on CFU counts. Error bars are standard deviation from the mean of 9 repeats

2.3.1.2. Effect of sensor reagents on early stage biofilm growth

2.3.1.2.1. Biofilm biovolume

Biofilms grown after no reagent treatment had an average biovolume of 7.3 $\mu m^3 \cdot \mu m^{-2}$. Treatment with Griess reagent caused the greatest reduction in biofilm growth, with an

average biovolume of 0.1 $\mu m^3 \cdot \mu m^{-2}$ (Figure 2.2). This is an approximate 97% reduction in biovolume.

Comparisons of biofilm biovolume after treatment with different reagents (Figure 2.2) revealed significant reduction in biovolume as a result of the reagents ($p < 0.001$). Post-hoc Tukey HSD tests showed that HCl, Griess reagent and Ammonium reagent (with OPA) caused significant reductions in biovolume ($p < 0.001$ for all 3 reagents).

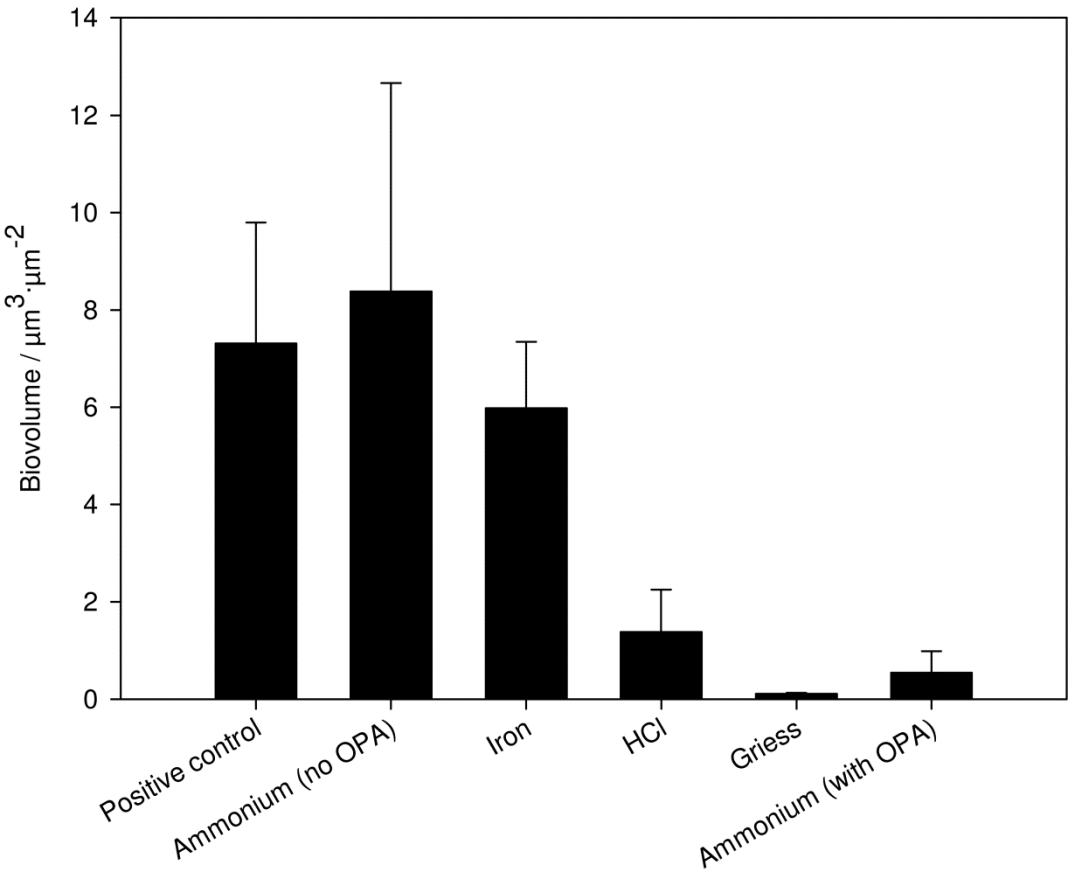


Figure 2.2: The biovolumes of biofilms grown for 5 days after treatment with marine sensor reagents. Griess reagent, ammonium reagent (with OPA) and HCl all significantly reduced biofilm formation after 5 days, while iron reagent and ammonium reagent (without OPA) have no significant effect on biofilm formation. Error bars are standard deviation from the mean of 9 repeats.

2.3.1.2.2. Biofilm absorbance

Biofilms grown after no reagent treatment had an average absorbance of 1.1 optical density units (ODU). Treatment with Griess reagent caused the greatest reduction in biofilm growth, with an average absorbance of 0.1 ODU. This is an approximate 91% reduction in absorbance.

Comparisons of absorbance after treatment with different reagents (Figure 2.3) revealed significant reduction in absorbance as a result of the reagents ($p < 0.001$). Post-hoc Tukey HSD tests showed that HCl, Griess reagent and Ammonium reagent (with OPA) caused significant reductions in absorbance ($p < 0.001$ for all 3 reagents).

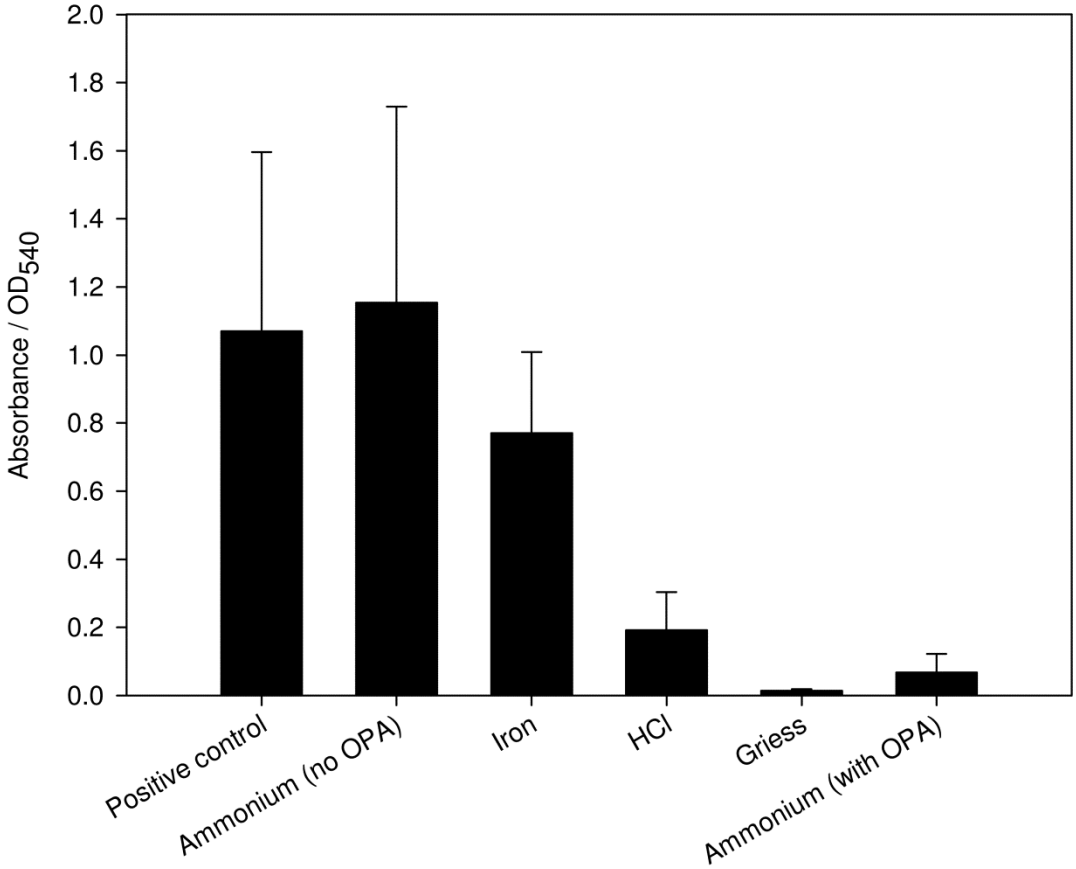


Figure 2.3: The optical density of re-dissolved bound crystal violet, indicating biomass of biofilms grown for 5 days after treatment with marine sensor reagents. Griess reagent, ammonium reagent (with OPA) and HCl all significantly reduce biofilm formation after 5 days, while iron reagent and ammonium reagent (without OPA) have no significant effect on biofilm formation. Error bars are standard deviation for the mean of 9 repeats.

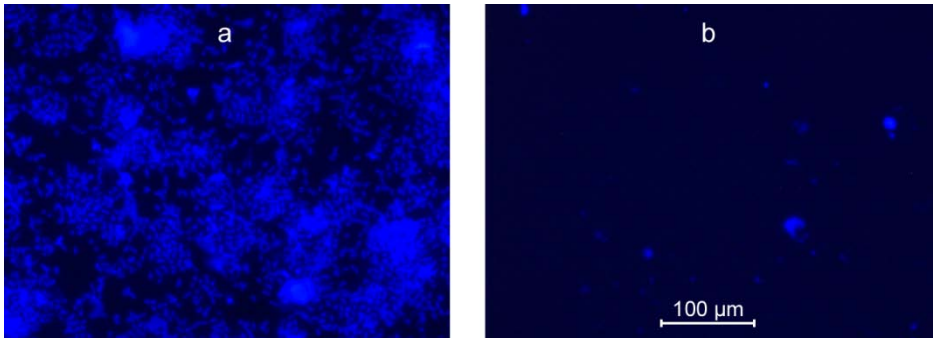


Figure 2.4: EF microscopy images (500x magnification) of 5 days growth biofilms. After initial inoculation, the samples were exposed to (a) filtered seawater and (b) Griess reagent. Biofilm formation after treatment with Griess reagent was much lower than that of the seawater control.

2.3.1.3. Effect of sensor reagents on established biofilm growth

2.3.1.3.1. Biofilm biovolume

The smallest amount of additional growth observed for 5-day old biofilms treated with reagents was for those treated with HCl, with an average change in biovolume of just 0.4 $\mu m^3 \cdot \mu m^{-2}$. This is a 97.6% reduction in growth relative to the control, which had an average change in biovolume of 15.3 $\mu m^3 \cdot \mu m^{-2}$.

Statistical analyses by ANOVA show there to be a significant effect of the reagents on the change in biovolume ($p < 0.001$). Post-hoc Tukey HSD test revealed that Griess reagent and HCl both significantly reduce biofilm growth ($p < 0.001$ for both), while the other reagents had no significant effect.

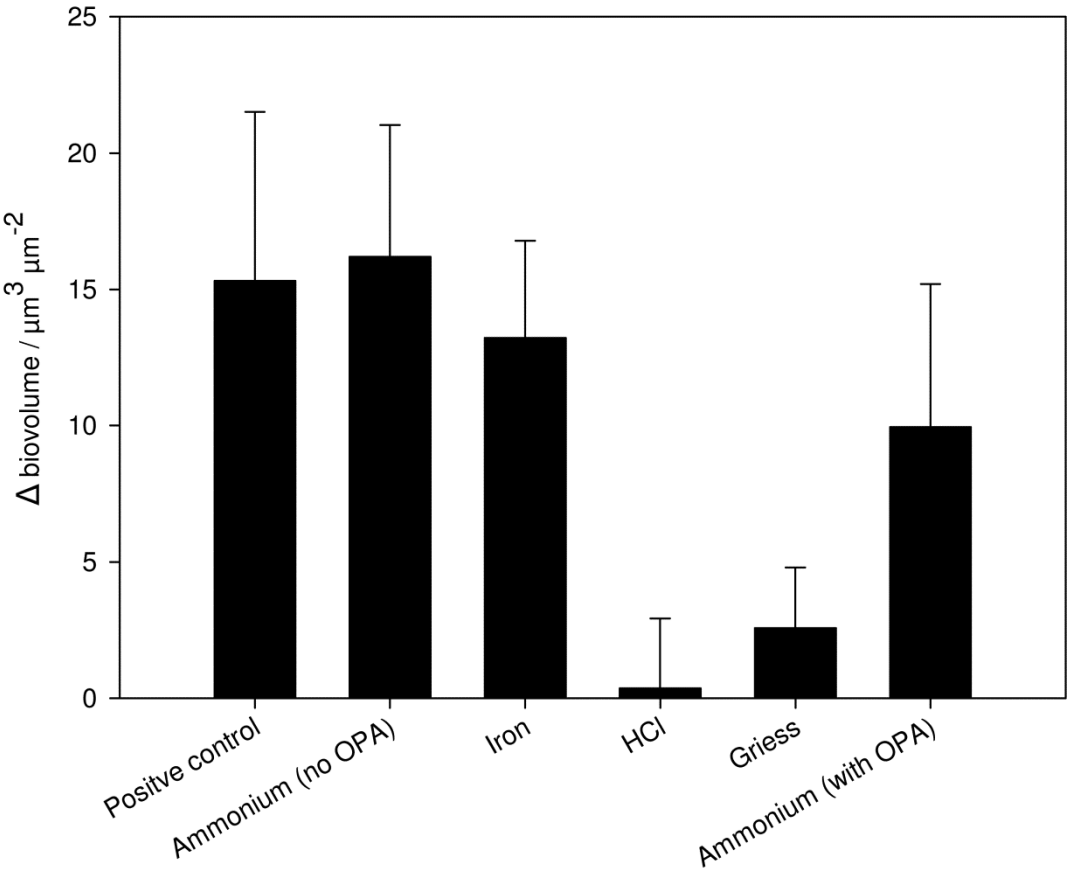


Figure 2.5: Five day old biofilms continued to grow with no significant difference from the control after treatment with ammonium reagent (with and without OPA) and iron reagent. However, Those biofilms treated with HCl and Griess reagent had significantly less biofilm growth than the control. Error bars are standard deviation form the mean of 9 repeats.

2.3.1.3.2. Biofilm optical density

The smallest amount of additional growth following treatment of 5-day old biofilms with reagents was for those treated with HCl, with an average change in absorbance of just 0.09

ODU. This is a 95.8% reduction in growth compared with the control, which had an average change in absorbance of 2.07 ODU.

Statistical analyses by ANOVA show there to be a significant effect of the reagents on the change in absorbance ($p < 0.001$). Post-hoc Tukey HSD test revealed that Griess reagent and HCl both significantly reduce biofilm growth ($p = 0.004$ and < 0.001 for Griess and HCl respectively), while the other reagents had no significant effect.

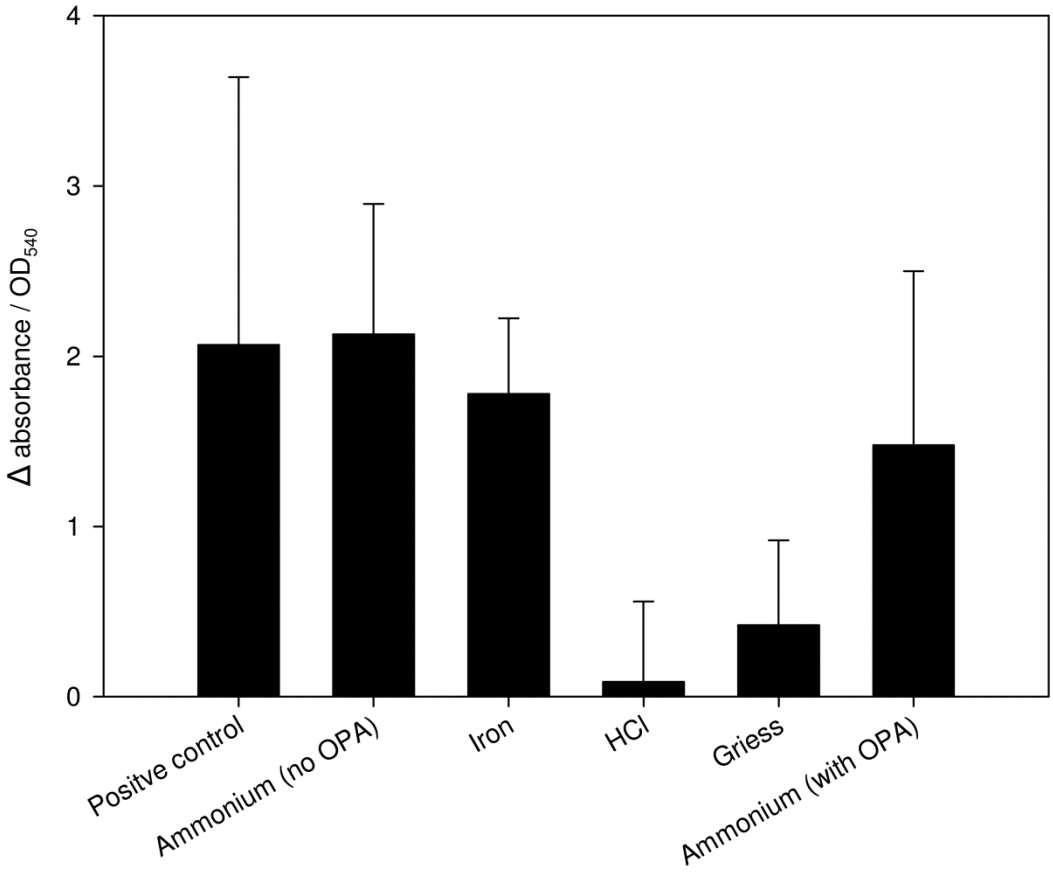


Figure 2.6: Similarly to biovolume measurements, five day old biofilms continued to grow with no significant difference from the control after treatment with ammonium reagent (with and without OPA) and iron reagent. However, Those biofilms treated with HCl and Griess reagent had significantly less biofilm growth than the control. Error bars are standard deviation form the mean of 9 repeats.

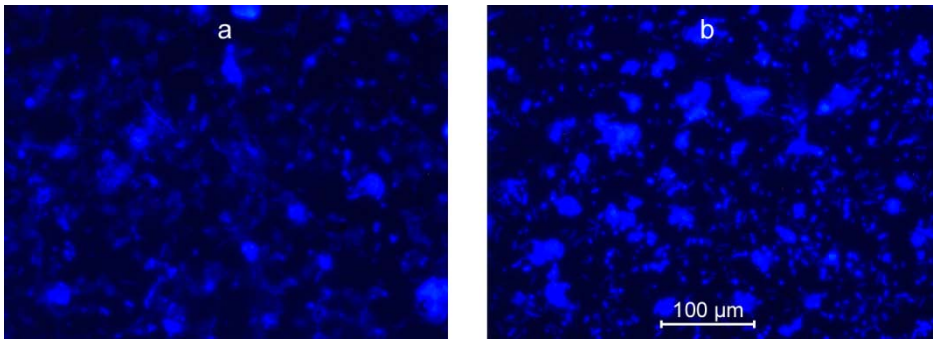


Figure 2.7: After 5 days growth, a biofilm was clearly visible in the 6-well plate wells. Treatment with HCl (b) did not cause any visible immediate reduction in the amount of biofilm on the surface compared to the control (a).

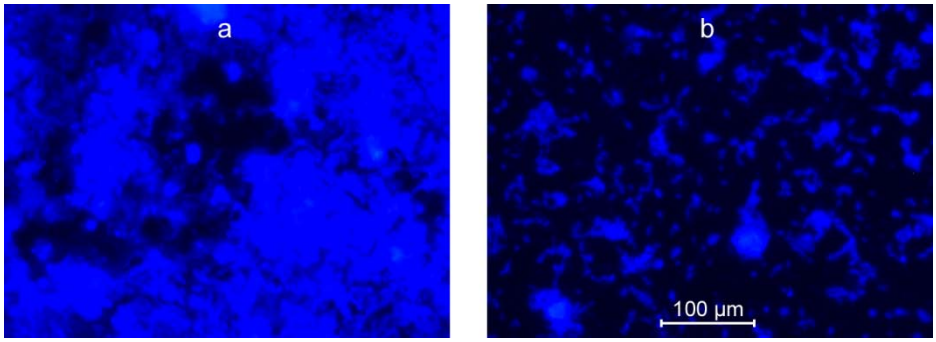


Figure 2.8: After an additional 5 days growth, the amount of biofilm in the wells treated with HCl (b) did not appear to have changed much, while the control (a) wells had much more growth.

2.3.1.4. Validation of the crystal violet assay for the measurement of biofilm biomass

All biovolume and absorbance data for the experiments presented in this chapter were compared with each other to determine whether they correlated (Figure 2.9). A Pearson's test revealed a correlation of 0.96 with a significance of < 0.001.

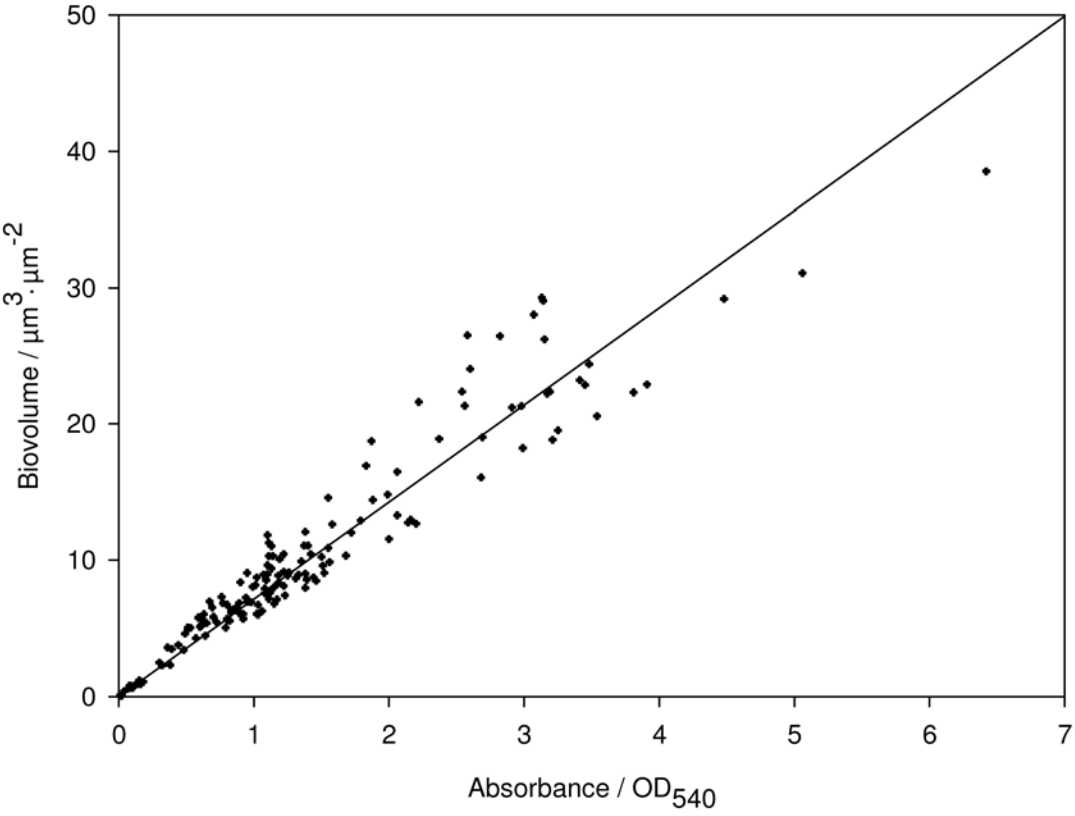


Figure 2.9: A strong correlation was apparent between the derived measure of biovolume (the product of coverage and thickness) and the absorbance of redissolved crystal violet.

2.4. Discussion

In this study, reagents used in the operation of marine environmental micro-sensors were investigated. Additionally, the commonly used crystal violet absorbance assay was tested to determine its suitability for use with marine biofilms. The strong correlation between biovolume and re-dissolved crystal violet indicate that the crystal violet assay gives a reliable indication of the amount of biofilm present in the system used here. This is the first time that this method has been used to measure biofilm growth by mixed marine communities and indicates that the crystal violet assay method can be applied to several different types of biofilm.

Some sensor reagents have a large impact on the development of marine biofilms. Specifically Griess reagent and ammonium reagent (including OPA) have toxic effects on planktonic bacteria and substantially reduce the development of attached microorganisms into biofilms. This is also true of HCl, which while not a sensing reagent, has been suggested for use as a cleaning agent between samplings. The clear effect of ammonium reagent containing OPA compared with the lack of effect of ammonium reagent not containing OPA indicates that it is the OPA in this reagent which imparts the antifouling properties. The use of 5 mM OPA in this study demonstrated similar effects to those observed by Walsh and colleagues (1999), who showed a log 5 reduction in *P. aeruginosa* at 0.13 mM OPA.

While OPA had a clear impact on the initial formation of biofilms by marine species, it had no significant effect on the continued growth of established biofilms. This indicates that the biofilms were more tolerant to OPA than were the planktonic bacteria. The tolerance of biofilms to toxic agents is a common trait, as reviewed by Gilbert and colleagues (2002), and is probably an important driver for microorganisms to adopt a sessile lifestyle. Simões and colleagues (Simões *et al.*, 2003a, Simões *et al.*, 2003b) showed *P. fluorescens* biofilms to be more resistant to OPA than their planktonic counterparts. OPA was shown to reduce activity of biofilms after prolonged contact (>30 minutes) but it is not clear whether any toxic effect resulted. The decreased susceptibility of biofilms to OPA was attributed to the interaction of OPA with proteins in the EPS of the biofilm. However, in the case of the ammonium reagent, where OPA is in a solution containing sodium sulphite, OPA does not react with amines and reacts specifically with ammonium ion (Kérouel and Aminot, 1997). It would therefore be expected that any tolerance created by interactions with proteins in the EPS would be reduced. Simões and colleagues (2011) found that biofilm cells without EPS and treated with OPA can be cultured unlike their planktonic counterparts, indicating that OPA tolerance may be due in part to the formation of persister cells (dormant variants of bacterial cells that are highly tolerant to antibacterials).

Unlike OPA both Griess reagent and HCl significantly reduced the continued growth of biofilms. This indicates that a mechanism exists by which Griess reagent and HCl can overcome biofilm tolerance and so have a toxic effect on the microorganisms within the biofilm structures. This effect is particularly interesting when we consider Griess reagent, whose effect might be assumed to be dependent on the anti-bacterial sulphanilamide. While no studies on the tolerance of biofilms to sulphanilamide have been reported, there is no reason to assume that it would be any more effective against biofilms than any other antibacterial. As well as sulphanilamide, Griess reagent contains HCl albeit at a much lower concentration than that tested in this study (50 mM compared with 1.2 M), however this concentration of HCl is high enough to lower the pH of seawater from 8.2 to 2.4. This change in pH by nearly 6 units is more than enough to disrupt the normal functioning of most organisms and so almost certainly played a significant role in the effect of Griess reagent on biofilms.

Previous studies have shown some biofilms to be tolerant to acidic environments. Most of these studies looked at biofilms that occupy intermittently acidic environments such as the human mouth (Welin-Neilands and Svensater, 2007), or extremely acid environments such as acid mine drainage sites (Smucker and Vis, 2011). In these cases one would expect the organisms involved to be tolerant to low pH and so able to survive in such conditions. In the marine environment, where the pH is typically between 7.9 and 8.25 (Raven *et al.*, 2005), biofilms would not usually encounter pHs as low as <0.01 (1.2M HCl) or 2.4 (Griess reagent) as in this study and so would likely not have evolved to be tolerant to such conditions. For the majority of environments where marine microsensors will be deployed therefore, the acidic nature of Griess reagent or HCl will likely provide a good level of protection from biofouling. However in areas of the oceans where the pH of the water is typically low, such as around hydrothermal vents, we would expect to find acidophilic microorganisms (Reysenbach *et al.*, 2006, Simmons and Norris, 2002). If these organisms enter the microsensors, then the antifouling action of HCl or Griess reagent may be significantly reduced.

Ferrozine had a small effect on the growth of planktonic bacteria enumerated by colony counts. This investigation appears to be the first to show ferrozine having any inhibitory effect on bacterial growth, but while no direct studies on the toxicity of ferrozine are apparent in the literature, it is used to determine the level of iron (III) reduction in cultures grown in the presence of ferrozine (Dailey and Lascelles, 1977, Dobbin *et al.*, 1995, Dobbin *et al.*, 1996). The fact that cultures of bacteria can be grown in the presence of ferrozine without any apparent inhibitory effects suggests that ferrozine is not toxic, at least to the bacteria used in those studies.

Iron sensors, which use ferrozine as their sensing reagent will not have the benefit of such reduced fouling as is evident from the minimal effect that ferrozine has on planktonic

bacteria and biofilm formation. In this case, it seems likely that bacteria will be able to establish themselves on the walls of the sensor channels and form biofilms. In the majority of ocean sites where the pH is around 8, it is likely that the areas of the sensors where OPA and Griess reagent are used are not at risk of major biofouling. This is due to the anti-fouling effect of these reagents on planktonic and newly attached cells, therefore reducing the establishment of mature biofilms. However, in other parts of the sensors, where OPA and Griess reagent are not present (such as seawater inlets), no antibacterial effect of the reagent will be present, thus creating potential 'hotspots' for biofouling within chemical sensors.

The chemical sensors will need additional antifouling methods regardless of any antibacterial effect that the reagents may have. HCl could prove to be very useful in this respect, due to its effectiveness for reducing planktonic bacterial growth as shown here. However, microscopy indicated that there was no immediate reduction in the amount of biofilm on a surface after treatment with HCl. For this reason it is important that any additional antifouling strategy used in the sensors is used from the beginning of operation to reduce microbial settlement and prevent the establishment of biofilms.

Due to the existence of different chemical environments in the oceans, it is possible that relying solely on the antifouling effects of HCl will not be sufficient for the entire operating range of the micro-sensors. Further work using prototype sensors deployed at sites such as hydrothermal vents could confirm or disprove this. HCl would also not be suited to cleaning of pH sensors and so looking for other antifouling methods that can be applied to a range of sensors perhaps in a battery of antifoulants is desirable.

Chapter 3 Effect of substratum material on biofilm formation

3.1. Introduction

Lab-on-chip devices can be constructed from a range of different bulk materials, each selected for their machinability, durability, chemical compatibility, optical properties and cost and in recent years the use of glass has largely been replaced by thermoplastics (Ogilvie *et al.*, 2010). Recent advances in fabrication methods for lab-on-chip devices using thermoplastics have allowed rapid prototyping and construction of devices at a much lower cost than was previously possible (Boulart *et al.*, 2008, Floquet *et al.*, 2011, Ogilvie *et al.*, 2011, Ogilvie *et al.*, 2010). However, as well as considering the mechanical properties of the bulk materials, it is also necessary to consider their fouling potential. Previous studies have shown that different polymers exposed in the marine environment foul to different extents. For example Kerr and colleagues (2001) demonstrated that after a short exposure to the sea, poly-ethylene terephthalate (PET) had a surface coverage of 3.62%, while poly-methylpentene (PMP) had a surface coverage of 51.01%. So, while a material may have ideal mechanical properties for a particular application, it may foul very readily rendering it less ideal for prolonged exposure to the marine environment. Alternatively there may be different materials with slightly less ideal mechanical properties, but which fouls much less readily. One might then decide to use the latter material to construct a sensor, as a compromise of all of the properties that must be considered.

The physicochemical properties of the material surface play an important role in the level of fouling that develops on a surface. The most important properties that determine fouling appear to be the surface energy or wettability along with the surface roughness. Several studies indicate that reducing the surface energy of a substratum increases its susceptibility to fouling (Allion *et al.*, 2006, Ista *et al.*, 1996, Kerr *et al.*, 2001, Wiencek and Fletcher, 1995). Studies have shown that increasing surface roughness also increases fouling potential (Allion *et al.*, 2006, Hilbert *et al.*, 2003, Kerr *et al.*, 2001, Verran and Boyd, 2001).

In this study, the formation of biofilms on two organic polymers, cyclic olefin copolymer (COC) and poly-methylmethacrylate (PMMA), deployed in the marine environment was investigated. These polymers are used in the construction of lab-on-chip devices, and have been used by CMM in the prototyping of marine microsensors. No literature was found that had previously compared the fouling of these two polymers. However, a study by Kerr and colleagues (1999) compared the fouling of glass and PMMA in water collected from the Firth of Clyde (Scotland) and found that PMMA fouled less than glass. In order to allow comparison of the current study with other studies, glass was used as a control surface. To determine whether any differences in marine biofilm formation on these materials were due to the physicochemical properties of the materials, surface roughness and surface energy were measured.

3.2. Materials and methods

3.2.1.1. Substratum materials

The materials used were glass (Fisher Scientific, UK product # FB58622), Topas 5013 COC (Topas) and Plexiglas XT PMMA (Amari plastics). All of the coupons used were 76 x 26 mm and were cleaned prior to deployment by ultrasonication in water for 15 minutes at room temperature. They were then rinsed with isopropanol and dried with nitrogen gas.

3.2.1.2. Material properties

To determine the hydrophobicity (ΔG_{sws}^{TOT}) of the materials used in these experiments, contact angles of 1 µl drops of reference liquids were measured using a Kruss DSA30 goniometer. The van Oss model (van Oss *et al.*, 1988) was used to calculate surface free energy as it is generally considered to provide the most accurate results.

Water and formamide were used as polar liquids and for the non-polar liquid, α-bromonaphthalene was used with glass and PMMA, while diiodomethane was used with COC (this is because α-bromonaphthalene dissolves COC). The measurements were repeated five times for each material and analysis liquid.

Table 3.1: Physical properties of liquids used for the calculation of surface energies of 4 different materials. Data were taken from van Oss (2006).

	Liquid surface tension components / mJ·m^{-2}			
Liquid	γ_l^{TOT}	γ_l^{LW}	γ_l^+	γ_l^-
Water	72.8	21.8	25.5	25.5
Formamide	58.0	39.0	2.28	39.6
α-bromonaphthalene	44.4	44.4	-	-
diiodomethane	50.8	50.8	-	-

To calculate hydrophobicity using the van Oss model, the measured contact angles where used in the equations below, along with known physical properties of the analysis liquids as outlined in Table 3.1.

$$\Delta G_{sws}^{TOT} = \Delta G_{sws}^{LW} \qquad \text{[Equation 2]}$$

where:

ΔG_{sws}^{TOT} = Total free energy/hydrophobicity (mJ/m^2)

$$\Delta G_{sws}^{LW} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2$$

$$\Delta G_{sws}^{AB} = -4\left[\left(\sqrt{\gamma_s^+\gamma_s^-}\right) + \left(\sqrt{\gamma_w^+\gamma_w^-}\right) - \left(\sqrt{\gamma_s^+\gamma_w^-}\right) - \left(\sqrt{\gamma_w^+\gamma_s^-}\right)\right]$$

where:

$\gamma_w^{LW} = \boldsymbol{\gamma_l^{LW}}$ for water

$\gamma_w^+ = \gamma_l^+$ for water

$\gamma_w^- = \gamma_l^-$

$\gamma_s^{LW} = \frac{\boldsymbol{\gamma_l^{TOT}}}{4}(1 + \cos\theta)^2$

$\gamma_s^+ \; and \; \gamma_s^-$ = acid and base components of material surface tension derived by solving Equation 3 with measurements using two polar liquids.

where:

θ = contact angle for analysis liquid (radians)

and where:

$$\gamma_l^{TOT}(1 + \cos\theta) = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^-\gamma_l^+} + \sqrt{\gamma_s^+\gamma_l^-}\right) \qquad \text{[Equation 3]}$$

The surface roughness of glass, PMMA and COC were measured using a Taylor Hobson Talysurf 120L contacting profilometer. Three profiles were measured over 30 mm transects for each material. The roughness of the surface was calculated as the arithmetic mean of the profile amplitude using Equation 4.

$$R_a = \frac{1}{n}\sum_{i=1}^{n} y_i \qquad \text{[Equation 4]}$$

where:

R_a = Average roughness (μm)

y = Absolute amplitude (μm)

3.2.1.3. Sampling

Coupons of glass, PMMA and COC were deployed in marine exposure tubes (MET) (Figure 3.1) at the National Oceanography Centre in Southampton (NOCS) (50°53'28.36"N, 1°23'37.56"W (WGS-84)) dock for 3 days, 14 days and 28 days. For each material and time point, 3 coupons were used. The first deployment was carried out between 15th April 2011 and 27th May 2011. The experiment was repeated once more between 13th May 2011 and 10th June 2011.

Once removed, samples were dip rinsed in 0.2 µm filter-sterilised sea water (SSW) to remove loosely attached microorganisms. They were then fixed in 4% (w/v) paraformaldehyde for 1 hour and stored at 4°C until needed.

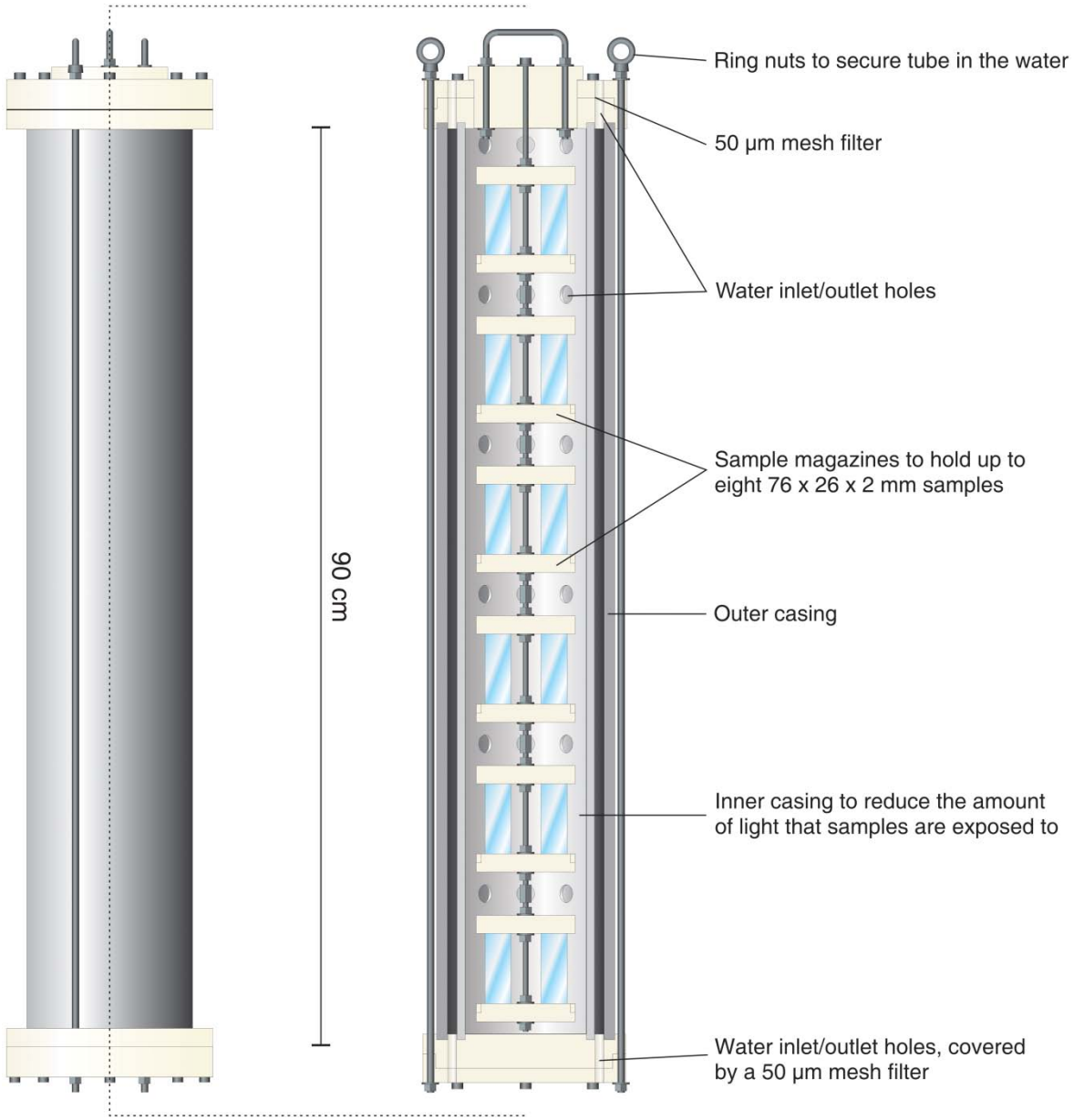


Figure 3.1: The marine exposure tube (MET) was designed in such a way as to allow water to flow freely over the samples, while reducing the amount of light that reached the samples. Filters with a mesh size of 50 µm excluded larger particles that might otherwise have settled on the samples.

3.2.1.4. Measurement of fouling

Before the samples were analysed, they were rinsed with sterile deionised water to remove paraformaldehyde and salt deposits which would have made visual analysis problematic. Biofilm thickness was measured using EDIC microscopy at 1000x magnification. This was achieved by measuring the difference in focal distance between the coupon surface

and the top most part of the biofilm in the field of view using the StagePro application within ImagePro. Thickness measurements were made for ten random fields of view for each coupon.

To the each sample, 0.5 ml of 5 μg/ml DAPI in PBS was added and left for 15 minutes in the dark. The DAPI stain was then aspirated and the samples were rinsed with sterile distilled water. These were allowed to dry before being viewed under epifluorescence microscopy at 500x magnification. For each sample, 10 images in randomly selected locations were taken. Using ImageJ image analysis software, the percentage surface coverage of biofilm in each image was calculated. A three-dimensional measure of biovolume was calculated as the product of thickness (μm) and coverage (μm^2) for each sample.

Averages were calculated for the thickness, surface coverage and biovolume data for each sample. All statistical analyses were performed in the IBM SPSS statistics software package (version 17). Data were tested for normality using the Shapiro-Wilk test, and those data sets that were found not to have a normal distribution were $\log_{10}$ transformed. Differences in thickness, surface coverage and biovolume between materials and deployment times were tested using one-way ANOVA tests. Tukey HSD post-hoc tests were used to test pair-wise differences in fouling between materials and deployment times.

3.2.1.4.1. Optical density

After microscope based analyses were completed, the coupons were stained with 2% (w/v) crystal violet for 10 minutes, and rinsed carefully with deionised water to remove the excess stain. They were then allowed to dry before their optical densities at 540 nm were measured on a BioTek ELx800 plate reader. To achieve this, the coupons were placed on a plate reader adapter as shown in Figure 3.2. The plate reader adapter was able to make measurements for 3 coupons simultaneously, however only the centre eight readings for each coupon were used to avoid edge effects.

Absorbance measurements were also made for coupons that had not been exposed to the sea, but which had been stained as detailed above. The absorbance data were standardised by subtracting the average absorbance for each blank material from the corresponding sample absorbance.

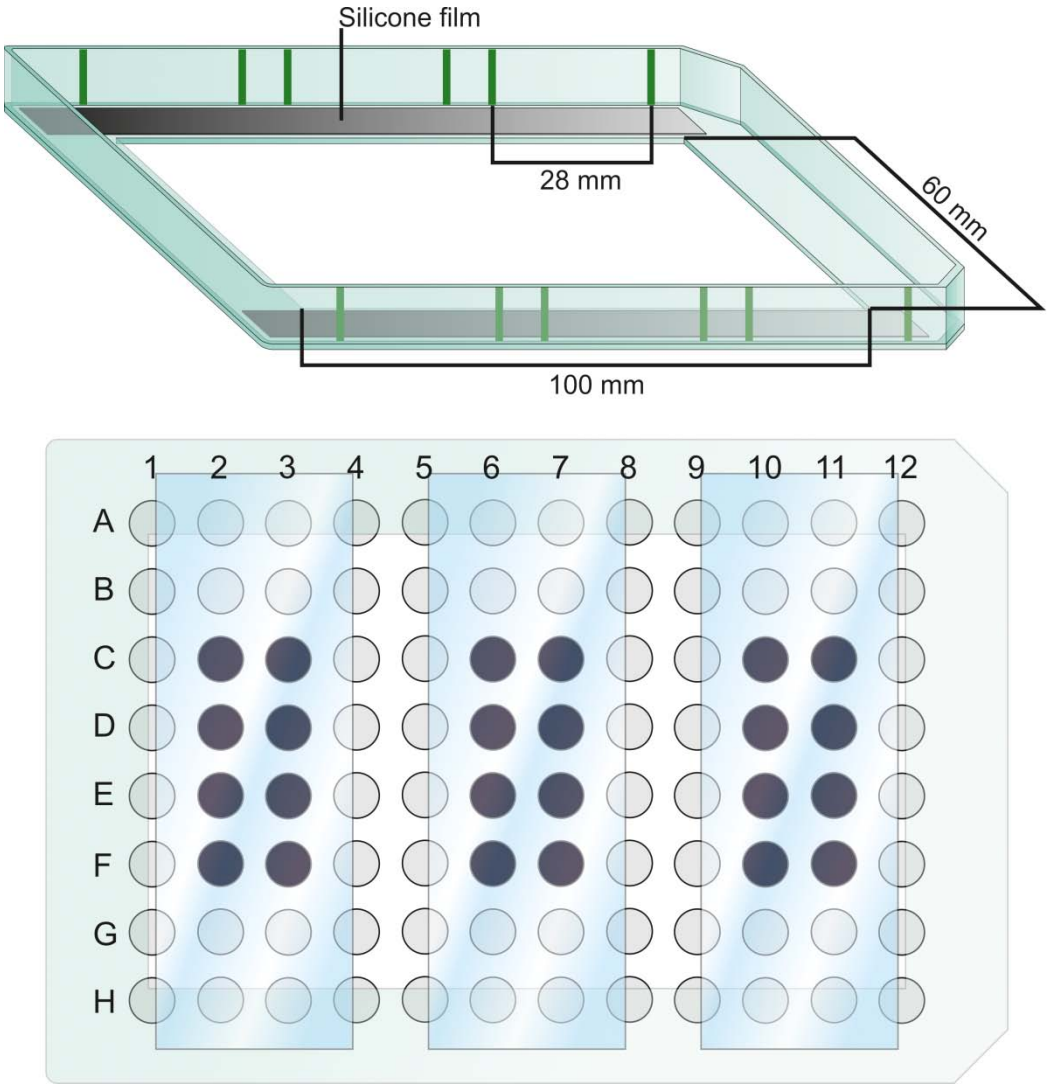


Figure 3.2: A microplate reader modification was used to allow the optical densities of microscope slides to be measured. A 60 x 100 mm hole in the centre of a microplate lid allows the sample absorbance to be recorded. The silicone film strips act to increase grip on the samples, keeping them within the marked spaces. Eight measurements were taken for each sample. These measurements were in the centre of the coupon to avoid problems such as edge effects and areas where measurement points did not fully occupy the coupon.

3.2.1.4.2. Fouling rate

The rates of fouling were calculated for each material by dividing the change in thickness, coverage, biovolume or absorbance by the number of days between deployments. This was repeated for the fouling between 0 and 28 days to allow comparison of the overall growth rates, as well as fouling between 0 – 3 days, 3 - 14 days and 14 - 28 days to allow comparison of fouling rates at different stages of the deployment. Overall fouling rates were compared between materials using a one-way ANOVA test. Changes in fouling rates for 0 – 3 days, 3 - 14 days and 14 - 28 days were tested for significance using a two-way ANOVA test to test the effects of material and deployment period.

3.2.1.5. Comparison of measurement parameters

To determine whether there was a relationship between optical density and biovolume that would allow prediction of one measurement from the other, all biovolume and absorbance data from this chapter were compared. Curve estimation was run on untransformed data in SPSS and the curve type that had the greatest R^2 value was taken as the best model for the relationships between the data. Data were then transformed depending on the shape of the relationship between optical density and biovolume, and a Pearson's correlation was run to determine the significance of the relationship.

3.3. Results

3.3.1.1. Material properties

Surface energies calculated from contact angle measurements showed that COC had a surface energy of 22.4 mJm $^{-2}$ and had the lowest surface energy of the four materials tested, while glass had a surface energy of 46.6 mJm $^{-2}$ which was the highest (Table 3.3). ANOVA tests revealed that the surface energies varied significantly between materials ($p < 0.001$), and Tukey HSD tests revealed all pairwise comparisons of materials to be significantly different ($p < 0.001$ in all cases).

Surface roughness measurements revealed that COC had the roughest surface with a R_a value of 0.012 μm. ANOVA ($p = 0.009$) and Tukey HSD tests showed that COC was significantly rougher than all the other materials ($p = 0.020$ and 0.009 for glass and PMMA respectively). However, glass and PMMA did not have significantly different roughnesses ($p = 0.937$).

Table 3.2: Measured contact angles and calculated hydrophobicities for 3 materials measured showed that glass was hydophillic (ΔG_{sws}^{TOT} >0 mJ·m^{-2}) while COC and PMMA were hydrophobic. θ_w, θ_f and θ_p are contact angles for water, formamide and the non-polar liquid respectively. Non-polar liquid used for glass was α-bromonaphthalene, and diiodomethane for COC and PMMA.

Material	**Mean contact angles / °**			**Surface tension parameters (mJ·m^{-2})**			**Hydrophobicity**
	θ_w	θ_f	θ_p	γ_s^{LW}	γ_s^+	γ_s^-	ΔG_{sws}^{TOT} / mJ·m^{-2}
Glass	31.4	17.5	25.0	40.3	1.41	39.65	13.6
COC	98.3	71.2	51.7	33.3	0.05	0.38	-88.1
PMMA	76.1	58.1	34.3	42.3	0.02	9.47	-45.3

Table 3.3: Roughness (R_a) varied between the materials tested, with COC having highest roughness. The average roughness (R_a) of 3 different materials were all in the nm range. SD is standard deviation from the mean of 5 repeats.

Material	**Mean R_a / nm**	**SD R_a / nm**
Glass	7.5	0.4
PMMA	6.9	0.2
COC	12.1	2.8

3.3.1.2. Measurement of fouling

ANOVA tests revealed that there were no significant differences in either coverage or biovolume between materials after 14 and 28 days exposure ($p = 0.566 – 0.774$ and $0.390 – 0.653$ respectively) and no significant differences in thickness ($p = 0.238 – 0.706$) or absorbance ($p = 0.113 – 0.520$) between any materials at any time point. However, a

significant difference in the biofilm coverage ($p = 0.009$) and biovolume ($p = 0.015$) between materials was found after 3 days deployment. Post-hoc Tukey HSD tests showed that glass had significantly less coverage ($p = 0.007$) and biovolume ($p = 0.014$) than COC.

Additional ANOVA tests to determine if there were differences in fouling between deployment times were conducted on $\log_{10}$ transformed data to increase homogeneity of variances. It was demonstrated that there were significant differences in fouling between all deployment times on all materials and for all measurement parameters ($p < 0.001$ in all cases). Post-hoc tests revealed that in the majority of cases there were significant increases in fouling in successive deployment times on all materials ($p = <0.001 – 0.038$). However, no significant difference was observed between 14 and 28 days deployment for COC thickness and coverage ($p = 0.159$ and 0.078 respectively); glass coverage and biovolume ($p = 0.924$ and 0.151 respectively); and PMMA coverage, biovolume and absorbance ($p = 0.301$, 0.063 and 0.242 respectively).

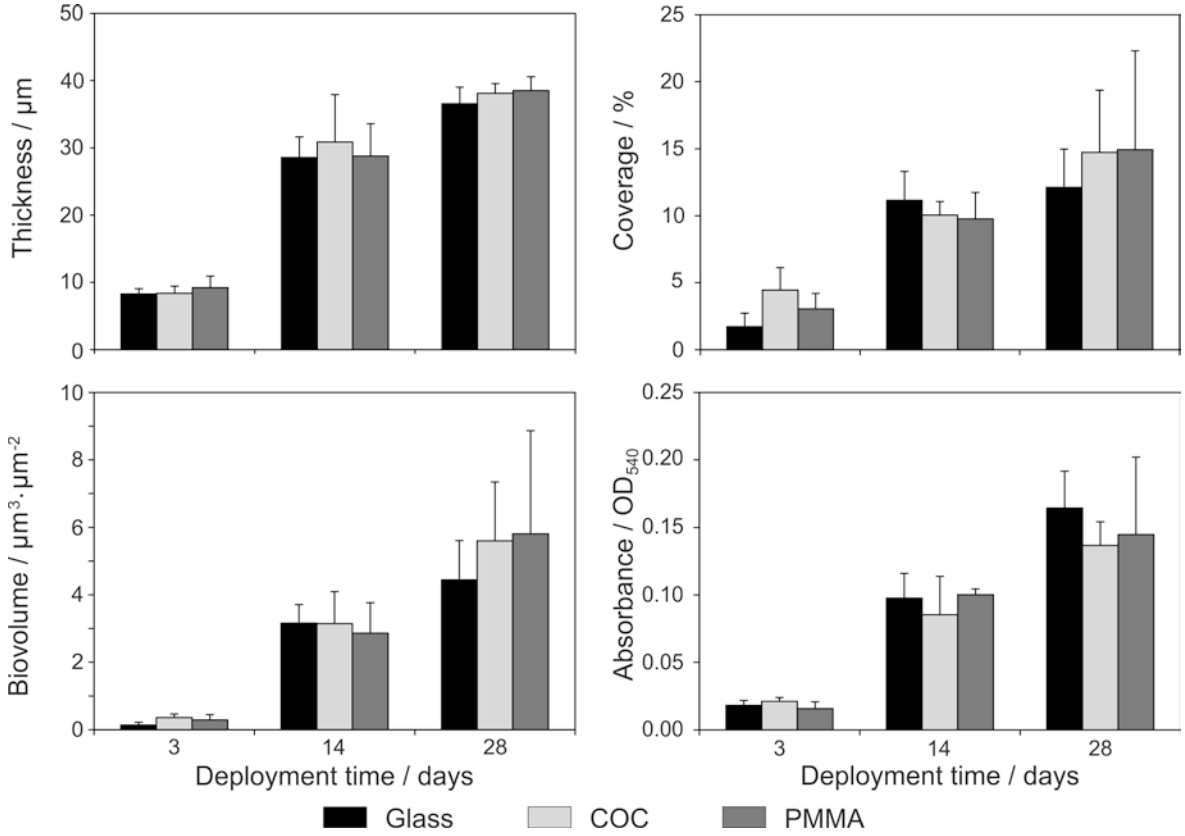


Figure 3.3: Only the coverage and biovolume of glass and COC after 3 days differed significantly from each other. No other effect of substratum material on the amount of biofilm was observed. Significant differences were found between the time points, with an overall trend of increased biofilm at each time point. Error bars are standard deviation from the mean of 6 repeats.

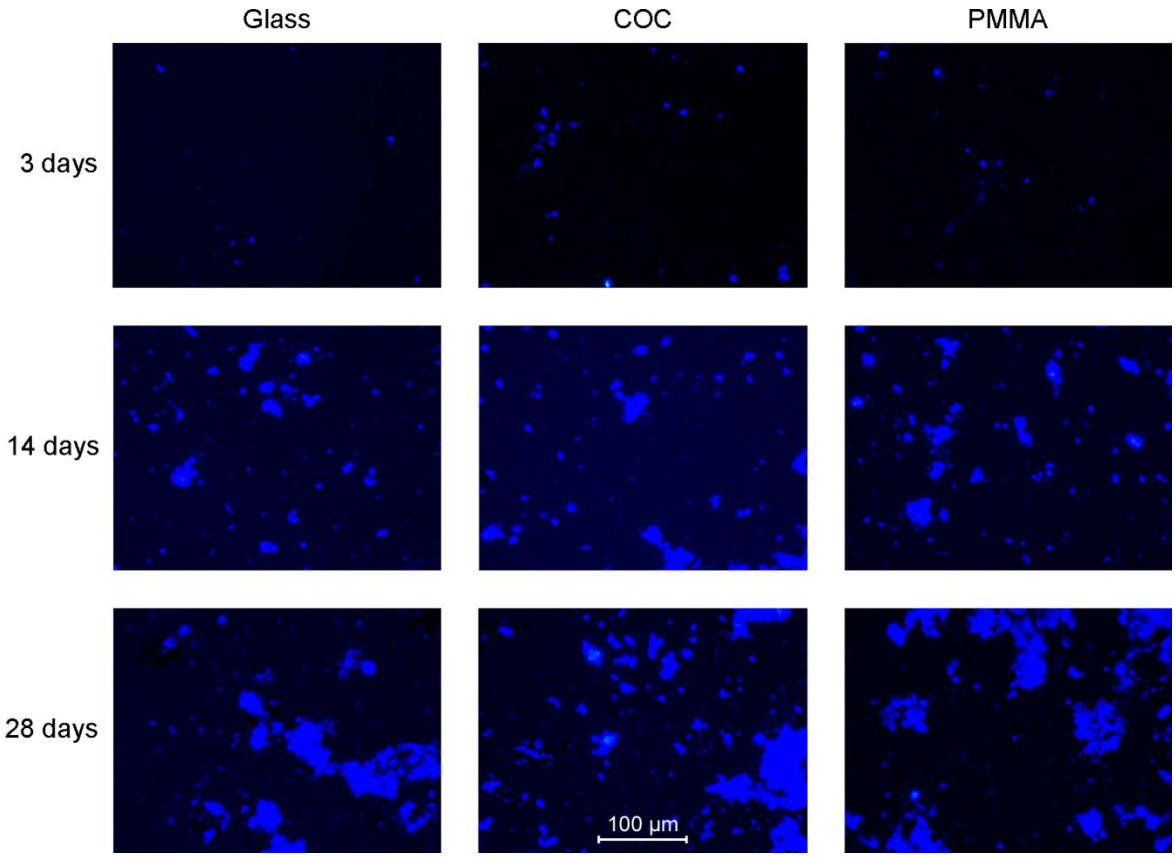


Figure 3.4: Over 28 days, the amount of biofilm on the surfaces of glass, COC and PMMA did not vary across the materials. However a clear increase in biofilm coverage was seen at each time point.

3.3.1.2.1. Fouling rate

Overall accumulation rates from day 0 to day 28 did not vary significantly between materials for any of the measurement parameter tests (P=0.280, 0.653, 0.566 and 0.503 for thickness, coverage, biovolume and absorbance, respectively). Additional ANOVAs to test for changes in fouling rate over time revealed that there was a significant difference in the fouling rates between materials but only between day 0 and day 3 and only for the coverage ($p = 0.009$) and biovolume ($p = 0.016$) measures. Post-hoc tests showed that the rate of increase in coverage and biovolume between day 0 and day 3 was significantly greater for COC than for glass ($p = 0.007$ and 0.014 for coverage and biovolume respectively).

ANOVA tests run on $\log_{10}$ transformed data showed that the rate of increase in thickness ($p < 0.001$ for all materials) and coverage ($p = <0.001$, 0.002 and 0.024 for COC, glass and PMMA respectively) changed significantly on all materials throughout the deployments. Additionally, the rate of increase in biovolume changed significantly throughout the deployments, but only on glass ($p < 0.001$). Post-hoc tests revealed that the rate of increase in thickness was reduced significantly on all materials between all successive time points except between days 0-3 and 3-14 ($p = 0.136$) on COC.

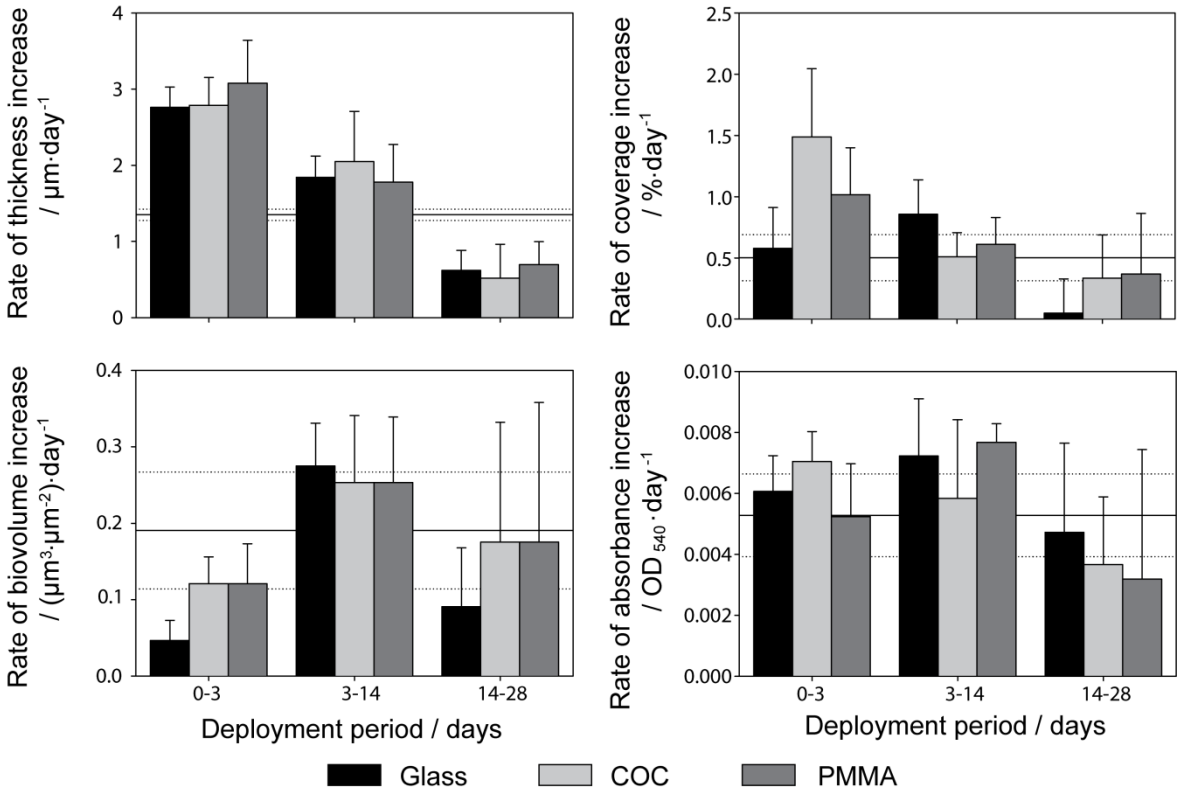


Figure 3.5: Overall accumulation rates did not vary between materials for any of the measurements made. However the rate of increase in thickness and coverage varied significantly between time periods in most cases. In these cases there appeared to be an overall reduction in the rate of increase in thickness and coverage. Error bars and dotted lines are standard deviation over 6 repeats.

3.3.1.3. Comparison of measurement parameters

Curve estimation was run on untransformed biovolume and optical density data (Figure 3.6) and the power curve was shown to have the greatest accuracy, with an R^2 value of 0.885 and a p value of <0.001. A Pearson's correlation was run on square root transformed data and showed a significant ($p < 0.001$) linear correlation of 0.921.

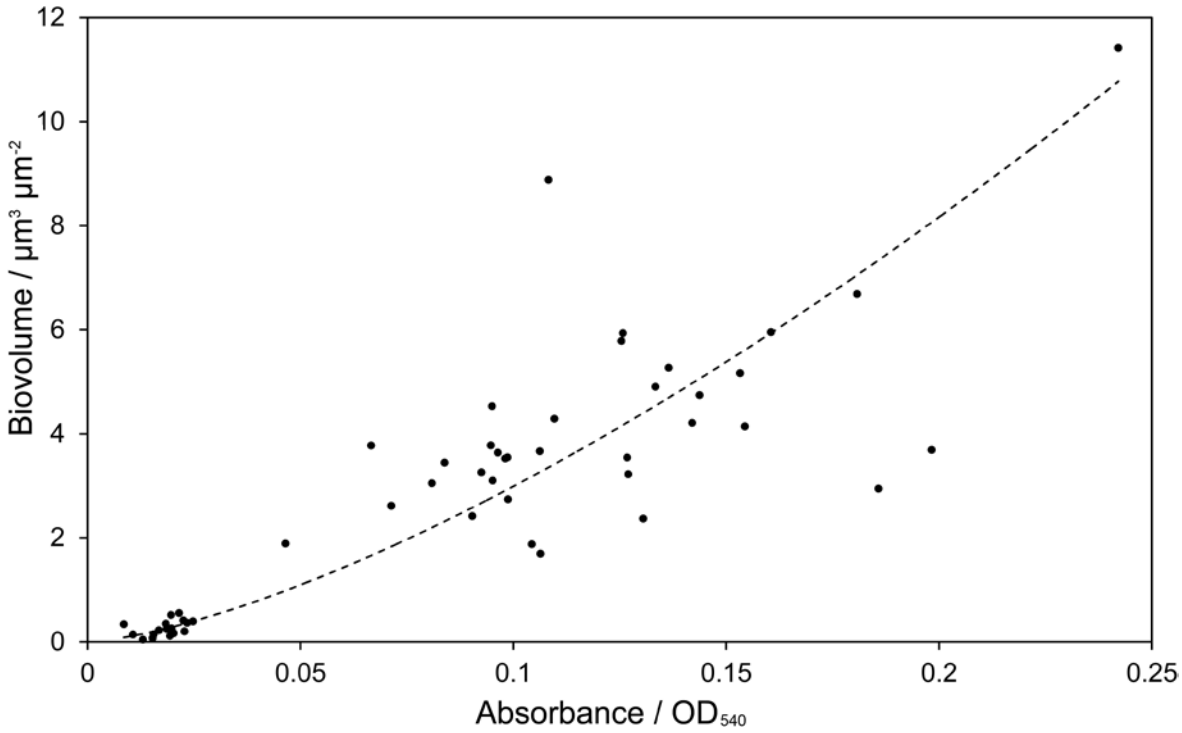


Figure 3.6: A power relationship was apparent between the derived measure of biovolume (the product of coverage and thickness) and the absorbance of crystal violet stained biofilms.

3.4. Discussion

The total amount of biofouling measured by thickness and absorbance showed no significant differences between any of the materials or time points tested. However, some differences were found in coverage and biovolume. Glass was found to have less biofilm coverage than COC after 3 days. Due to the lack of differences in thickness between materials at day 3, it is assumed that the difference in biovolume was in the most part due to the change in coverage. The differences were no longer apparent after 14 days indicating that whatever factor or factors had caused the differences between fouling on glass and COC was no longer present by 14 days exposure. Initial attachment of cells to a substratum is mediated in part by physicochemical properties of the material. In general, increasing roughness of a substratum material is found to result in an increase in the level of biofilm accumulation. However, Hilbert and colleagues (Hilbert *et al.*, 2003) found that below 0.9 μm, further reducing the R_a of stainless steel made no difference to bacterial attachment. The R_a values measured on the substrata in this study were much lower than 0.9 μm. Therefore, even though COC was significantly rougher than the other materials tested, it is likely that it was still too smooth to cause a significant difference in fouling from the other materials.

Many studies have also shown that an increase in hydrophobicity of a surface often results in a higher degree of microbial attachment (Allion *et al.*, 2006, Callow *et al.*, 2000, Ista *et al.*, 2004, Ista *et al.*, 1996, Wiencek and Fletcher, 1995, Wiencek and Fletcher, 1997). Ista and colleagues (Ista *et al.*, 2004, Ista *et al.*, 1996) in particular have shown that the marine bacterium *Cobetia marina* attaches more readily to hydrophobic (low energy) surfaces than to hydrophilic (high energy) surfaces, suggesting that this trend is applicable to the marine environment.

So it is perhaps therefore not surprising to find that there was more biofilm coverage on the lower energy COC than on glass. However in all of the previous studies, it was the initial attachment of bacteria to a surface that was under investigation, and as such the length of exposure was limited to between 20 minutes and 2 hours. Given the large difference in exposure times between these experiments and those in the literature, it would be unwise to make conclusions based on the findings of those studies.

It is likely that the surface energies of the substratum materials played a more important role at the earlier stages of biofilm formation than at the later stages. As the biofilms matured, the properties of the substratum material had less impact on the continued formation of biofilms and so by day 14 the initial differences in coverage between glass and COC were no longer apparent.

As the biofilms did not reach 100% confluence on any of the substrata, this opens the question of what changed on the surfaces between day 3 and 14 to stop biofilm coverage continuing to increase at a greater rate on COC than on glass. The differences in the physical

properties of glass and COC may have resulted in the attachment of different bacterial species of primary colonisers. Differences in primary colonising communities between hydrophobic and hydrophilic surfaces have been shown in previous studies (Jones *et al.*, 2007, Lee *et al.*, 2008). If the primary colonisers on COC provided more favourable conditions for secondary settlement compared to the primary colonisers on glass, then the settlement of secondary colonisers may have occurred earlier on COC than on glass (Martiny *et al.*, 2003). This hypothesis could be tested by studying the succession of bacterial communities associated with the different surface types.

A precursor to most biofilms is the formation of a conditioning film on the substratum (Jain and Bhosle, 2009). This is largely made up of polysaccharides, proteins, lipids, nucleic acids and amino acids (Siboni *et al.*, 2007), although its composition depends largely on the nature of the surrounding liquid and the substratum (Hood and Zottola, 1995, Whitehead and Verran, 2009). The conditioning film may provide additional binding sites for colonising bacteria (Lappin-Scott and Costerton, 1995) and may also alter the physical properties of the substratum surface (Schneider, 1996). It is possible that after 3 days exposure, the conditioning film on COC and glass differed, thereby affecting the successive attachment of biofilm forming bacteria. Indeed, it is known that biological polymers adsorb less readily to hydrophilic surfaces (Sigal *et al.*, 1998). In this case one might expect that the conditioning film on hydrophilic glass be less developed, thereby reducing the attachment of primary colonising bacteria. By 14 days, the conditioning film on glass may have had the time to mature, thus allowing the biofilm coverage to increase to the same level as on COC. Study of the development of the conditioning films (in isolation from the bacterial biofilm) on these materials may yield important insight into the functioning of biofilms in the marine environment.

The lack of a significant difference in the biofilm coverage on both glass and COC between days 14 and 28 suggests that the biofilms had reached a maximum carrying capacity in terms of coverage on both materials. At this stage any additional increase in the total amount of biomass of biofilm (as measured by biovolume or absorbance) was due to increasing thickness. The fact that the biofilms never reached 100% confluence on any of the surfaces before the increase in coverage ceased, suggests that there were interactions between different areas of the biofilms that limited their horizontal expansion. A well-known potential mechanism for such control is that of quorum sensing, a process by which signalling molecules, when present above a threshold concentration, have an effect on microbial physiological processes (Brown and Johnstone, 2001, Fuqua *et al.*, 1994).

No differences in fouling were found between glass and PMMA at any time point. This is in contrast to what was found by Kerr and colleagues (1999), who found that glass fouled to a greater extent than PMMA after 22 days exposure to water from the Firth of Clyde. Their

findings were in contrast to the general trend seen by other investigators where hydrophilic surfaces such as glass tend to foul less than hydrophobic surfaces such as PMMA. This unexpected result was attributed to PMMA being very slightly soluble. The dissolved PMMA was washed away, resulting in a form of foul release. No details were given about the specific type of PMMA or glass used in the Kerr study, and it is possible that variations in the manufacturing processes between those samples used by Kerr and in this study caused the difference in the comparative fouling of the materials.

Biofilm coverage is an important consideration in the construction of microsensors, especially for those where optical measurements require a clear window for reliable measurement. However, while glass had less biofilm coverage initially, it is COC and PMMA which are of the most interest in this project, as these are the materials that will be used for the bulk of the sensor bodies. The lack of significant differences between any of the measurement parameters at any time point between COC and PMMA suggests that the choice of either material will not be important when considering their direct biofouling potential. This does not mean however that the difference observed between glass and COC are of no value. Two important points have been identified in this study. Firstly, it has already been determined that the choice of material can affect the rate of biofilm formation in the marine environment. It is likely that this difference is a result of the differences in surface energies between materials. This means that investigation into modifying the surface energies of the sensor materials that will be in contact with biofilm forming organisms could help to provide some degree of fouling protection for the sensors. Such modifications could be achieved through the use of self-assembling monolayers (SAMs) or nano-structuring of materials as reviewed by Callow and Callow (2011). Secondly, we know that any reduced fouling potential imparted by the physical properties of the materials is only present at the early stages of a deployment, and so other antifouling strategies will be needed to maintain a low level of fouling throughout the sensors' deployment time.

The use of the crystal violet absorbance assay in this study as a semi-quantitative measure of biomass has proven to be somewhat representative of the biofilm formation on these materials. A power relationship was observed between the absorbance of the stained biofilms and their measured biovolume. This is in contrast to the microtitre plate assays used in chapter 2, where a strong, linear relationship was seen between biovolume and absorbance of redissolved crystal violet. This non-linear relationship between the measures indicated that for samples with a high level of biovolume, the absorbance measure will give a less accurate indication of the true level of biomass present, and so it can only be used reliably for short exposure periods. However, the results of the crystal violet absorbance assay in this study missed the significant difference in fouling at the early stages of deployment between glass and COC, indicating that it is only capable of showing large differences in fouling such as between

time points as opposed to between materials at the same time point. While the crystal violet assay has a place in biomass measurement where large differences are expected and a fine level of resolution is not necessary, it should not be relied upon as the only measure of biofilm accumulation.

In conclusion, it appears that either of the polymers COC and PMMA that have been proposed for the construction of microsensor devices can be used without the need to consider which fouls more or less. On the other hand, it may be expected that any components made with glass would foul to a lesser extent. This means that any decisions about the material to use in the construction of microsensors can be based on other factors such as machinability, chemical compatibility and optical properties without consideration of fouling potential. Additionally, when choosing the material with which to construct microsensors, one may also consider the compatibility of the material to antifouling techniques such as SAMs and nano-structuring.

Chapter 4 Effect of low-concentration diffusible molecules on biofilm formation and community structure

4.1. Introduction

Biofilms are complex structures which utilise chemical signalling to regulate many of their biological processes. Over the last 15 years many studies attempting to understand these processes have been published (Davies *et al.*, 1998, Moller *et al.*, 1998, Nobile and Mitchell, 2005, Sudarsan *et al.*, 2008, Teasdale *et al.*, 2009, Vejborg and Klemm, 2008, Xavier and Bassler, 2005). As a result there are now many known chemicals that are used to regulate the lifecycles of biofilms, some of which modifying the formation and development of biofilms at a physiological level. The application of these compounds, herein referred to as low-concentration diffusible molecules (LCDMs), represents an alternative approach to the control of biofilm formation instead of antibiotics or bactericides which induce cell death. By using non-toxic LCDMs instead of chemicals which induce cell death, problems such as the development of antibiotic resistance will be overcome. In addition, the use of non-toxic chemicals to control biofouling will negate the need to introduce toxic compounds into the potentially fragile ecosystems in which marine microsensors will be deployed.

One such chemical is nitric oxide (NO), a water-soluble free radical reactive gas known to play a role in many biological processes. The addition of low levels of NO reduces the intracellular concentration of the signalling molecule cdGMP, which in turn triggers an innate dispersal mechanism (Barraud *et al.*, 2006, Barraud *et al.*, 2009b). NO has been found to induce dispersal of several single species biofilms, as well as mixed species biofilms (Barraud *et al.*, 2009b).

Another example of a signalling molecule found to effect biofilm formation is *cis*-2-decenoic acid (CDA). CDA is a short chain, mono-unsaturated fatty acid produced by *P. aeruginosa* which has been shown by Davies and Marques (2009) to induce biofilm dispersal in several single species systems as well as reduce the formation of *P. aeruginosa* biofilms. The exact mechanism by which CDA reduces biofilm formation is not understood. However, it is structurally related to other signalling molecules known as diffusible signalling factors which appear to act as cell to cell signalling molecules in both Gram negative and Gram positive bacteria, as well as fungi (Boon *et al.*, 2008, Dow *et al.*, 2003, Torres *et al.*, 2007, Vílchez *et al.*, 2010).

Patulin is a mycotoxin produced by fungi of the genera *Penicillium* and *Aspergillus* (Bergel *et al.*, 1943). It inhibits the growth of both Gram positive and Gram negative bacteria as well as some fungi and protozoa (Bergel *et al.*, 1943, Kavanagh, 1947, Klemmer *et al.*, 1955, Lee and Röschenthaler, 1986, Raistrick, 1943). Rasmussen and colleagues (2005) showed that patulin inhibits quorum sensing in *P. aeruginosa* and increases the susceptibility to the antibiotic

tobramycin. Liaqat and colleagues (Liaqat *et al.*, 2008, Liaqat *et al.*, 2010) studied the effect of patulin on *Klebsiella sp.*, *B. subtilis*, *Bacillus cereus* and *P. aeruginosa* biofilms. It was found that Patulin increased the formation of *B. cereu*s and *P. aeruginosa* biofilms at concentrations between 0.25 and 25 μM. Patulin alone was not shown to affect either *Klebsiella sp.* or *B. subtilis* biofilms. However, when treated with a combination of patulin and EDTA, *B. subtilis* biofilm formation was increased.

In natural ecosystems many biofilms do not exist as single species structures, but rather consist of multiple species. The combined populations of microorganisms found in such a system is referred to as a community. The biofilm community members (i.e. microbial populations) often do not live independently of each other, but instead interact to create a biofilm ecosystem with each member filling a niche. The interaction between populations may be mutually beneficial. For example Palmer and colleagues (2001) showed that the oral bacteria *Streptococcus oralis* and *Actinomyces naeslundii* did not form biofilms when grown independently, but did when grown together. However, the interactions between biofilm populations are not always mutually beneficial, with some populations interacting in a commensal fashion – a benefit to one population but no effect on the other, or in competition (Molin *et al.*, 2004).

Some LCDMs such as nitric oxide and CDA have been shown to effect the formation and dispersal of multi-species biofilms, however no investigations have been carried out to investigate the effects of these molecules at a community level. Given that such molecules affect different species to different extents (Barraud *et al.*, 2009b, Davies and Marques, 2009), it is likely that different LCDMs will result in a shift in the species composition and abundance within biofilm communities, rather than a uniform dispersal or disruption of the entire community.

Therefore, in order to begin to understand the effects that LCDMs have on multispecies biofilms, it is desirable to determine the community structure of the biofilms. One technique that is commonly used to study microbial communities is denaturing gradient gel electrophoresis (DGGE) (Diez *et al.*, 2001, Muyzer *et al.*, 1993, Muyzer and Smalla, 1998, Sahan *et al.*, 2007). This technique profiles the species diversity of a system by separating PCR amplicons of different species in a polyacrylamide gel based on their cytosine (C) and guanine (G) contents. Unlike many other profiling methodologies, DGGE allows the separated PCR products to be sequenced and therefore allows taxonomic investigation of a system.

In this study, three LCDMs, NO, CDA and patulin were investigated for their effect on mixed marine biofilm communities grown from natural microbial assemblages taken from the sea. Firstly the effect on biofilm formation was measured using optical density and microscopy techniques. Additionally, their effects on biofilm communities were investigated using DGGE.

4.2. Materials and methods

4.2.1. Effect of varying LCDMs on biofilm formation

The effects on biofilm formation of three different LCDMs, the NO donor, sodium nitroprusside (SNP), CDA and patulin were investigated in a titreplate microcosm, using a standard inoculum (section 2.3.1), and filter sterilised seawater (SSW) as the growth medium. This microcosm was chosen, as it allowed the LCDMs to be applied in known concentrations, under controlled conditions.

4.2.1.1. Preparation of LCDM solutions

Five stock solutions of 100 – 0.01 mM CDA (Advanced Synthesis Technologies, CA) and patulin (Sigma-Aldrich, UK) were created in neat ethanol or ethyl acetate respectively and stored at -20°C until needed. CDA stocks were stored under argon. To create growth media doped with CDA or patulin, 1 μl of stock solution was added to 2 ml of SSW to give final concentrations of between 5 nM and 50 μM. Each solution was sterilised through a 0.2 μm pore sized filter. To control for the effects of the carrying solvents, ethanol and ethyl acetate, control solutions were also created using 1 μl of pure solvent in 2 ml of SSW.

Solutions of the NO donor sodium nitroprusside (SNP, Sigma-Aldrich, UK) were created daily as they were needed, due to the instability of SNP over long periods. SNP solutions were created by serially diluting 50 mM SNP (in SSW) with SSW to create five final concentrations of SNP of 500 nM to 5 mM. These solutions were sterilised through a 0.2 μm pore sized filter before use. SNP was used at a higher concentration range than the other LCDMs because its active component, NO, has previously been shown to be released by SNP at an efficiency of about 0.1% (Barraud, 2007).

4.2.1.2. Growth of biofilms

Standard inoculum (section 2.3.1.) was diluted 100x in the LCDM doped SSW solutions to give two sets of five inocula with final CDA and patulin concentrations between 5 nM and 50 μM, and five inocula with final SNP concentrations between 500 nM and 5 mM. To the wells of two 96-well titreplates 180 μl of each inoculum was added, giving two plates with 5 repeats for each treatment and control. Both plates were left at 22 °C for 4 hours to allow the attachment of cells to the surface of the titreplates. To test the effect of light on the action of the LCDMs, one plate was exposed to light from a Philips Master TL-D super 80 fluorescent tube lamp (Appendix 6), while the other plate was left in the dark during attachment. Both plates were static during attachment.

After attachment, the suspensions in the wells were removed, and the wells were rinsed carefully with SSW and new LCDM solutions were added. Plates were then left in the same conditions as for attachment for 5 days, with solutions being changed every 24 hours. After the 5 day growth period, the solutions were removed from the plates and the wells were rinsed with SSW before being fixed with 4% PFA. The biomass of the biofilms was measured as in section 2.2.5.2, using 180 μl crystal violet and 180 μl acetic acid. The effects of each LCDM in both the light and the dark were tested using a one-way ANOVA on log transformed data, and a Dunnett's t post hoc test was performed to compare the effects of each concentration used with the control. To determine the effect of light on biofilms treated with LCDMs, the absorbance data for biofilms grown in the presence of LCDMs were normalised against their respective controls, to give the data as a proportion of the control. A two-way ANOVA was then performed to test for any effects of light.

Further biofilms were grown in 6-well titreplates to allow visualisation by microscopy. These biofilms were treated in the same way as above, but using 2 ml of media per well instead of 180 μl. Only one concentration of each LCDM was investigated further as follows: 500 μM SNP, 5 μM patulin and 50 μM CDA. These concentrations were selected due to following the results of previous tests on a range of concentrations. After 5 days growth, the biofilms were fixed with 4% PFA and measured and analysed as in section 2.2.5.

4.2.2. Toxicity of LCDMs

To determine whether the effects of the LCDM were due to changes in biofilm physiology or toxicity, colony based assays were performed. Samples of standard inoculum were diluted 1000x and treated with a final concentration of 500 μM SNP, 50 μM CDA or 5 μM patulin in SSW. The samples were then left in either the light or the dark for 24 hours.

After this time the suspensions were diluted 100x, 1000x and 10000x in SSW before 100 μl of each was spread on to three 90mm marine agar plates. SSW was used as a negative control. These plates were left in an incubator at 22°C for 3 days before the colonies were counted. Plates with >300 or <30 CFU were not counted.

4.2.3. Effects of variable growth conditions on biofilm formation

4.2.3.1. Effect of different growth vessel sizes

To determine whether the amount of biofilm growth was affected by the size of the growth vessel used, absorbance data obtained using the 6-well plate assay were compared with absorbance data obtained using the 96-well plate assay at the appropriate LCDM concentration. Two-way ANOVAs were run comparing the proportional change in

absorbance for biofilms grown with different LCDMs and in different sized plate wells. This showed whether there was any interaction between LCDM effect and growth vessel size.

4.2.3.2. Effect of different growth substrata

The effect of the substratum material on which the biofilms were grown was investigated by growing biofilms in additional 6-well titreplates. However, at the base of each well in these plates, a 20 mm x 20 mm coupon of PMMA (Plexiglas XT) was stuck with a silicone-based adhesive and allowed to cure for 3 days to allow any volatile liquids to evaporate. Biofilms were grown in these plates in SSW in the same way and at the same time as those in section 4.2.1.2. The samples were also analysed the same way as those in section 4.2.1.2, with the exception that no crystal violet assay was used.

4.2.4. Community analysis

4.2.4.1. Sample collection and DNA extraction

Biofilms were grown in the presence of the molecules SNP (500 μM), CDA (50 μM) and patulin 5 μM) in the light and the dark in the same way as in section 4.2.1.2, but with 3 repeats for each treatment. After 5 days growth, the wells were rinsed with SSW. DNA was extracted and purified using a modified protocol for the Qiagen DNeasy blood and tissue kit. One gram of sterile 100 μm diameter glass beads and 180 μl of enzymatic lysis buffer (Appendix 9) were added to the wells, which were then sealed with an adhesive lid to avoid contamination between wells while the plate was shaken for 15 seconds on a Tecan Sunrise microplate absorbance reader. The plates were then left at 37°C for 15 minutes. The shaking and heating process was repeated 3 times, with an additional shaking step after the final heating. The solution was pipetted into clean microfuge tubes and remaining biofilm was removed from the wells using sterile cotton swabs. The swab tips were placed into the microfuge tubes. DNA purification was then continued following the standard procedure for the Gram-positive protocol as per the manufacturer's instructions.

4.2.4.2. PCR

Universal bacterial PCR primers U968f-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and U1401R (5'-CGG TGT GTA CAA GAC CC-3') were used to amplify partial 16S rDNA sequences from the V6 region (Nubel *et al.*, 1996, Piquet *et al.*, 2011). The first 40 bases of the primer U968f-GC was a GC clamp to stop complete denaturation of dsDNA strands during DGGE. PCRs were run for each sample using the following conditions. Each reaction contained Promega PCR master mix (1x), 3 pmol of each primer, 10 μl DNA template and water to make up the

reaction mixture to a volume of 25 µl. The PCR was run in a MJ Research Tetrad 2 thermal cycler with a denaturation at 94°C for 5 minutes followed by 10 touchdown cycles, reducing the annealing temperature by 0.5°C per cycle from 60 to 55°C for 1 minute and an extension at 72°C for 2 minutes. This was followed by a further 20 cycles with an annealing temperature of 55°C. The amplification was finished with a final extension step of 72°C for 30 minutes to reduce artifactual double bands in DGGE gels (Janse *et al.*, 2004). PCR products were visualised on agarose gel stained with ethidium bromide and DNA concentrations were calculated using the ImageJ software.

4.2.4.3. DGGE and cloning

DGGEs were performed using the BioRad Dcode system. Three hundred ng of each PCR product was run through a 6% polyacrylamide gel with a denaturant gradient from 46 to 60% (based on a 100% denaturant gel in Appendix 10) for 18 hours at 60°C at 100 V. Three lanes in each gel contained a standard created from bands extracted from previous DGGEs to allow comparisons between gels.

The gels were stained in a 0.5 mgml $^{-1}$ ethidium bromide / TAE solution for 15 minutes and then destained with fresh TAE buffer for a further 15 minutes. The stained gels were imaged on a Syngene G:Box UV transilluminator. Dominant bands were extracted, reamplified and run through a second DGGE gel to confirm their position and that they were single bands. Products with multiple bands were re-extracted, amplified and run through DGGE again until a single band was obtained. Single bands were reamplified using the same primers but without the 40 base pair GC clamp. PCR products were purified using the Qiagen QIAquick PCR purification kit before being sequenced by Source Bioscience (Oxford) using the Sanger sequencing technology. Those bands that gave poor sequence data were cloned by first ligating into a pDrive cloning vector (Qiagen). Competent cells were prepared by culturing *E. coli* Xl-1 blue cells in lysogeny broth (LB, Oxoid) overnight at 37°C. The cultures were then pelleted and resuspended in transformation buffer (50 mM $CaCl_2$, 10 mM Tris-HCl pH 7.4). Plasmids were transformed into the competent cells using a heat shock technique according to the plasmid manufacturer's instructions. Transformed cells were placed in SOC medium (Appendix 3) for 2 hours at 37°C before being used to inoculate selective LB agar plates containing 100 mg·l^{-1} ampicillin (Fisher Scientific, UK) and incubated at 37°C overnight. Three colonies from each plate were picked randomly and used to inoculate 5 ml of LB. These cultures were again left overnight at 37°C before being pelleted and the plasmids extracted using the Sigma-Aldrich (UK) GenElute Plasmid Miniprep kit according to the manufacturer's instructions. Purified plasmids were sent to Source Bioscience (Oxford) to be sequenced using the standard T7 promoter (F) primer. Sequences were aligned using the MEGA 4 software package, and redundant plasmid DNA sequences were trimmed from both ends of the 16S

sequences. These sequences were combined with the earlier sequence set and submitted to the EMBL database under accession numbers HE818046 to HE818071.

4.2.4.4. DGGE and sequence data analysis

DNA sequences were aligned using the NAST program on the GreenGenes database website (DeSantis *et al.*, 2006a, DeSantis *et al.*, 2006b) and classified using the Classify program.

The banding patterns of the gels were compared using the Phoretix 1D software to obtain a matrix of bands present in each sample and their relative intensities. These data were further analysed using DGGEstat software (van Hannen, 2000) to create a qualitative Dice similarity matrix and a quantitative Euclidean similarity matrix. The similarity data were visualised with a multidimensional scaling (MDS) plot using the ALSCAL program within the SPSS 19 software. Species richness (number of species) and Shannon diversity indices (Shannon, 1948) were calculated for each sample, and paired t-tests were used to compare the species richness and diversity of each treatment to the control.

4.3. Results

4.3.1. Effect of carrying solvents on biofilm formation

To determine whether any potential effect of the LCDMs was caused by the carrying solvent, the controls were compared using a two-way ANOVA test. No differences between any of the controls were observed ($p = 0.107$). However, those biofilms grown in the light had between 34% and 42% less biomass than those grown in the dark, which was shown to be significant ($p < 0.001$) (Figure 4.1). No significant interaction between light and carrying solvent was observed ($p = 0.609$).

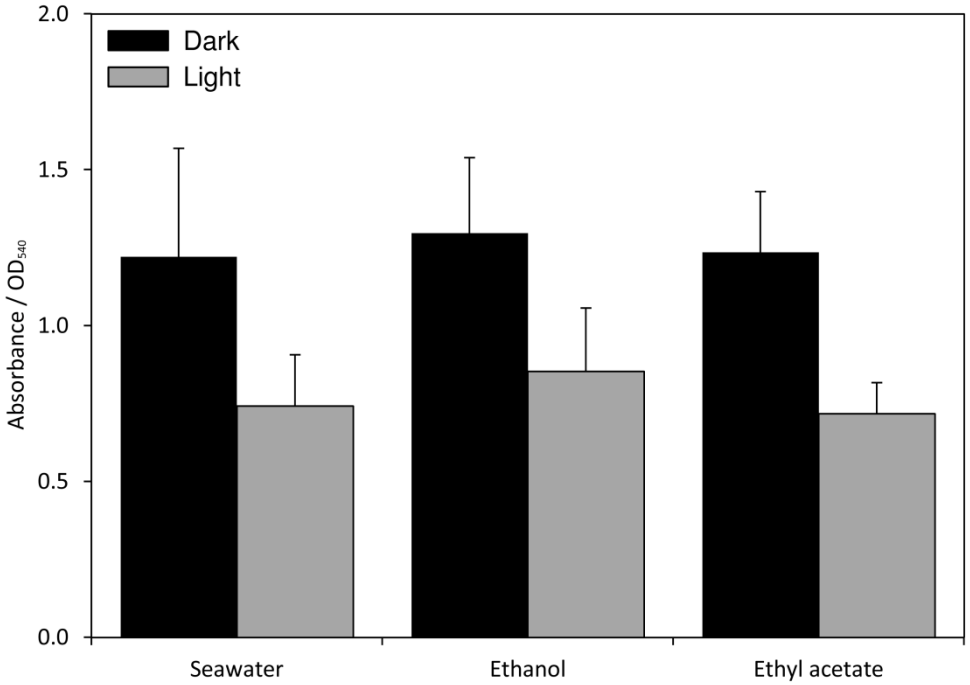


Figure 4.1: Comparisons of the different carrying liquids for the LCDMs showed that while dilute ethyl acetate and ethanol had no effect on biofilm formation, those grown in the light had less growth than those grown in the dark. Error bars are standard deviation form the mean of 15 repeats.

4.3.2. Effect of LCDMs on biofilm formation

4.3.2.1. SNP

One-way ANOVA tests showed that there was a significant effect of changing concentration of SNP on biofilm formation in both the light and the dark ($p < 0.001$). Two-way ANOVAs showed that there was also a significant reduction in the formation of biofilms in light conditions compared with formation of biofilms in the dark ($p < 0.001$). This effect of light was apparent even when data normalised against their respective controls were compared (Figure 4.2). Biofilms grown in the dark in 50 μM SNP showed a 33% reduction in growth compared with the control and was the lowest concentration at which SNP had a significant

effect in the dark ($p < 0.001$). The formation of biofilm was reduced by 90% at 5000 mM SNP in the dark. The concentration at which SNP had caused a significant ($p < 0.001$) reduction in biofilm formation in the light was 5 μM, 10 times lower than in the dark. Biofilm formation was reduced by 82%, 69% and 59% in the light at 50, 500 and 5000 μM. Further Dunnett's tests with 50 μM SNP as the control group showed that the level of biofilm formation at 500 and 5000 μM SNP was significantly greater than at 50 μM ($p < 0.001$ in both cases).

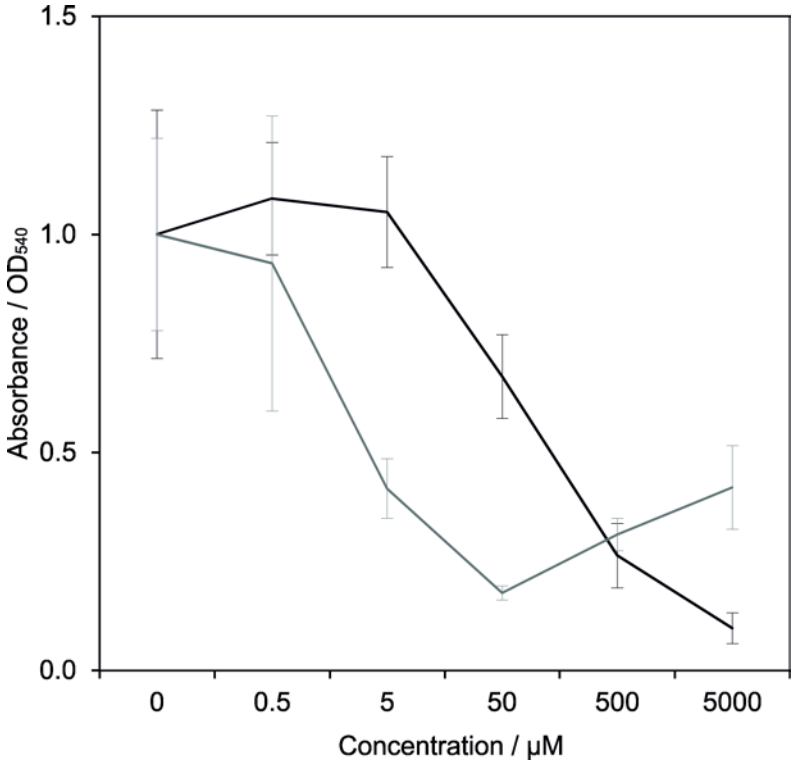


Figure 4.2: SNP reduced the formation of *in vitro* grown marine biofilms at concentrations of 50 μM and above in the dark (black line) and 5 μM and above in the light (grey line). In the light, 50 μM SNP had the greatest effect on biofilm formation, with greater concentrations having a reduced inhibitory effect. Error bars are standard deviation from the mean of 15 repeats.

4.3.2.2. CDA

One-way ANOVA tests showed that CDA significantly changed the amount of biofilm formed in both the light and the dark ($p < 0.001$ in both cases). Two-way ANOVA tests also showed that the presence of light significantly reduced biofilm formation ($p < 0.001$) this was true even when data proportional to the light and dark controls were compared. Dunnett's post-hoc tests showed that those biofilms grown in the light and the dark showed significant reduction in biofilm formation ($p < 0.001$ in both cases) by around 27% when treated with 0.005 μM CDA. A significant increase in biofilm formation ($p = 0.031$) of 15% was observed at 0.5 μM CDA when grown in the dark, and at 50 μM CDA, there was another reduction in biofilm of approximately 18% ($p = 0.022$). No significant increase in biofilm formation was

observed for biofilms grown in the light, but biofilm formation decreased significantly ($p < 0.001$) at concentrations of 5 μM and above.

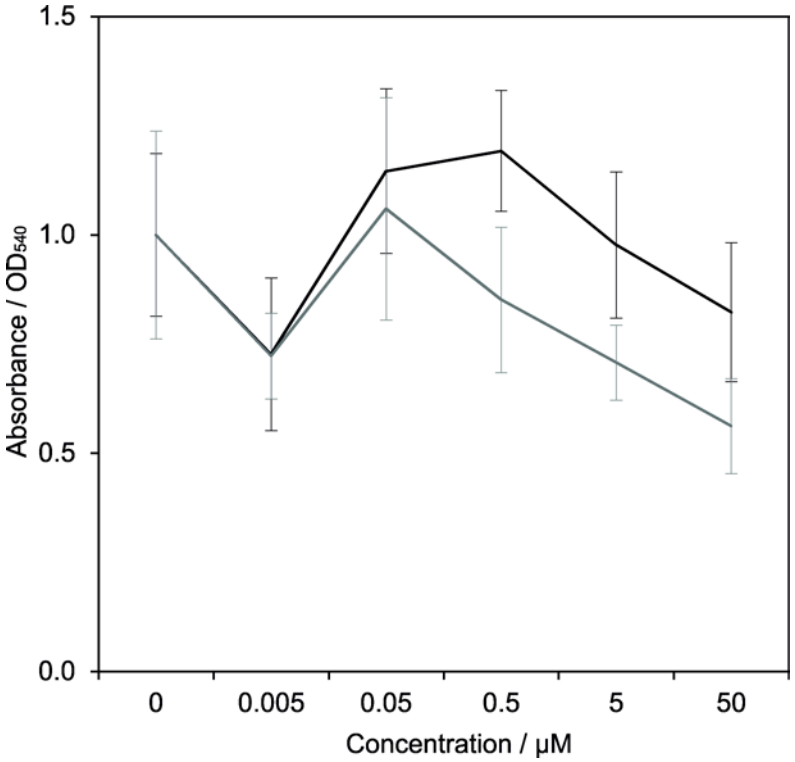


Figure 4.3: CDA altered the formation of marine biofilms grown *in vitro*. At 0.005 μM CDA, biofilm formation decreased in both the dark (black line) and light (grey line), but returned to normal at 0.05 μM in both cases. In the dark, biofilm formation increased significantly at 0.5 μM but was reduced at 50 μM. No increases in biofilm formation were seen in the light. There was however significantly less biofilm growth overall in the light than in the dark. Error bars are standard deviation form the mean of 15 repeats.

4.3.2.3. Patulin

One-way ANOVA tests showed patulin significantly reduced biofilm formation in the light and the dark ($p < 0.001$ in both cases). Two-way ANOVAs showed no significant interaction between patulin and the light ($p = 0.123$). However, the lowest concentration at which patulin had a significant effect in the dark was 0.5 μM ($p < 0.001$) while in the light it was 0.05 μM ($p = 0.008$). Patulin reduced biofilm formation at all concentrations higher than this with the greatest reduction being 95% in the dark and 82% in the light.

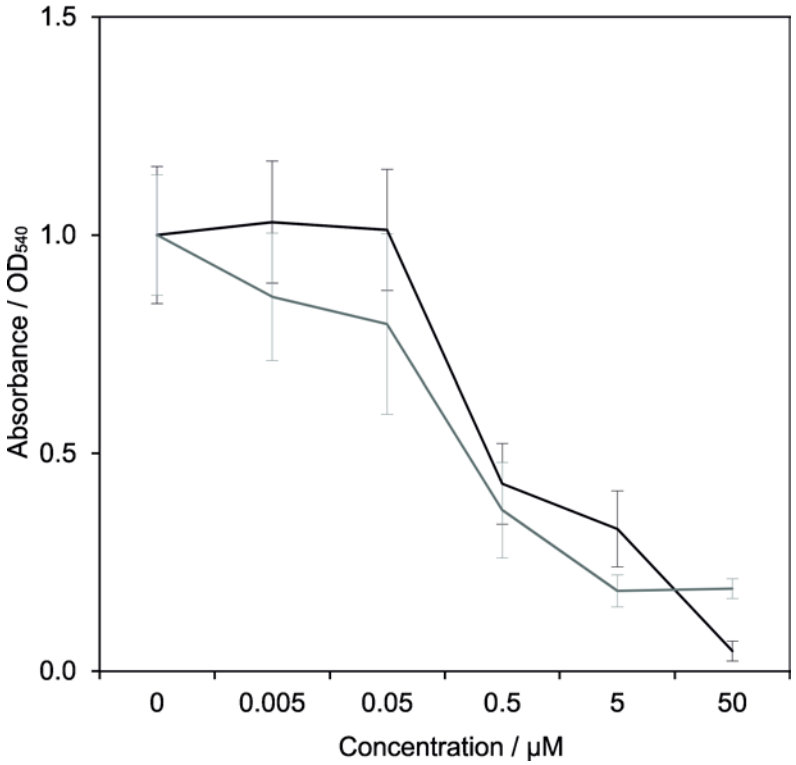


Figure 4.4: Patulin reduced bioilm formation in both the light (grey line) and dark (black line). In the light, biofilm formation was significantly reduced at concentrations of 0.05 µM and above. In the dark, biofilm formation was significanlty reduced at concentration of 0.5 µM and above. No significant diffrences in overall fouling in the light and dark was seen. Error bars are standard deviation form the mean of 15 repeats.

4.3.2.4. Growth of biofilms in 6-well plate microcosm

Biofilms were grown again in the presence of the LCDMs SNP, patulin and CDA, but only at a single concentration and in larger vessels to allow analysis by microscopy. A significant reduction in biofilm formation was seen with both 500 µM SNP ($p < 0.001$) and 5 µM patulin ($p < 0.001$) at concentrations shown previously to significantly reduce biofilm formation (Figure 4.5 and Figure 4.6). For 50 µM CDA however, where there was a significant reduction in biofilm formation in the 96-well plate assays. In the 6-well plates there was a significant increase in biofilm formation by 221% in the light and 92% in the dark.

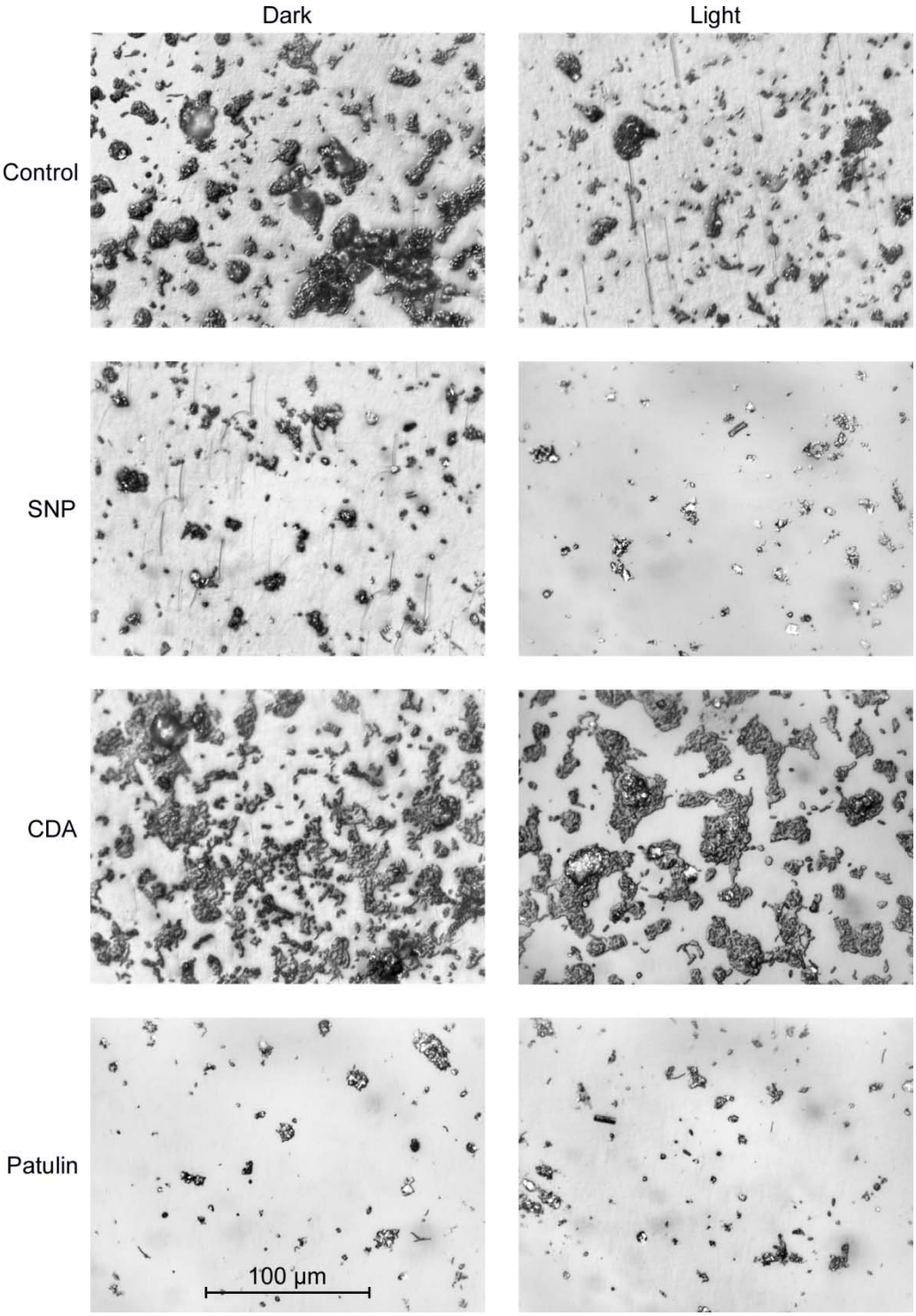


Figure 4.5: Marine biofilms grown in 6-well microplates had significantly less formation when grown in the presence of 500 µM SNP and 5 µM patulin. However treatment with 50 µM CDA increased biofilm formation instead of reducing it as was seen in previous tests.

Two-way ANOVAs revealed that there was indeed a significant difference in the amount of biofilm formation between 6-well plates and 96-well plates ($p < 0.001$) and there was a significant interaction between plate type and LCDM ($p < 0.001$) and between plate type, LCDM and light ($p < 0.001$).

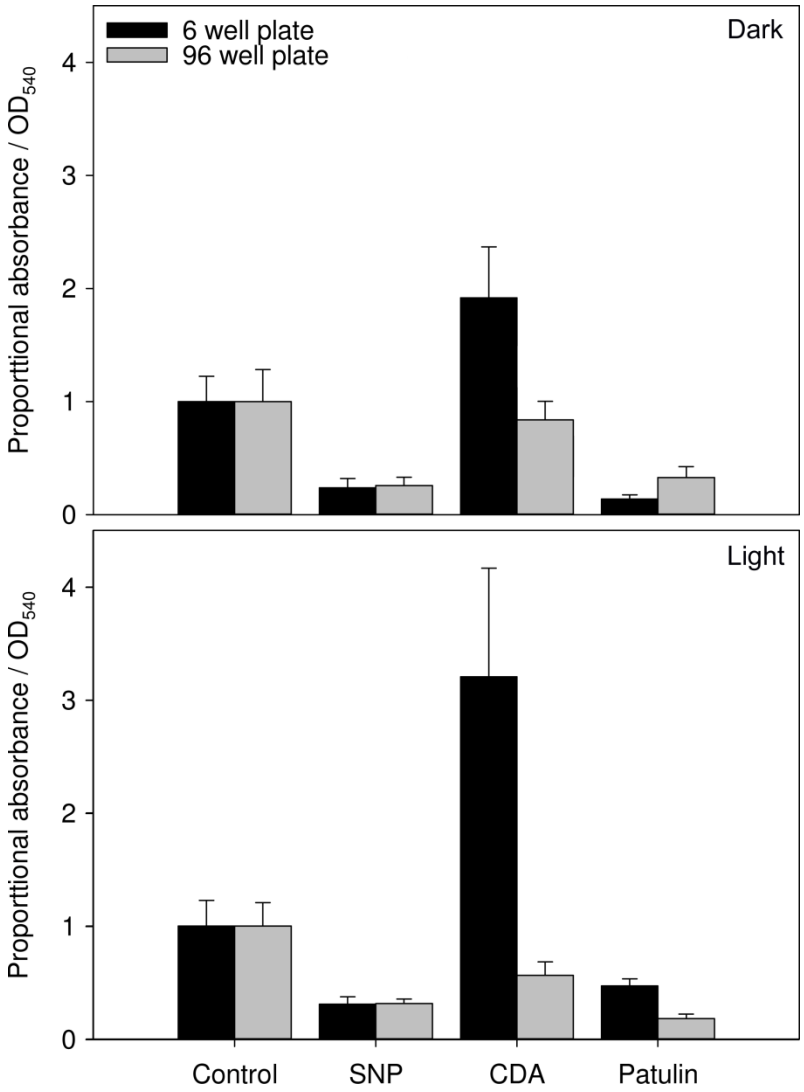


Figure 4.6: When grown in the presence of SNP or patulin, the size of the growth vessel did not appear to cause a great difference in the amount of biofilm that formed. However the difference between the growth of biofilm in the presence of CDA is very great between the 96-well plates and the 6-well plates. Error bars are standard deviation form the mean of 9 or 15 repeats (for 6 and 96-well plates resectively).

To determine the source of these interactions, t-tests were used to make pairwise comparisons between biofilms in either the light or the dark for each LCDM in both 96-well and 6-well plates. The amount of biofilm growth relative to the control varied significantly between 96-well and 6-well plates in the light and dark for all both CDA and patulin ($p < 0.001$ in all cases), but not for SNP ($p = 0.338$ and 0.837 for the dark and light respectively). Growth in a 6-well plate increased biofilm formation in all significant cases except for patulin in the dark, where there was significantly lower biofilm formation in the 6-well plate.

4.3.3. Toxicity of LCDMs

Marine inoculum suspensions grown on agar plates showed marked differences in growth depending on the LCDM with which they were treated before plating. While there was no difference in CFU counts between light and dark in the control or CDA, the effect of SNP in the presence of light was markedly increased. Patulin reduced colony formation from around $1.2x10^7$ CFU·ml^{-1} to an average of around $5.2x10^5$ CFU·ml^{-1} in the dark, and completely stopped colony formation in the light.

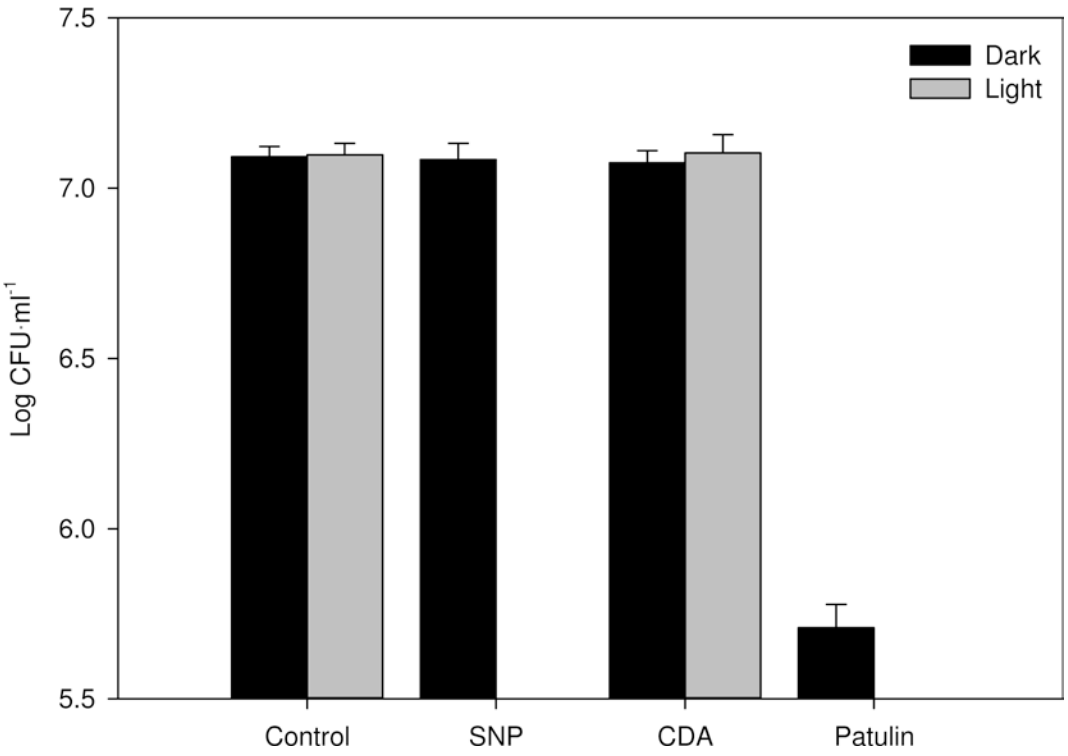


Figure 4.7: Marine inocula treated with different LCDMs and spread over Marine Agar plates after 24 hours exposure to the LCDM showed marked differences in colony formation. Patulin had the greatest effect, reducing colony formation in both the light and the dark. SNP also had a great effect, but only in the light. Error bars are standard deviation form the mean of 9 repeats.

4.3.4. Effect of different growth substrata

To determine whether the substratum material had any effect on the level of biofilm formation, a two-way ANOVA was run comparing the biofilm biovolumes on PMMA and polystyrene in both light and dark conditions. A significant effect of both light ($p < 0.001$) and substratum material ($p < 0.001$) were found. Additionally, a significant interaction between light and substratum material was found ($p < 0.001$).

This was further investigated using t-tests for pairwise comparisons of each light and substratum condition (Figure 4.8). No significant differences between biofilms grown in the dark on polystyrene and biofilms grown in both the light and the dark on PMMA were

observed. However, biofilms grown on polystyrene in the light had 57% less growth than those grown on polystyrene in the dark and 64% less than the equivalent biofilms on PMMA. Both of these differences were significant ($p < 0.001$ in both cases).

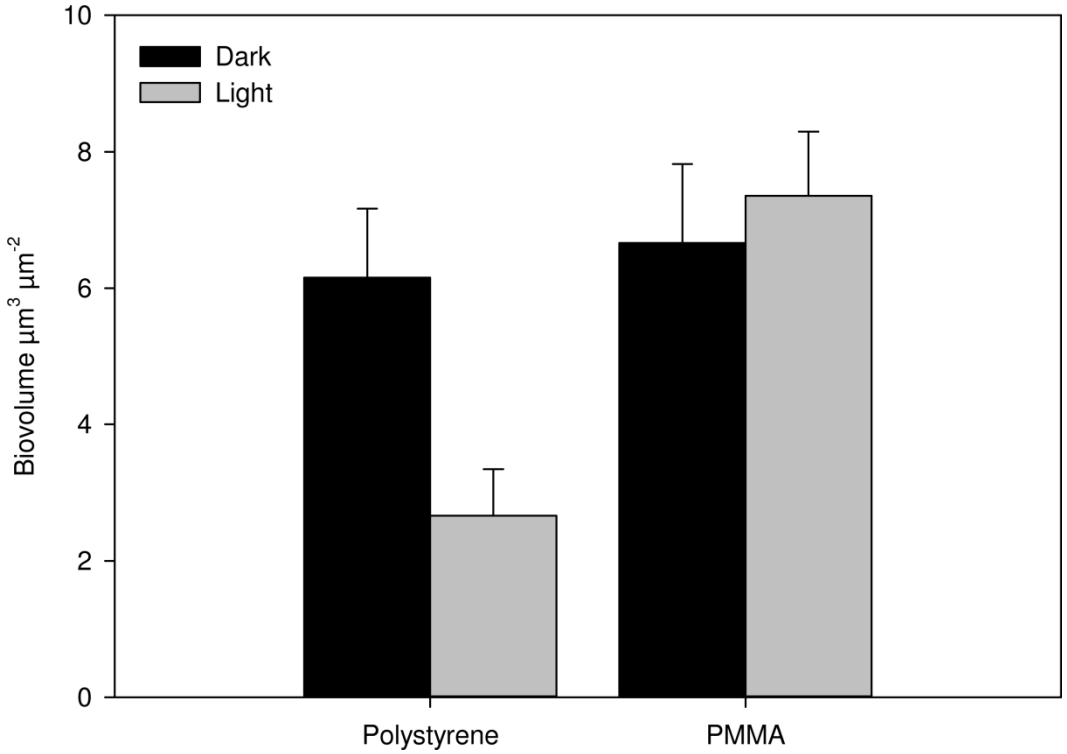


Figure 4.8: Biofilms grown in-vitro on polystyrene or PMMA show different levels of growth depending on whether grown in the light or dark. While the presence of light reduces biofilm formation on polystyrene, the same is not true of PMMA. Error bars are standard deviation of 9 repeats.

4.3.5. Community analysis

Phylogenetic analyses of extracted DGGE bands indicated that 19 of the 26 identified biofilm forming organisms in this system were in the class Gammaproteobacteria (Table 4.1). Other organisms included members of the Alpha and Beta proteobacteria, and only one organism, *Luteolibacter* of the phylum Verrucomicrobia, was identified that did not belong to the Proteobacteria phylum. Eighteen unique identifications were made. Bands 02, 07, 11, 16, 17 and 18 were represented by more than one sequence and showed that the same banding position can represent more than one species. Bands 02, 16 and 17 contained species from different classes, while bands 07 and 18 contained organisms from the same class.

Table 4.1: DGGE bands were sequenced and compared to the GreenGenes database to identify the taxonomy of the organisms present in the system. Four organisms were identified to species level and most organisms were identified to the level of genus. Some banding positions were represented by more than one sequence (indicated by letters), and revealed that the same banding position could represent more than one species and in some cases those different species were from different classes.

Band	Identification	Taxonomic level of identification	Class
05	Rhodobacteraceae	Family	Alphaproteobacteria
06	*Loktanella*	Genus	Alphaproteobacteria
13	*Hyphomicrobium*	Genus	Alphaproteobacteria
02b	Alcaligenaceae	Family	Betaproteobacteria
16b	*Delftia*	Genus	Betaproteobacteria
17b	*Delftia*	Genus	Betaproteobacteria
01	*Colwellia*	Genus	Gammaproteobacteria
02a	*Pseudoalteromonas issachenkonii*	Species	Gammaproteobacteria
03	Altermonadaceae	Family	Gammaproteobacteria
04	*Pseudoalteromonas*	Genus	Gammaproteobacteria
07a	*Glaciecola*	Genus	Gammaproteobacteria
07b	*Pseudoalteromonas issachenkonii*	Species	Gammaproteobacteria
07c	*Marinobacterium*	Genus	Gammaproteobacteria
07d	*Marinomonas mediterranea*	Species	Gammaproteobacteria
08	Oceanospirillaceae	Family	Gammaproteobacteria
09	*Colwellia*	Genus	Gammaproteobacteria
10	*Pseudomonas pachastrellae*	Species	Gammaproteobacteria
11a	Thiotrichacaea	Family	Gammaproteobacteria
11b	Sinobaceraceae	Family	Gammaproteobacteria
12	*Pseudoalteromonas*	Genus	Gammaproteobacteria
14	*Simidua*	Genus	Gammaproteobacteria
16a	*Pseudoalteromonas*	Genus	Gammaproteobacteria
17a	*Marinobacter*	Genus	Gammaproteobacteria
18a	*Marinobacter*	Genus	Gammaproteobacteria
18b	*Marinobacter*	Genus	Gammaproteobacteria
15	*Luteolibacter*	Genus	Verrucomicrobiae

A total of 30 unique DGGE banding positions were found in analysis of samples treated with different LCDMs. The maximum number of bands found in an individual sample was 12; this was in a sample grown in the presence of SNP in the dark. In contrast, two samples (CDA in the dark and patulin in the light) had only 1 band and had the lowest species richness and Shannon diversity. A sample grown with SNP in the light had a Shannon diversity of 1.99 and had the highest diversity. However, comparisons of the species richness and Shannon diversities of the marine biofilm communities by paired t-tests revealed no significant differences ($p = 0.132$-0.477) between the control and any of the LCDMs.

There were no bands that occurred in all of the samples and 3 bands were found only in single samples. One band was unique to the control, 1 band was unique to SNP, 1 band was unique to CDA and no bands were unique to patulin. However, the bands unique to the control, SNP and CDA were the same ones which occurred in only one sample. None of these 3 bands could be isolated for reliable identification by sequencing.

Comparisons of samples grown in the dark and the light revealed that there were a total of 25 different bands in samples grown in the dark and 28 different band in samples grown in the light. Five bands (including bands 01 and 09 and 3 un-sequenced bands) were unique to those samples grown in the dark and of those, 1 band was unique to the control, 1 band was unique to SNP and no bands were unique to CDA or patulin. Two bands were unique to those samples grown in the light and of those, 1 band was unique to CDA and no bands were unique to the control, SNP or patulin.

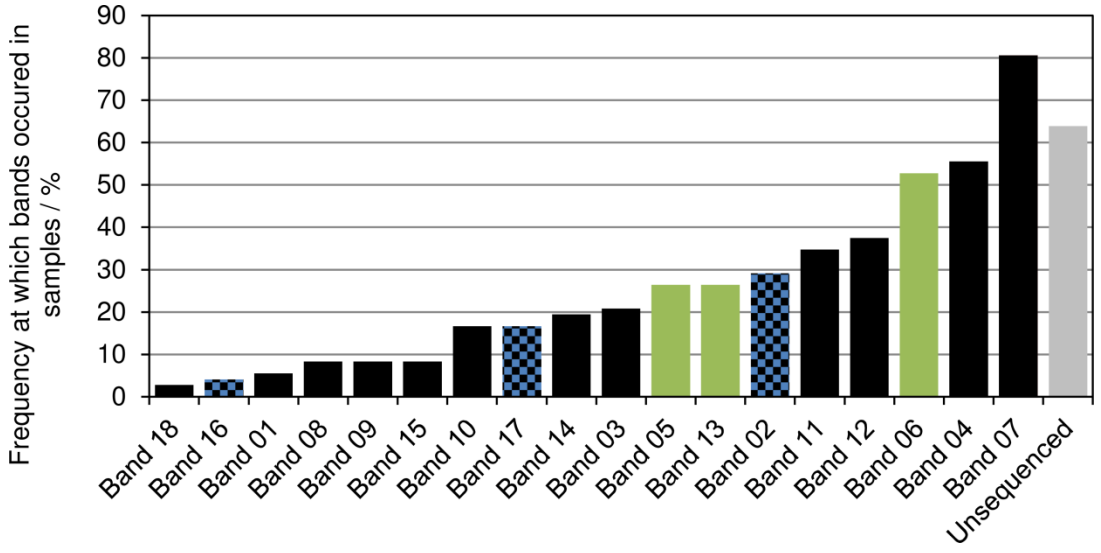


Figure 4.9: Analysis of the frequency at which each band occurred in all of the samples analysed showed that band 07, was the most frequently occurring band. Gammaproteobacteria (black) and Alphaproteobacteria represented the three most frequently found bands that were sequenced. Three bands, 02, 16 and 17 were identified as both Betaproteobacteria and Gammaproteobacteria (blue and black cheques) in separate sequencing reactions.

Between 63% and 86% of the bands observed had intensities below 5% of the total lane intensity. Band 07 had the greatest overall intensity and accounted for 39% of the band signal in all of the samples combined. Band 07 also occurred in 81% of the samples analysed (Figure 4.9) and was the most frequently observed band. Band 06 had the next highest combined intensity of 20% and occurred in 53% of samples. Band 03 had a total intensity of 7% and was the only other band which accounted for more than 5% of the total intensity for all band combined. Comparisons of band intensities across the treatments (Table 4.2) showed that band 07 had the highest intensity in most treatments, accounting for between 39% and 49% of the total intensity for each treatment. Only for SNP in the dark did band 07 not have the highest intensity. Instead band 02 had the highest intensity, accounting for 25% of the total intensity.

Table 4.2: Biofilms grown in the presence of 3 different LCDMs in light and dark conditions were analysed by DGGE and show similarities in those bands which had the greatest intensities in the gels. Below are all of the bands which accounted for more than 5% of total band intensities for each treatment. Only one sample, SNP dark, had a band of greater than 5% intensity that could not be sequenced (U).

Dark				Light			
Control	SNP	CDA	Patulin	Control	SNP	CDA	Patulin
Band 02	Band 07	Band 07	B07	B07	B07	B07	B07
Band 03	Band 06	Band 04	B03	B06	B06	B06	B03
Band 12	Band 05	Band 06	B06	B17	B05		B06
Band 06	U	Band 12	B04		B04		B13
Band 07		Band 02					
Band 05							

MDS plots of Dice and Euclidean distance data (Figure 4.10) showed that the DGGE profile patterns did not cluster based on similar treatments as would have been expected. This was even true for the control profiles, which did not cluster any more with each other than with the profiles of those communities treated with LCDMs.

Comparisons of the community structures between treatments at the taxonomic level of class showed little difference between treatments when only considering quantitative presence/absence data (Figure 4.11). Notably though, samples treated with SNP and grown in the dark did not contain Betaproteobacteria or Verrucomicrobiae. Quantitative comparisons of the total band intensity of each class showed that those samples that were not sequenced did not represent a large proportion of the community members. However, Gammaproteobacteria was numerically dominant in all samples. In those samples grown in the light, Alphaproteobacteria became more prevalent than in those samples grown in the dark.

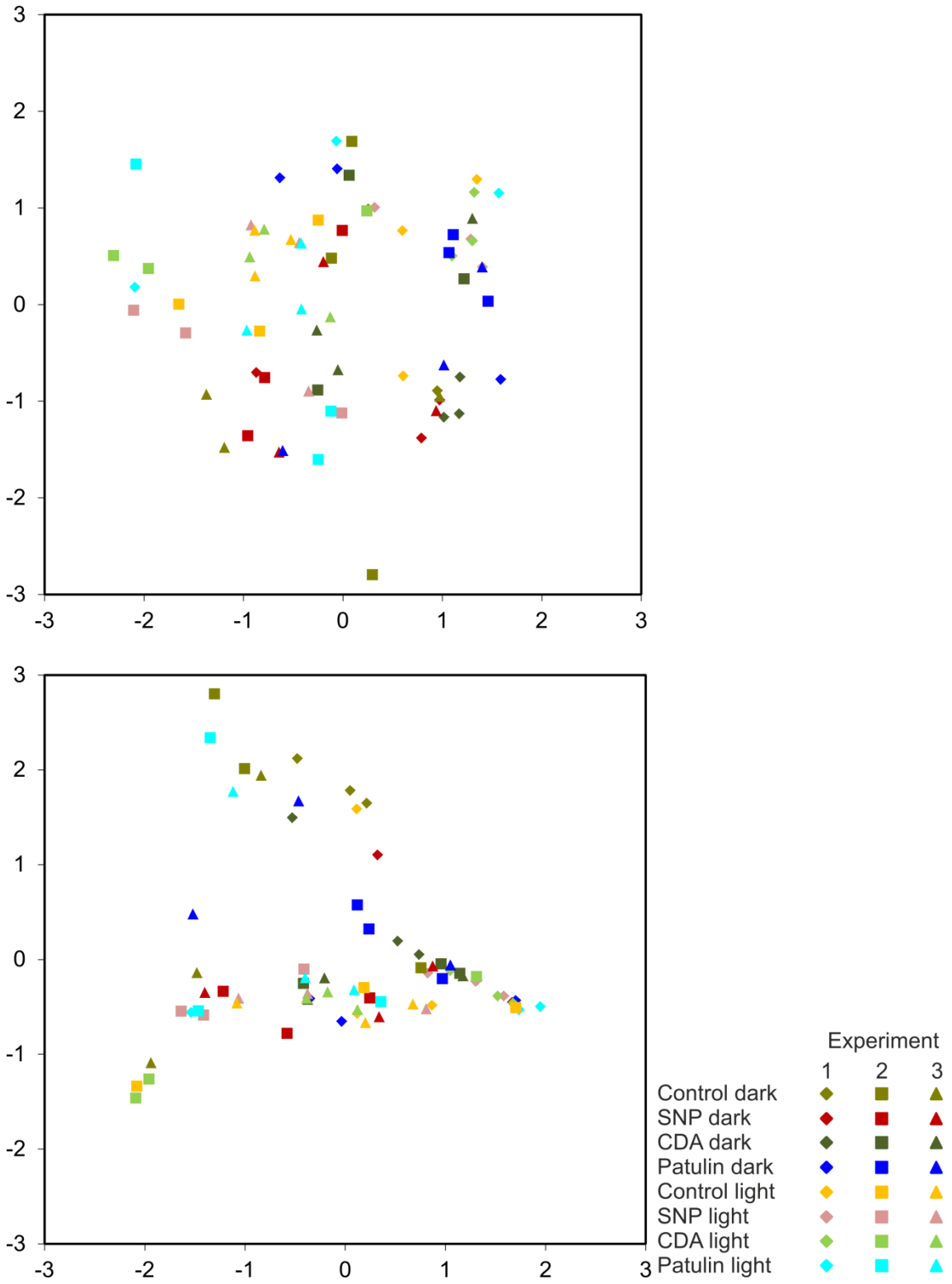


Figure 4.10: An MDS plot of Dice similarities (above) of DGGE profile species presence/absence data revealed that there was no clustering of communities treated with LCDMs. This was also true for MDS plots Euclidean similarities (bottom). Axis scales are arbitrary distance units calculated by the ALSCAL program within the SPSS 19 software.

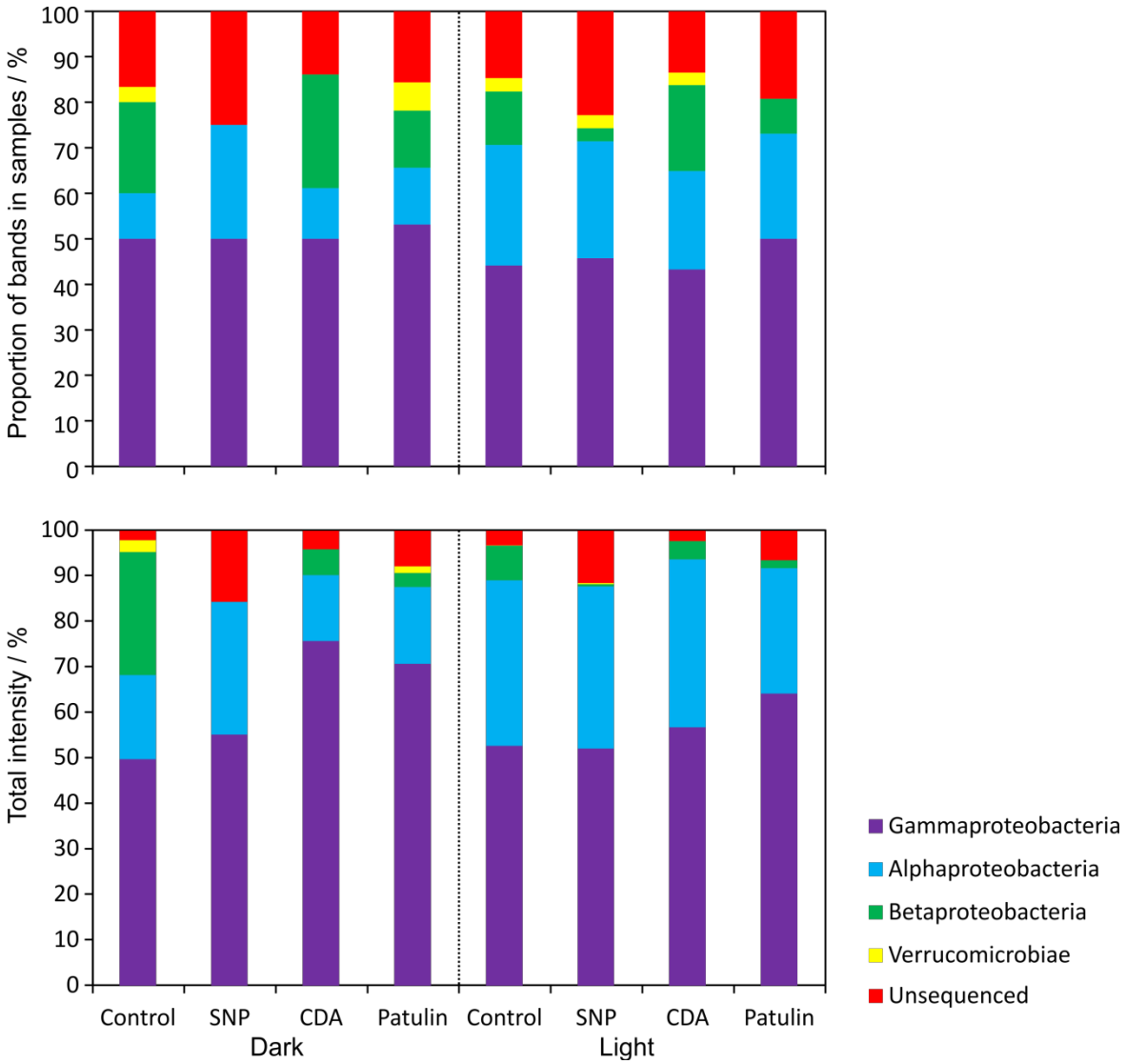


Figure 4.11: Comparisons of the proportion of bands present in samples (top) that were identified as belonging to the class Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Verrucomicrobiae or otherwise unidentified, showed little difference between treatments. However a notable exception to this was the lack of Betaproteobacteria and Verrucomicrobiae in SNP samples grown in the dark. Quantitative comparisons of the contribution of classes to the total band intensity for different treatment (bottom) showed that those bands that were not sequenced represented only a small proportion of the total communities. Additionally the predominance of Alphaproteobacteria appeared to be greater in those samples grown in the light than those grown in the dark.

4.4. Discussion

4.4.1. Biomass measurements

4.4.1.1. Effect of LCDMs

This study aimed to investigate the effects of three LCDMs on marine biofilms. These LCDMs have previously shown efficacy against biofilms formed by medically important bacteria and have not previously been tested for their effects on marine species.

Previous studies investigating the effects of NO on biofilms have shown the ability of this LCDM to trigger dispersal (Barraud, 2007, Barraud *et al.*, 2006, Barraud *et al.*, 2009b). One of the aims of this study was to determine whether the cell signalling properties of NO could be exploited to reduce the amount of marine biofilm that formed on surfaces.

This work has shown that NO reduced the growth of biofilms when it was present from the beginning of biofilm development. It is interesting to note that the SNP concentrations that caused a reduction in biofilm formation in this study were much greater than those concentrations found to be effective in previous studies. While Barraud and colleagues (2006) reported dispersal events occurring at SNP concentrations of 500 nM, no significant decrease in biofilm formation was seen in this study until an SNP concentration of 50 μM was used in low light conditions. This means that an increase of 2 orders of magnitude was needed in this system compared with Barraud and colleagues. The reason for this increase in the amount of SNP needed for an effect on biofilms is not clear, but may be due to the difference in the nature of biofilm formation as studied here, and biofilm dispersal as studies by Barraud and colleagues. Preliminary studies by R. Howlin (personal communication) suggest that in order for SNP-derived nitric oxide to trigger dispersal of *P. aeruginosa* biofilms, the biofilm must first be well established. If this is also true of marine biofilms, then we would not expect nitric oxide to trigger dispersal in this system where it was present from the beginning of the biofilm growth. However the lack of toxicity shows that there could be another physiological effect causing the reduction in biofilm formation. Microarray studies by Barraud and colleagues (2009a) showed that the addition of nitric oxide to *P. aeruginosa* biofilms altered the expression of several genes associated with the sessile mode of bacterial life. For example, the *pilA* gene which is linked to twitching and swarming motility was upregulated. If homologous genes in the marine communities in this system were upregulated, or at least stopped from being down regulated, then the maintenance of motility could reduce the formation of biofilms. However, Stabb and Ruby (2003) showed that a *Vibrio fischeri pilA* knockout mutant colonised *Euprymna scolopes* squids poorer than wild type *V. fischeri*, indicating that *pilA* plays a role in allowing *V. fischeri* to colonise surfaces. Since *pilA* is involved in the production of pili and motility, which

aid the initial attachment of bacteria to a surface, it is possible that the upregulation of *pilA* could actually increase initial biofilm formation by some Gram-negative species.

Additionally, the *morA* gene which is involved in the expression of adhesive surface structures and flagella (Choy *et al.*, 2004) was down-regulated in Barraud's investigations. If this was the case in this study we may expect that less attachment, both initial and irreversible, of bacteria would occur, which could lead to less biofilm formation or a reduction in biofilm stability. In the latter case, one can envisage the biofilms being removed during growth by the action of replacing growth media.

Another possible explanation for the difference in the concentration at which SNP is effective between Barraud's investigations and this present work is the difference in media in which SNP was diluted: seawater in this study and M9 medium in Barraud's studies. No investigations have been undertaken to determine whether the release kinetics of nitric oxide from SNP varies due to salinity or other chemical factors. If seawater had a buffering effect on the release of nitric oxide from SNP, then a higher concentration of SNP would be needed in order to introduce the same level of NO into solution. In order to test this hypothesis, a nitric oxide probe should be used to measure the release of nitric oxide from SNP in seawater and M9.

The effect of CDA on the formation of biofilms, while statistically significant, was much lower than that of SNP and patulin. The reduction in biofilm formation appeared to begin at the lowest concentration of 0.005 μM. However, further tests using even lower concentrations may reveal a greater effect at lower concentrations. Davies and Marques (2009) showed that CDA was effective at inducing dispersal of many single species biofilms as well as an undefined mixed culture of airborne bacteria. The study by Davies and Marques did not show any data relating to the effects of biofilm formation in the presence of different concentration of CDA, but dispersal of established biofilms was shown to occur in a range of 0.001 μM to 10 mM. As with this study, the Davies' and Marques' investigations showed an effect of CDA at the lowest concentration used, and so it is possible that it is effective at lower, unstudied concentrations.

There was also a significant increase in biofilm growth at 0.5 μM CDA in the dark. This could suggest that CDA became a carbon source for some organisms at this concentration, but the lack of increase in biofilm formation at 5 μM and above, combined with the lack of effect of CDA on planktonic cells does not support this. Alternatively, CDA may act to increase biofilm formation at a physiological level at 0.5 μM, giving it two peak concentrations of activity in opposite directions.

The reduction in biofilm formation caused by patulin is somewhat surprising because previous studies have shown that it increases the formation of biofilms (Liaqat *et al.*, 2008, Liaqat *et al.*, 2010). However, due to the relatively high concentrations at which patulin was

shown to reduce biofilm growth (5 to 50 μM), it is possible that it had an antibacterial effect on the marine bacteria, reducing their ability to grow. The range of concentrations at which patulin had an effect on the formation of biofilms was similar to those concentrations that have previously been shown to have an inhibitory effect on bacterial growth (13 to 650 μM) (Kavanagh, 1947, Klemmer *et al.*, 1955, Raistrick, 1943). This is supported by colony counting tests which showed that the inhibitory effect of patulin occurred in the planktonic phase at 5 μM, and so may not be a biofilm effect. In this case, patulin appears to be around 15 times more effective at reducing planktonic growth than other biocides such as OPA when used at similar concentrations (Simões *et al.*, 2007). So it could be argued that patulin has potential as an antifoulant by killing microorganisms before they get a chance to attach to surfaces. However, to achieve a similar level of antibacterial activity, the cost of patulin is over 300 times that of OPA (correct on 06/03/2012), and is therefore not a viable option for antifouling especially in a system where OPA is already in use (such as the ammonium sensor).

4.4.1.2. Effect of light

The comparisons of biofilms grown in the light and dark showed that there was less biofilm formation in the light. At first glance it might appear that this effect is due to inhibition by ultraviolet (UV) light (Elasri and Miller, 1999). However, this effect did not exist when the same test was carried out on PMMA. This suggests that the reduction in biofilm formation in the light is related to the nature of the polystyrene material from which the titreplates were constructed. It is possible that the interaction of light with polystyrene caused the release of chemicals that reduced biofilm formation.

Studies of light induced degradation of polystyrene have shown that in the presence of UV light, polystyrene releases molecular and atomic (radical) hydrogen, water, carbon monoxide, carbon dioxide and a variety of organic compounds including aromatic and short chain hydrocarbons (Achhammer *et al.*, 1952, Biederman and Osada, 1990, Wells *et al.*, 1994). Due to the mixed nature of the chemicals released when polystyrene is exposed to short wavelength light, it is possible that one of those chemicals may be toxic or at least inhibitory to biofilm forming bacteria.

Linquist and colleagues (1998) found that polystyrene pipettes used for handling PCR products released PCR inhibitors after exposure to UV light. The chemical nature of these inhibitors was not studied, but it does indicate that polystyrene releases chemicals when exposed to shortwave length radiation. In this study, biofilms grown in the light were exposed to the artificial light of a plant growth room. The level of UV light that the microplates were exposed to was much lower than that of the pipettes in the study by Linquist, but it is possible that in order to inhibit biofilm growth, either lower concentrations of break down product are

needed than for PCR inhibition or different products are released that inhibit biofilm formation.

Another possible cause for the light effect seen only on polystyrene is a difference in the community structure that is associated with polystyrene and PMMA. For example, if there was preferential attachment of certain types of communities to the different plastics it is possible that the polystyrene community happened to be sensitive to light, while the PMMA community was not. However, this hypothesis is not supported by the community analysis data obtained in this study, as the lack of a specific community forming on polystyrene indicates that there is no preferential attachment by different species in this system.

From the data obtained in this study, it is impossible to determine whether a reduction in biofilm growth in the light as seen on polystyrene or the unchanged biofilm growth on PMMA is normal. In order to fully understand the effect of light on the formation of biofilms, it would first be necessary to repeat these experiments on a range of surfaces including chemically inert materials. Depending on the results of those studies it may then be worthwhile to isolate and characterise any biofilm inhibitor (or promoter) molecules that may exist in the system for possible exploitation for biofilm control.

The inhibiting effect of light on biofilm growth was also apparent when they were grown in the presence of both SNP and CDA. This effect was still apparent even when the effect of light on the controls was taken into account. This indicates that light increased the inhibitory effect of the LCDMs either directly, through increasing the activity of the LCDMs, or indirectly through a synergistic effect between the LCDMs and the polystyrene-light inhibition effect.

In the case of SNP, planktonic bacteria showed no growth in those samples that were exposed to the light, suggesting that SNP became toxic in the light, while it remains non-inhibiting to planktonic bacteria growth in the dark. It is likely that the presence of light directly increased the release of nitric oxide (Arnold *et al.*, 1984). Hetrick showed that nitric oxide can be toxic to several species of microorganisms (Hetrick *et al.*, 2009). However, it is more likely that most of the toxicity was caused by cyanide. A molecule of SNP contains 5 cyanide molecules, which can be released when a solution of SNP is exposed to light. The rate at which this breakdown occurs is greatly increased when high frequency light waves such a UV are present (Arnold *et al.*, 1984).

The change in efficacy of CDA under light conditions is less likely to be due to a direct increase in activity of the LCDMs on biofilm formation because CDA is not known to breakdown into more toxic molecules in the light. Colony counts of bacteria treated with CDA in the light and dark support this, as no difference in toxicity was evident in the light and dark. It therefore seems likely that the increased biofilm inhibition in the light for CDA was due to a synergistic interaction between the polystyrene-light effect and the LCDMs. However,

further investigation into the polystyrene-light effect would be needed before any conclusions could be drawn regarding the nature of this synergism.

4.4.1.3. Effect of growth vessel

The effect of the growth vessel was also tested. Biofilms grown in the light showed an increase in growth in the 6-well plates relative to the 96-well plates. This trend was not seen for biofilms grown in the dark, suggesting that the differences between the vessels were at least in part related to light. It has already been shown that light affects biofilm growth on polystyrene. If we assume that this is due to the release of toxic breakdown products, then this suggests that more of the breakdown product is released in the 96-well plate system than in the 6-well plate system relative to the volume of media used. A simple calculation shows that with the volumes of media used in these experiments, the surface area to volume ratio of the 96-well plates is 1.3 times greater than the 6-well plates. We would therefore expect the dilution of the breakdown products in the 96-well plates would be smaller. However, given the large difference in fouling between the two systems, a 1.3 times difference in surface area to volume ratio does not appear to be sufficient to explain the large difference.

Alternatively, the lower level of apparent biofilm formation in the smaller well sizes could be an effect of increased shear forces exerted when changing the growth media. In the smaller wells, the force of growth media being ejected from a pipette may have been sufficient to remove some of the attached biofilm. In the larger sized well, the same force would be spread over a larger area, decreasing the average shear force experienced at each point of the well. If detached biofilms were then not able to re-establish themselves on the growth surface, this would result in an overall decrease in biofilm biomass by the time the analyses took place.

4.4.2. Community analysis

The DGGE band (07) that was the most prevalent band across contained several co-migrating species, all of which were Gammaproteobacteria. The next most intense band (06) was identified as belonging to the Alphaproteobacteria genus *Loktanella*. The *Loktanella* genus contains 11 known species, all of which have been isolated from marine environments (Hosoya and Yokota, 2007, Ivanova *et al.*, 2005, Lau *et al.*, 2004, Lee, 2012, Moon *et al.*, 2010, van Trappen *et al.*, 2004, Weon *et al.*, 2006, Yoon *et al.*, 2007). Alphaproteobacteria have been shown previously to be important marine biofilm formers (Dang and Lovell, 2000, Dang and Lovell, 2002, Jones *et al.*, 2007, Lee *et al.*, 2008). Previous studies of marine biofilms have shown that it is the Gamma and Alphaproteobacteria that tend to predominate, which is supported by this study. However, a study by Lee and colleagues (2008) showed that the while the Gammaproteobacteria are most prominent after 3 hours of exposure of an acrylic surface, they become less prominent as the biofilm becomes established. After 6 hours of exposure,

Alphaproteobacteria begin to dominate the biofilm. The importance of both Alpha and Gammaproteobacteria in this study is demonstrated by the high frequency of occurrence and over all band intensity of both groups. Betaproteobacteria are also commonly found in marine biofilms (Edwards *et al.*, 2010, Jones *et al.*, 2007, Lee *et al.*, 2008), but occur at much lower levels than Alpha and Gammaproteobacteria. In this study all three DGGE bands that were identified as belonging to Betaproteobacteria occurred at the same position as other bands which were identified as Gammaproteobacteria. It was therefore not possible to confidently assign identities to those banding positions and as a result it was not possible to determine the numerical importance of Betaproteobacteria in this system.

Analyses of communities at the taxonomic level of class showed some variation in community structure between treatments, most notably with an increase in the numbers of Alphaproteobacteria in those samples grown in the light compared with those samples grown in the dark. No evidence in the literature could be found to indicate that Alphaproteobacteria have increased rates of growth compared with Gammaproteobacteria in the light. Lee and colleagues (2008) showed that Gammaproteobacteria tend to be among the first organisms to colonise artificial surfaces, and dominate early stage marine biofilms, which indicates that biofilms grown in the dark resemble early stage biofilms while those grown in the light resemble later stage biofilms. Unlike this study, in which biofilms were grown in a static microcosm, Lee's biofilms were grown in an open marine system. This means that while Lee's biofilms had a constant influx of new colonisers from the water column, the biofilms in this system were only formed from those organisms that were able to attach to the titreplate surface during the initial attachment period. Assuming similar growth rates for both Gamma and Alphaproteobacteria, this means that Alphaproteobacteria were better able to attach in the harsher environment of high light intensities (and the growth inhibitors released by polystyrene). This increased attachment by later stage biofilm community members may indicate a phenotypic plasticity that allows them to adapt to harsh environments by reverting to a sessile biofilm state.

The MDS plots of DGGE banding similarity showed no clustering among treatments for either the qualitative (presence/absence) or quantitative (% band intensity) data. This indicates that the LCDMs did not have an effect on the species present in biofilm communities. This is an unexpected result, as absorbance and microscopy analyses revealed differences in biofilm formation due to treatment with LCDMs. This suggests that the LCDMs are effective at reducing biofilm formation irrespective of the species present in these assays. The lack of effect on the species within biofilms treated with different LCDMs was further supported by the richness and diversity data, which showed that none of the treatments significantly altered the diversity of species in biofilms. Again, this was unexpected, as one might expect that the

LCDMs would have a greater effect on some species than on others, resulting in an increase in the occurrence of some species over others.

These are important findings if the LCDMs are to be applied to marine micro-sensors. The sensors will be deployed throughout the oceans and therefore encounter a wide range of microbial species, and it is therefore vital that any antifouling strategy has broad-range effectiveness.

While little community effect was seen using DGGE analysis, the true nature of the biofilm ecosystem may not be at the community level. In traditional models of the formation of biofilm communities, members of certain taxonomic groups will colonise a surface in a particular order to fulfil a specific niche (Hansen *et al.*, 2007, Hassell *et al.*, 1994). The niches will vary depending on the physical and chemical environment, thereby modifying the structure of the biofilm community in a predictable way. In neutral community models (NCM) however, the membership of a community is determined largely by chance (Bell, 2000, Hubbell, 2001). Niches are colonised randomly by whichever species are present that can occupy that niche. In any given system there may be a large number of different species that are capable of exploiting the same resources, and this group of species is known as a guild (Root, 1967). Members of guilds may not be taxonomically related which means that in two communities grown under exactly the same conditions, the species composition may vary considerably due to chance, while the community function may remain similar. This concept was demonstrated in biofilm communities by Burke and colleagues (2011) with bacterial species associated with the green macroalga, *Ulva australis*. The assemblage of bacterial communities on *U. australis* was observed to vary significantly even under similar conditions. However, profiling of the functional systems of the bacterial biofilms showed a large degree of similarity.

Application of NCM to this study explains why there were no distinct biofilm communities based on 16S rDNA profiling even when the effects of the physiological treatments were so great. It is possible that had functional genes been profiled, then there would have been a much greater degree of similarity among treatments.

This raises the question of how valid it is to use a species concept when studying marine prokaryotic systems. If the similarities between the ecologies of taxonomically similar organisms (such as species or genera) is no greater than between the ecologies of taxonomically dissimilar organisms, then the species concept would appear to serve little purpose. Further study could be conducted to determine the species that occur within different guilds involved in the formation of marine biofilms, and so use guild as an operational unit in marine microbiology. However the value of such work is unclear, as there is likely to be overlapping of species in different guilds. This may be especially true within biofilm communities where it is well known that the transfer of genetic information, and so

functionality, is enhanced (Angles *et al.*, 1993, Hausner and Wuertz, 1999, Molin and Tolker-Nielsen, 2003) and so membership of a guild may be temporary and unpredictable.

For this reason, investigations of biofilms at a functional level may serve to increase the understanding of biofilm biology more effectively than community profiling. Methods including DNA stable isotope probing (DNA-SIP) (Radajewski *et al.*, 2000), microautoradiography and fluorescence *in-situ* hybridisation (MAR-FISH) (Lee *et al.*, 1999), mRNA FISH (Femino *et al.*, 1998, Huang *et al.*, 2009) and prokaryotic *in-situ* reverse transcriptase PCR (PI-RTPCR) (Hodson *et al.*, 1995) allow the functionality of groups of microorganisms within complex communities to be determined. Using methods like these allows the user to target specific biological functions of interest and determine which groups of organisms, be it taxonomically or spatially, are performing those functions. Additionally, the use of methodologies such as RNA-seq (Siezen *et al.*, 2010, van Vliet, 2010, Wang *et al.*, 2009) to uncover the transcriptomes of whole biofilms may reveal differences in whole biofilm functionality without the need for supporting taxonomic information.

It is clear that much work is still needed to understand the processes involved in marine biofilm formation, the interactions between organisms in mixed communities and the effect that LCDMs such as NO and CDA have. However, this study has shown that NO and CDA can be used to indiscriminately reduce biofilm formation by mixed communities at the physiological level instead of relying on toxicity.

4.4.3. Application of LCDMs to marine microsensors

The reduction in marine biofilm formation shown by the LCDMs tested indicates that they have potential for application as antifouling agents in marine microsensors. The LCDMs in this study appeared to have a broad spectrum effect on biofilm species which makes them ideally suited to use in marine sensors that will be deployed in different environments and will have different species within them.

In addition to this, an advantage that LCDMs have over other chemical based anti-fouling techniques is that their reduced toxicity lends them particularly well to use in sensitive environments. In the case of environmental sensors it is undesirable to introduce any agent to the environment that will alter the conditions that are being measured, and so any means to eliminate the need for toxic chemicals is welcome. However, caution should be taken when introducing any novel chemical to the environment and further study into the wider environmental impacts of LCDMs should be undertaken before committing to full deployment.

If LCDMs were found to be environmentally viable, then different application methods should be considered to determine the most feasible method for delivery of the LCDMs to the sensors.

One possible delivery method for LCDMs is by mixing LCDMs in reagent reservoirs within the sensors. In this way, the sensors could be treated with LCDMs intermittently when reagents are pumped through the channels. This may be particularly effective in those sensors which have very frequent usage and will in turn be exposed to LCDMs frequently. However if the period between sensor operations is extended, there may be time for biofilms to develop and these biofilms may be difficult to remove once established.

In the case of NO, where the breakdown of donor molecule is required to deliver the LCDM to solution, selection of a donor molecule that is suited to the sensor in question is very important. For example, bis-N-nitroso-caged nitric oxides (BNN) release NO when exposed to 300-360 nm wavelength light (Namiki *et al.*, 1999). It is mostly nitrate sensors that operate in this range of wavelengths (Taberman, 2010), in which sensors the use of nitric oxide may not be suitable. However, other groups have developed methods for detecting phosphates using sub 400 nm wavelength light (McGraw *et al.*, 2007), and so BNN may have some potential applicability to these technologies. Additionally, a 2,6-dimethylnitrobenzene-based compound known as Flu-DNB was synthesised by Hishikawa and colleagues (2009) who showed it to release NO when exposed to light of with wavelengths of 720 nm. Any future developments of sensors that utilise light at these wavelengths may consider using Flu-DNB or related chemicals to deliver anti-fouling NO. Weyerbrock and colleagues (2012) studied the NO donor JS-K for its use to treat malignant gliomas. JS-K releases NO in the presence of glutathione and glutathione S-transferases and so NO release may be controlled by introducing these agents when NO is needed.

An alternative strategy delivering LCDMs by doping sensor reagents is to embed the materials from which sensors are manufactured with LCDMs. This allows the chemicals to be released passively over time so that the sensors are constantly treated with LCDMs.

Cai and colleagues (2012) developed a NO releasing film by doping a layer of poly(lactic-co-glycolic acid) with the NO donor dibutyhexyldiamine diazeniumdiolate. It was found that glass surfaces coated with this film showed a 98.4% and a 99.9% reduction formation of biofilms *S. aureus* and *E. coli* respectively. However, the length of time over which NO was released was only 15 days, which would not be long enough to provide the long-term antifouling protection needed by in-situ marine sensors. Additionally, as LCDMs embedded in a surface are depleted there is a gradual reduction in the level of LCDM released, leading to a gradual reduction in the antifouling efficacy of these materials.

Once the LCDMs are delivered to the sensing environment their diffusion throughout the sensor will be affected by the hydrodynamic scheme within in the sensor channels. Any 'dead zones' in the channels, where flow is reduced, may receive lower levels of LCDMs and therefore any microorganisms which were able to attach a these points may not be affected to the same extent as those parts of the sensors with higher flow rates. In addition to this, a

barrier of slow moving fluid adjacent to the biofilm, known as a concentration boundary layer or mass transfer boundary layer (Stewart, 2012) reduces the transport of solutes into and out from biofilms (Kühl and Jørgensen, 1992, Wäsche *et al.*, 2002). A reduction in the Reynolds number of the system reduces the movement of solutes further still (Zhu and Chen, 2001). Therefore the bulk fluid velocity, as well as the morphology of the sensor channel (which will influence the Reynolds number) will also affect the transport of LCDMs into biofilms.

Therefore, in order that the use of LCDMs is affective at reducing the formation of biofilms in marine micro-sensors, investigations into how the varying hydrodynamic schemes within the sensor affect the movements of LCDMs would need to be conducted.

Any strategy that is used to deliver antifouling LCDMs to microsensors will require careful consideration of the balance between potential benefits and pit falls. Therefore while the use of LCDMs in microsensors may prove to be beneficial for antifouling, much work is needed before they can be practically applied.

Chapter 5 General discussion

Marine biofouling is a complex phenomenon and there is still much to be uncovered about its complexities. As such strategies to remediate fouling should take such complexities in to account if they are to be effective. This study attempted to determine some of the potential problems associated with biofouling in lab-on-chip sensors and it has shown that fouling is likely to be a significant problem in any attempt to deploy such sensors for extended periods in the sea. It has also shown that the LCDMs NO and CDA may have potential applications for reducing marine biofouling. Here the main conclusions of each chapter are summarised and further work that is needed is discussed.

5.1. Sensor reagents

Three of the reagents that will be used in the sensors under development by the CMM were tested for their effects on marine biofilms. Griess and OPA were found to reduce fouling, while ferrozine was not. Griess however was the only reagent tested that reduced the growth of established biofilms. Additionally, 1.2% HCl also reduces the growth of established biofilms. These results show that for some sensors, specifically those using ferrozine and those without chemicals reagents (such as cytometers), additional antifouling strategies will be needed throughout the sensors.

HCl has been proposed as a cleaning reagent to be used between measurements in the sensors. This will certainly help to reduce fouling, but there will be areas in the sensors where it will not be possible to use HCl, causing fouling hotspots. Future studies should investigate which parts of the sensors are most prone to fouling. This could be performed simply by directly viewing biofilm accumulation throughout the sensors under a microscopy. During the course of this investigation attempts were made to construct a model sensor which had transparent walls that were thin enough to view biofilm accumulation *in situ*. However, technical challenges that were beyond the scope of this project to overcome closed this line of investigation. Further collaboration with experts in the construction of microfluidic devices would allow these investigations to proceed.

No evidence was found to suggest that applying HCl to established biofilms will reduce the biomass on the surface, and therefore the use of antifouling chemicals should be used from the very start of operation.

Due to the varying nature of the oceanic environment in which the sensors will be deployed, it is likely that the types of organisms and there susceptibility to different chemicals will also vary considerably. For example, if the sensors are deployed in acidic environments such as around sulphurous hydrothermal vents, one might expect that the biofilms that form would be acidophilic to a higher degree than in other areas of the oceans. Therefore it would

also be expected that these biofilms would be less susceptible to treatment by HCl. If this was the case, then using HCl in the sensors may not have any beneficial effect in terms of biofouling, and other strategies would need to be considered. To test this hypothesis, laboratory based experiments using acidophilic bacteria isolated from hydrothermal vents and other acidic environments could be conducted in a similar manner as in this investigation. However, while laboratory based experiments provide the investigator with the ability to control the entire growth environment, more meaningful data may be obtained by deploying prototype devices in acidic environments and studying biofilm formation in devices that have and have not been treated with HCl or other chemicals.

In addition to nitrate/nitrite, ammonia and iron sensors whose reagents were tested in this study, the CMM are also developing sensors to measure pH, manganese and phosphate. Further studies should expand these investigations by looking at the effects of the reagents used in those sensors on biofouling.

5.2. Substratum materials

Glass, COC and PMMA were compared to determine whether there were any differences in the level of fouling that occurred on the different materials. When the thicknesses of the biofilms were compared, no differences were seen between the materials, and after 2 weeks, the biofilms had reached a thickness of around 30 μm. At these thicknesses, the biofilms could have a large effect on flow though the sensors, which have channels that can be as narrow as 10 μm.

While no differences in thickness were observed, glass was shown to have less surface coverage than COC. While glass is not used to manufacture the micro-sensors, this result indicates that at the early stages of exposure to the marine environment, different surface properties can have some effect on fouling. In this case it was likely that the higher hydrophilicity of glass resulted in the greater degree of fouling. Future studies should investigate the use of modified surfaces with increased hydrophilicity. This may be achieved using surface modifications such as polyethylene glycol chains (Tao *et al.*, 2008).

Future studies should also investigate the biofilm communities found on the different materials to improve the understanding of fouling on different materials. Additionally, by increasing the range of times that the substrata are exposed to the sea to include very short exposures (minutes or hours) to much longer exposures (weeks or months) in the same experiment, a much more detailed understanding of the process of biofouling of different materials could be attained. In order to obtain the most useful information from these investigations, any biofouling should be studied at both the gross morphological level and the

molecular level. Community analyses of biofilm samples from these investigations were attempted, but were not successful due to insufficient DNA extraction. Optimisation of this first and most crucial step in community analysis must be carried out for any future study to be successful. Additionally, proteomic and transcriptomic investigations of biofilms that have formed at different stages of exposure would help to achieve an understanding of the physiological processes that occur in marine biofilm formation.

No differences in fouling were found at all between COC and PMMA, which are two materials used in the construction of microsensors. Further investigations should be carried out to determine the extent of fouling on other materials used in sensor construction, including Viton, which is used for the manufacture of micro-valves inside the sensor channels.

5.3. Validation of crystal violet assays for biomass measurements

The commonly used crystal violet assay for measuring biofilm biomass in titreplate systems was tested for its suitability for mixed marine biofilms. Such absorbance-based methodologies provide a high throughput methodology for determining biofilm formation. It was shown that this assay can be used to reliably predict the biovolume of those mixed communities grown in a titreplate microcosm. However, further validation of this method should be carried out to ensure its reliability. Such validation might include comparisons of the crystal violet absorbance with the dry mass of the biofilms. Direct measurements of biomass are difficult in this type of system due to the low mass of biofilm which is at the edge of detectability by standard laboratory equipment. However, indirect measurements of biomass, such as measuring evolved CO_2 emissions from incinerated biofilms could help to overcome problems associated with direct measurements.

Unlike the assay used for measuring biomass in titreplates, the assay used to measure biomass on coupons exposed to the marine environment did not give a linear correlation. This assay was different in that the bound crystal violet was not redissolved in acetic acid before absorbance measurements were taken. This was due to the nature of the coupons which would have given false data due to edge effects had the crystal violet been redissolved. The non-linear correlation shown in this case indicates that at higher levels of biofilm formations, this assay becomes less able to resolve differences in fouling and therefore is not suitable for a long time course. Future investigations should look at the suitability of related methods such as fluorescence based assays like those used by Honraet and Nelis (2006), who used the nucleotide stain Syto 9, which fluoresces when bound to nucleic acids.

Fluorescence based assays provide a vast pool of assays which would allow a large range of biological information to be gained in a high throughput fashion. Such information includes but is not limited to the proportion of live to dead cells (Alakomi *et al.*, 2005, Boulos *et al.*, 1999), the proportion of different taxonomic groups (Amann and Fuchs, 2008) and the level of expression of specific genes (Femino *et al.*, 1998, Huang *et al.*, 2009).

5.4. Physiological effectors

All three of the LCDMs tested were shown to reduce the formation of biofilms by mixed marine communities. However, NO and patulin were found to be particularly effective. NO reduced biofilm formation at concentrations that had no effect on planktonic bacteria. The concentration at which NO is effective at reducing biofilm formation is much greater than that previously reported to trigger dispersal, and the cause of this needs more investigation. One piece of research that should be conducted is to determine the release kinetics of NO from SNP and from other NO donors. Due to the differences in the chemical nature of seawater to M9 and other media used in previous NO studies, there is a possibility of an alteration in the release of NO from SNP and other donors. Before NO can be applied to any system, a full study of the best donor molecule is vital.

The mode of action of patulin appeared to be one of toxicity as opposed to a cell signalling process. CDA was shown to reduce biofilm formation at low concentrations and also increase biofilm formation at a higher concentration. The low concentration at which CDA had a negative effect on biofilm formation concurs with that found by Davies and Marques (2009) who determined that CDA was effective at increasing dispersal in the nanomolar range of concentration. Reduced biofilm formation was found at the lowest concentration of CDA tested, and it is possible that it would reduce biofilm formation at even lower concentrations not studied. Further investigations should include a reduction in the concentration of CDA to determine the minimum inhibitory concentration. Additionally, investigations should be carried out to determine in detail the mechanism by which CDA has its effect. Such investigations should use molecular methods such as RNAseq to determine which genes have differential expression in the presence of CDA.

The presence of light was shown to have a large effect on biofilm formation, but the source of this effect was shown to be from the polystyrene growth substratum. Future studies that use the titreplate microcosm as a growth system should take this into account and perhaps consider using other, less active substrata. The lack of effect from PMMA in the light suggests that this may be a good material from which to construct titreplates for these studies. While it is not clear what the root of the polystyrene-light effect is, further investigation into

this effect may prove useful as a potential source of antifouling chemicals. The cause of the light effect could be investigated by conducting biofilm growth experiments on several different materials both in the light and the dark. Any similarities in materials that have a light effect on biofilm formation could be investigated to narrow the focus of the investigation. Analytical chemistry techniques such as mass spectroscopy could then be employed to determine the chemical nature of any biofilm inhibitors present. Depending on the results of this line of investigation, novel antifouling chemicals could be discovered, which would justify these investigations in their self.

Analysis of the biofilm communities grown in the presence of LCDMs revealed a possible difference at the taxonomic level of class between those samples grown in the light and those grown in the dark. The limitations of DGGE, including the co-migration of different species in the same band, mean that the banding pattern may not be a reliable measure of the community diversity. However, even in a case where different taxonomic groups co-migrate, one would still expect to see similarities in DGGE banding patterns if the communities were forming due to taxon specific cues during biofilm formation. However, no major effect of the LCDMs on the community composition based on DGGE banding patterns was observed. The lack of effect on the overall biofilm communities suggest that the LCDMs have a broad effect and so may be applicable in a wide range of environments where one would expect to see variations in the species present. This also points to the possibility that the biofilm communities in this system form in a neutral fashion dictated mainly by chance. Further investigations looking at the functionality of biofilms may reveal correlations between biofilm function and LCDM treatment.

Further investigations should be carried out using technologies such as next generation sequencing, which would give a more complete and reliable community structure dataset, and allow more robust conclusions to be drawn about the nature of biofilm formation under different conditions. Such analyses would be particularly powerful if they were conducted over a time series to show successional events, and also if combined with FISH to show any positional trends that were missed.

5.5. General conclusion

The studies in this report have shown that biofilms will cause potential problems with marine sensors, but the application of LCDMs such as NO or CDA may help to reduce the formation of such biofilms. However, these investigations were limited to growth systems with little or no flow of water. Marine microsensors will have water and reagents flowed through them. This flow will produce hydrodynamics effects such as altered solute availability

and shear, as well as allowing the constant influx of new microorganisms which was not accounted for in these studies. Additionally, microsensors will contain structures such as valves that will disrupt flow, adding another level to the complexity of fouling within sensors. In those sensors where chemical reagents such as Griess reagent are to be used, the antifouling action of the reagents will be limited to the areas in which those chemicals are present. Even with the application of LCDMs, there will be areas of the sensors where it will not be practical to apply those molecules.

For these reasons it is vital that further work to study the fouling of sensors is conducted using a microcosm that more accurately represents the variable environments within the microsensors as well as the variable environments in which the microsensors with operate throughout the oceans. A microfluidic model for the growth and monitoring of biofilms developed by Drescher and colleagues (2013) may be a good starting point for such studies. With these systems in place, the logistics of how to apply LCDMs to sensor applications can then be studied be that by slow release materials, actively pumping the LCDMs into the sensor channels or some other means.

It is clear from the studies of the effect of LCDMs on biofilm formation, that the application of LCDMs alone will not completely stop biofilm formation, and so they will likely need to be combined with other methods such as nanostructuring or surface modification of the sensor materials. However, this study has shown that LCDMs have the potential to be applied as antifoulants, and further investigation into novel LCDMs may yet provide powerful, environmentally friendly antifouling technologies.

Appendices

Appendix 1. Minimal Marine Media with Nutrients (3MN)

Method

Working in a sterile environment (either in a laminar flow cabinet or under a Bunsen flame):

- Sterilise all component solutions by filtration through a 0.2 μm filter membrane, except for nutrient solution which should be sterilised by autoclaving at 121°C for 15 minutes.
- To a sterile bottle add 920 ml 1.1x NSS, 40 ml 1M MOPS and 10 ml tricine & 0.4M iron sulphate.
- Slowly add 10 ml 132 mM potassium phosphate while stirring
- Add 10 ml 952 mM ammonium chloride and 33.3μl of 33,333x nutrient solution.
- Top up to 1000 ml with sterile H_2O.

1.1x Nine Salt Solution (NSS)

NaCl	19.36 g
Na_2SO_4	1.617 g
$MgCl_2$	2.057 g
$CaCl_2$	0.451 g
$NaHCO_3$	0.088 g
KCl	0.275 g
KBr	0.044 g
$SrCl_2$	0.0088 g
H_2BO_3	0.0088 g
H_2O	800 ml

Adjust to pH 7.0, top up water to 1000 ml.

1M MOPS

3-morpholinopropane-1-sulfonic acid	209.26 g
H_2O	To 1000 ml

Tricine & 0.4M Iron Sulphate

Tricine	71.67 g
$FeSO_4 \cdot 7H_2O$	0.278 g
H_2O	To 1000 ml

132 mM Potassium Phosphate

K_2HPO_4	22.99 g
H_2O	To 1000 ml

952 mM Ammonium Chloride

NH_4Cl	50.92 g
H_2O	900 ml

Adjust to pH 7.8 and top up water to 1000 ml

33,333x Nutrients

Peptone	50 g
Yeast extract	10 g
H_2O	To 1000 ml

Appendix 2. Marine Agar

Method

- Combine the components as below with 15 g agar and top up water to 1 litre.
- Sterilise by autoclaving at 121°C for 15 minutes.

Nutrients

Peptone	5 g
Yeast extract	1 g

Gravimetric salts

NaCl	19.45 g
$MgCl_2$	8.8 g
$MgSO_4$	3.24 g
$CaCl_2$	1.8 g
KCl	0.55 g

Minor Salts

Salt	Volume of 0.1 $g \cdot ml^{-1}$ solution / ml
$FeC_6H_5O_7 \cdot 3H_2O$	1
$NaHCO_3$	1.6
KBr	0.8
$SrCl_2$	0.34
H_3BO_3	0.22
Na_2SiO_3	0.04
NaF	0.024
NH_4NO_3	0.016
Na_2HPO_4	0.08

Appendix 3. Iron reagent

Method

- Create the buffer as below
- Dissolve 1.25 g of ferrozine in 100 ml of buffer
- Add H_2O to 250 ml

Buffer

Sodium acetate	155.3 g
Acetic acid (glacial)	6.4 ml
H_2O	To 1000 ml

Appendix 4. Ammonium reagent

Method

- For ammonium reagent with OPA, mix 1 ml of solution A, 489 ml of solution B and 10 ml of solution C
- For ammonium reagent without OPA, mix 1 ml of solution A, 489 ml of solution B and 10 ml of methanol
- Protect from light and store at 4°C

Solution A

Na_2SO_3	0.08 g
H_2O	10 ml

Solution B

$Na_2B_4O_7$	15 g
H_2O	500 ml

Solution C

ortho-phthadialdehyde	8.38 g
Methanol	50 ml

Appendix 5. Griess reagent

Method

- Create the sulphanilamide solution as below
- To the sulphanilamide solution, add 0.5 g of N-(1-naphthyl)ethylenediamine dihydrochloride
- Add H_2O to 500 ml

Sulfanilamide solution

Sulfanilamide	0.5 g
H_2O	50 ml
HCl (37%, fuming)	5 ml

Appendix 6. Fouling on polystyrene

Polystyrene coupons were deployed in the MET for a two week exposure to the sea between 13th May 2011 and 27th May 2011. Pieces of polystyrene approximately 5cm x 5cm were cut from titreplate lids and stuck to glass slides with a silicone based adhesive. The coupons were left for 3 days to allow any volatile liquids in the adhesive to evaporate. Coupons were cleaned using the same method in section 3.2.1.1 before deployment along with glass, PMMA and COC coupons. The contact angle between water and polystyrene was measured as 89.8° (standard deviation = 1.3°) using a goniometer as detailed in section 3.2.1.2. No contact angle was measured for a non-polar liquid, as all of those that were tested dissolved the polystyene material.

After a 2 week exposure in the sea, the samples were prepared and analysed as with the other materials discussed in sections 3.2.1.3 and 3.2.1.4. However, due to the reduction in transparency of polystyrene coupons caused by the silicone adhesive, it was not possible to carry out optical density analyses.

Figure A6.1 shows the level of fouling on polystyrene relative to the other materials tested. One-way ANOVA and post-hoc Tukey tests on log transformed coverage data showed that polystyrene had significantly lower coverage than glass ($p = 0.006$). Polystyrene was not found to have significantly different thickness or biovolume to any of the materials.

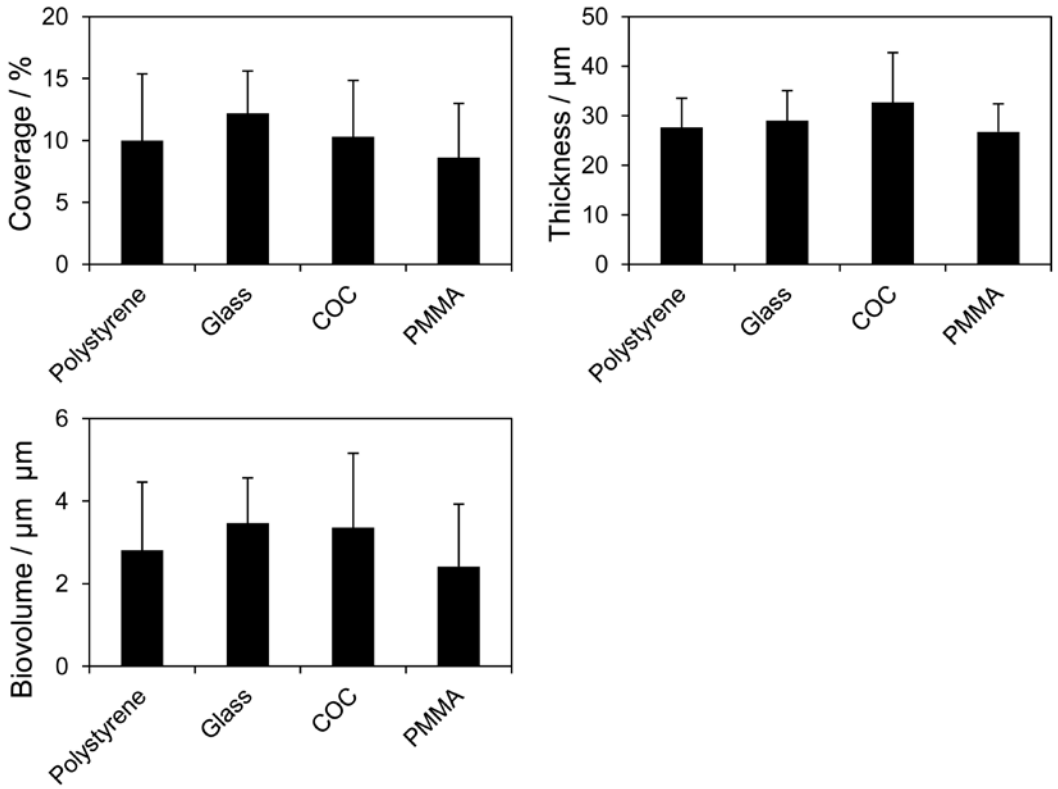
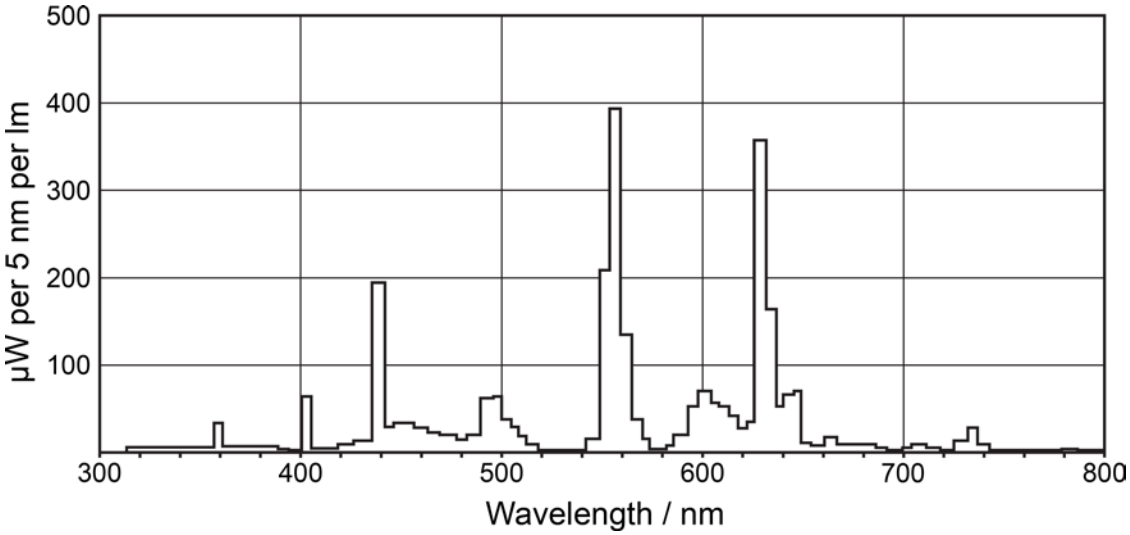


Figure A6.1: Fouling after 14 days exposure to the sea did not vary greatly between the four materials tested. However, both PMMA and polystyrene were found to have significantly lower coverage than glass.

Appendix 7. Philips Master TL-D Super 80 Fluorescent Tube Light Spectrum



Source: (Koninklijke-Philips-Electronics, 2013)

Appendix 8. SOC medium

Method

- Combine all components as below with 1 l of H_2O.
- Adjust to pH 7.0 using sodium hydroxide

Components

Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
KCl	0.186 g
$MgCl_2$	0.952 g
Glucose	3.603 g

Appendix 9. Enzymatic Lysis buffer

- Mix 4 ml 0.5 M Tris HCl (pH 8.0), 0.4 ml 0.5 M sodium EDTA and 2 ml 60% Triton X-100 (v/v in water).
- Bring volume to 100 ml with nuclease free water and filter sterilised through a 0.2 µm membrane.

Appendix 10. Denaturing gradient gels

- All gels had a final concentration of 6% (w/v) acrylamide.
- Acrylamide gels were polymerised using a final concentration of 0.09% for both TEMED and ammonium persulphate.

0% denaturant gel

40% acrylamide/bis	15 ml
50x TAE buffer	2 ml
H_2O	83 ml

100% denaturant gel

40% acrylamide/bis	15 ml
50x TAE buffer	2 ml
Deionised formamide	40 ml
Urea	42 g
H_2O	To 100 ml

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