

# **Marine Inspired Design for Antifouling Technology**

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to



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### **Declaration**

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Chloe Richards (Candidate) ID No.: 13391151 Date: 9<sup>th</sup> June 2022

Chloe Richards

*‘The only source of knowledge is experience’*

Albert Einstein

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### **List of Abbreviations**

| <b>Notation</b> | <b>Meaning</b>                         |
|-----------------|----------------------------------------|
| <b>EPS</b>      | Extracellular polysaccharides          |
| <b>AF</b>       | Antifouling                            |
| <b>SPC</b>      | Self-polishing copolymer               |
| <b>TBT</b>      | Tributyltin                            |
| <b>DLVO</b>     | Derjaguin-Landau-Verwey-Overbeek       |
| <b>SAM</b>      | Self-assembled monolayer               |
| <b>ps</b>       | picosecond                             |
| <b>SHS</b>      | Superhydrophobic surface               |
| <b>PREL</b>     | Proximity rolling-exposure lithography |
| <b>EMM</b>      | Electrochemical micromachining         |
| <b>ALD</b>      | Atomic layer deposition                |
| <b>PDMS</b>     | Poly(dimethyl)siloxane                 |
| <b>WCA</b>      | Water contact angle`                   |
| <b>AFM</b>      | Atomic Force Microscopy                |
| <b>SEM</b>      | Scanning Electron Microscopy           |
| <b>ANOVA</b>    | Analysis of Variance                   |
| <b>FR</b>       | Foul-release                           |
| <b>PMMA</b>     | Polymethylmethacrylate                 |
| <b>CAD</b>      | Computer aided design                  |
| <b>NaOH</b>     | Sodium hydroxide                       |
| <b>HCl</b>      | Hydrochloric acid                      |

|                      |                                    |
|----------------------|------------------------------------|
| <b>GF/F</b>          | Glass fibre filters                |
| <b>BSA</b>           | Bovine Serum Albumin               |
| <b>eDNA</b>          | environmental DNA                  |
| <b>DNA</b>           | Deoxyribonucleic Acid              |
| <b>RNA</b>           | Ribonucleic Acid                   |
| <b>WD</b>            | working distance                   |
| <b>FOV</b>           | fields of view                     |
| <b>LM</b>            | Light Microscopy                   |
| <b>N<sub>2</sub></b> | Nitrogen                           |
| <b>DILL</b>          | Dip-in Laser Lithography           |
| <b>STL</b>           | Stereolithography                  |
| <b>AO</b>            | Acridine Orange                    |
| <b>ITO</b>           | Indium Titanium Oxide              |
| <b>RPM</b>           | Revolutions Per Minute             |
| <b>Hz</b>            | Hertz                              |
| <b>PSA</b>           | Pressure Sensitive Adhesive        |
| <b>FMEA</b>          | Failure Modes and Effects Analysis |
| <b>ROI</b>           | region of interest                 |
| <b>STP</b>           | training set of pixels             |
| <b>LOD</b>           | Limit of Detection                 |
| <b>LOQ</b>           | Limit of Quantification            |
| <b>SR</b>            | <i>Scophthalmus rhombus</i>        |
| <b>RM</b>            | Replica Moulding                   |

|              |                                    |
|--------------|------------------------------------|
| <b>μCP</b>   | Microcontact Printing              |
| <b>MIMIC</b> | Micro-moulding in microcapillaries |
| <b>μTM</b>   | Micro-transfer moulding            |
| <b>SAMIM</b> | Solvent assisted micro-moulding    |
| <b>2PP</b>   | 2-Photon Polymerization            |
| <b>NIR</b>   | Near infrared                      |
| <b>EDX</b>   | Energy Dispersive X-ray Analysis   |
| <b>RBA</b>   | Raised Bar Array                   |
| <b>RCA</b>   | Recessed Cavity Array              |

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This thesis is dedicated to Conor Cronin

## List of Publications

Chloe Richards, Ciprian Briciu-Burghina, Matthew R. Jacobs, Alan Barrett, and Fiona Regan, *Assessment of Antifouling Potential of Novel Transparent Sol Gel Coatings for Application in the Marine Environment*, *Molecules* 2019, 24 (16), 2983.

Chloe Richards, Nicole O' Connor, Diveena Jose, Alan Barrett and Fiona Regan, *Selection and optimization of protein and carbohydrate assays for the characterization of marine biofouling*, *Analytical Methods*, 2020, 12, 2228 – 2236.

Chloe Richards, Asma Slaimi, Noel O'Connor, Alan Barrett, Sandra Kwiatkowska and Fiona Regan, *Bio-inspired Surface Texture Modification as a Viable Feature of Future Aquatic Antifouling Strategies: A Review*, *International Journal of Molecular Science*, 2020, 21(14), 5063.

## Oral Presentations

Chloe Richards, Adrián Delgado Ollero and Fiona Regan, *Characterization, replication and testing of designed micro-textures, inspired by the Brill fish, *Scophthalmus rhombus*, for the development of bioinspired antifouling materials*, *Environ* 2021, 16th – 18<sup>th</sup> June 2021, Ireland.

Chloe Richards, Fiona Regan, and Yan Delauré, *Next Evolution in Materials and Models for Ocean Energy*, NEMMO Month 6 Project Meeting, 6<sup>th</sup> November 2019, Technion – Israel Institute of Technology, Haifa, Israel.

Chloe Richards, Adrián Delgado Ollero, Yan Delauré and Fiona Regan, *Biomimetic texturing as an effective antifouling solution*, *Ocean Energy Europe* 2021, 8<sup>th</sup> December, Brussels, Belgium.

## Poster Presentations

Chloe Richards, Ciprian Briciu-Burghina, Alan Barrett and Fiona Regan, *Assessment of biofilm formation on novel transparent coatings for application to a low-cost optical sensor deployed in an estuarine environment*, Europt(r)ode XIV, 25th – 28th March 2018, Naples, Italy.

Chloe Richards, Ivan Maguire and Fiona Regan, *Characterization and testing of dermal scales of *Scophthalmus rhombus* for application in antifouling technology*, N.I.C.E. 2018, 14<sup>th</sup> – 17<sup>th</sup> October 2018, Nice, France (2<sup>nd</sup> Place Best Poster Award).

Chloe Richards, Alan Barrett, Ivan Maguire, Sandra Kwiatkowska and Fiona Regan, *Marine inspired textured materials for reduction of biofouling on surfaces*, OCEANS 2019, 17th – 20th June 2019, Marseille, France.

Chloe Richards and Fiona Regan, *Investigations of the performance of a marine antifouling material inspired by *Scophthalmus rhombus**, Environ 2020, 22nd – 24th October 2020, Ireland.

Chloe Richards, Adrián Delgado Ollero and Fiona Regan, *Characterization, replication and testing of designed micro-textures, inspired by the Brill fish, *Scophthalmus rhombus*, for the development of bioinspired antifouling materials*, DCU Chemistry Day 2021, 4<sup>th</sup> June 2021, Ireland (Best Poster Award).

## Workshops & Awards

1. Science@Sea Marine Science Training, 7<sup>th</sup> – 8<sup>th</sup> November 2018, Cork, Ireland.
2. N.I.C.E 2018, 2<sup>nd</sup> Place Student Poster, Nice, France.
3. The Alan Tetlow Memorial Bursary from Royal Society of Chemistry (Water Science Forum), 2018.
4. DCU Chemistry 2021, 1st Place Student Poster, Dublin, Ireland.
5. Arctic Frontiers Emerging Leaders 2022, 5<sup>th</sup> – 11<sup>th</sup> May 2022, Norway.

# Marine Inspired Design for Antifouling Technology

Chloe Richards, BSc

## Abstract

Biofouling occurs when artificial surfaces are immersed underwater, leading to the build-up of organic matter. Biofouling begins with the submersion of a clean surface, whereby micro-organisms will colonise and form highly intricate and dynamic microbial communities (i.e., bacteria, diatoms, barnacles and mussels), enveloped in a matrix of extrapolymeric substances (EPS) <sup>14</sup>. Currently, the application of biocidal coatings for biofilm prevention has been used, many of which are toxic to the aquatic environment. <sup>14,15</sup>

Surfaces that are capable of controlling cellular behaviour under natural conditions are challenging to design due to the diversity of attaching cell types in environments such as marine waters, where many variations in cell shape, size and adhesion strategy exist <sup>16</sup>. Nature has had billions of years of evolution to solve the fouling problem, for which scientists can use to gain insight into what common features exist on naturally non-fouling species, and then replicate or modify these features to produce natural antifouling (AF) mechanisms for commercial use <sup>17</sup>.

This thesis examines the influence of surface chemistry and surface topography on biofilm formation, looking at non-toxic strategies for antifouling technology in the marine environment. Many marine organisms have evolved to present physical, behavioral and chemical mechanisms that exhibit antifouling properties <sup>18</sup>. In that respect, the mechanisms used by marine organisms to prevent fouling on external surfaces are of huge interest to researchers of antifouling technology.

The natural surface of the Brill fish scale, has been characterized to determine its antifouling properties. An artificial surface, inspired by Brill has been reproduced using the latest microfabrication techniques and tested in the laboratory under controlled conditions. The results indicate that topography can have an influence on cell behaviour using two model organisms, *Amphora coffeaeformis* and *Nitzschia ovalis*. This work outlines a strategy for the future design of non-toxic antifouling materials.

# Chapter 1:

## Introduction

## 1.1 Introduction

---

Biofouling is a major problem in marine waters where most immersed surfaces become fouled to some extent, developing large amounts of biomass. In recent years, there has become a growing need for the development of antifouling technology to attenuate biofouling of marine monitoring equipment, ships, sensors, and buoys. The accumulation of large quantities of biomass on these surfaces cause substantial economic burdens within the marine industry <sup>19</sup>.

Advanced biofouling in marine waters can often accumulate such significant biomass that biofouling is often further subdivided into two subdivisions: microfouling and macrofouling. Microfouling is a type of fouling composed of microbial organisms such as bacteria and diatoms. Macrofouling is caused by the accumulation of larger life forms such as barnacles, bryozoans, polychaetes and macro-algae <sup>20,21</sup>. Microfouling is considered a necessary precursor to the development of a macrofouling community and can be detrimental to the deployment of sensitive equipment such as environmental sensors over time scales of just two weeks in areas of high fouling pressure <sup>22</sup>. The biofouling process is often very complex and occurs in several steps illustrated in Figure 1.1. The first step in biofilm formation is the attachment of cells to a surface. In the case of biofilm, a solid-liquid interface can provide an excellent environment for cells to grow as the constant flow of water past these surfaces provides fresh nutrients necessary for growth. Hydrophilic surfaces in particular have been shown to provide an optimum environment for cell adhesion, which improves the ability of cells to grow and proliferate <sup>23</sup>.

After this attachment step, cell proliferation leads to the formation of a micro-colony and cellular chemical signaling activates biofilm genes which enhance the ability of micro-colonies to form and adhere to solid interfaces. Mature biofilms can then detach from the solid interfaces to facilitate the multiplication and dispersal of cells and biofilm microcolonies<sup>2</sup>.



**Figure 1.1.** Primary steps in biofilm formation and dispersal (from left to right); a) conditioning, b) attachment, c) colonization and d) growth and detachment. The order of hard surface colonization during biofilm formation is as follows: seconds to hours (i.e., conditioning layer); hours to days (i.e., bacteria); days to months (i.e., diatoms) and months to years (i.e., larvae, spores) (Adapted from <sup>1</sup>).

It is important to note that this is only the early stages of biofilm formation in which microorganisms (i.e., bacteria, diatoms) will attach to the submerged substrate. However, the formation of this film will eventually lead to the process of macrofouling (occurring after the formation of an initial biofilm) in which larger organisms such as barnacles and mussels adhere to the surface <sup>24</sup>.

A biofilm is a vast chemical matrix consisting of proteins, DNA, RNA, polysaccharides and water. Water is the main component of this matrix accounting for almost 97% of the total biofilm's chemical composition. The structure of a biofilm can be subdivided into two components; a water channel (i.e., allowing for the transfer of nutrients through the matrix) and microbial cells (highly packed, no pores). Microbial cells that are present in the biofilm are often said to have enhanced characteristics which allow them to neutralize antimicrobial agents. Some even activate genes that are responsible for the expression of stress genes which, as a result, changeover to resistant phenotypes from environmental stresses (i.e., cell density, nutrition, temperature, pH and osmolarity) <sup>23</sup>.

Table 1.1 gives an overview of the biofilm matrix.

**Table 1.1.** The chemical composition of a biofilm matrix.

| Component       | Biofilm Composition (%)  |
|-----------------|--------------------------|
| Proteins        | <1 – 2 % (incl. enzymes) |
| DNA and RNA     | <1 – 2 %                 |
| Polysaccharides | 1 – 2 %                  |
| Microbial Cells | 2 – 5 %                  |
| Water           | 97 %                     |

After colonization occurs, cells will envelop themselves in a matrix of extracellular polymeric substances (EPS), polysaccharides, proteins, and nucleic acids, amongst other things. This highly dynamic and diverse matrix allows for the organization of microorganisms, resulting in biocidal resistance and the development of complex interactions<sup>2</sup>.

Extracellular polymeric substances (EPS) are notably the most important characteristic of a biofilm, enabling biofouling organisms to stay attached on a surface<sup>2</sup>. Environmental conditions such as porosity, density, water content, charge, hydrophobicity, and mechanical stability, are controlled by the EPS matrix, enabling biofouling organisms to survive <sup>25</sup>.

The EPS matrix is a diverse and highly hydrated environment, containing proteins, glycoproteins, glycolipids, and extracellular DNA, for the binding of cells and retainment of water. The attachment of an EPS matrix to the surface, allows for the transfer of essential nutrients from the environment to the enveloped organisms<sup>2</sup>. The EPS matrix is bound together by physiochemical interactions; hydrogen bonds, van der Waals forces and electrostatic interactions, providing mechanical stability for colonizing species <sup>26</sup>. An illustration of the EPS matrix structure is shown in Figure 1.2.

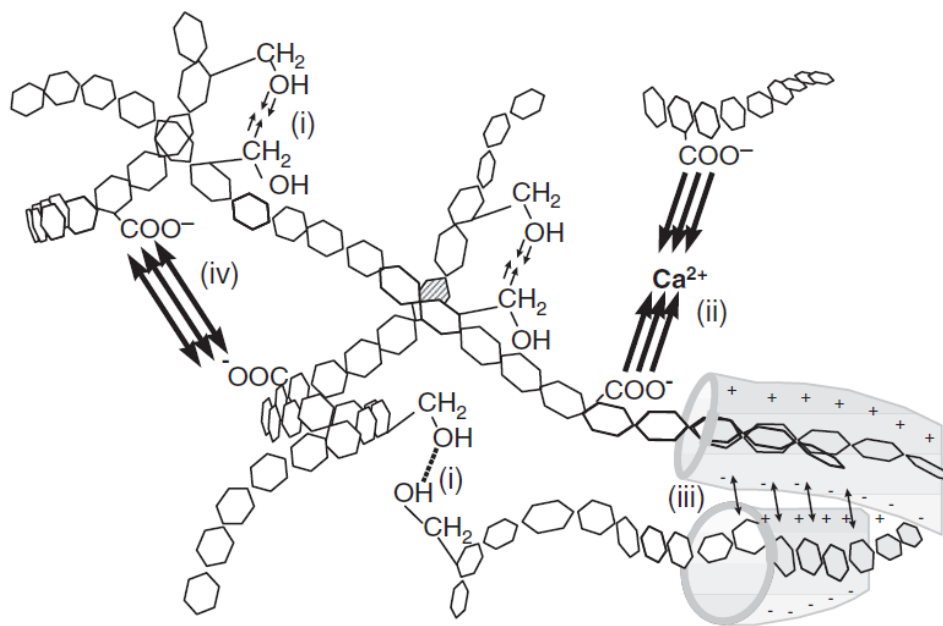


**Figure 1.2.** The forces that keep an EPS matrix together; (i) hydrogen bonding, (ii) cation bridging, (iii) van der Waals forces, and (iv) repulsive forces (Taken from <sup>2</sup>).

The studies carried out within this work look at the adherence of diatoms on artificial substrate submerged in the marine environment. Diatoms are a unique group of unicellular protists abundant in all aquatic environments but especially in the marine environment <sup>3,27</sup>. In general, diatoms can be split into two groups: planktonic and benthic. Planktonic diatoms are commonly found in lake environments and are suspended (unattached) in open water. Benthic diatoms have been classified as major foulers in the marine environment. Benthic diatoms grow on submerged underwater surfaces (i.e., sand, rock, marine monitoring equipment, any artificial surface), on macroalgae or even on 'large colonial mats' binding sandy surfaces <sup>28,29</sup>. The diatom frustule is made up of a 'highly ornamented siliceous cell wall', consisting of two thecae: an epitheca and a hypotheca). These thecae (which fit together like two jigsaw pieces), each contain a silica valve and one or more girdle bands that allow for cellular growth. The siliceous valves are

highly ornate (i.e., with defined pores/slits) which are species-specific. The siliceous valves have several functions including nutrient and gas exchanges with the external environment and the secretion of EPS. The raphe works closely with several other intercellular structures which come together for the secretion of adhesive mucilage – subsequently causing the cell-surface adhesion. A series of scanning electron micrographs highlighting some of the features of diatom structures is shown below in Figure 1.3 <sup>3</sup>.

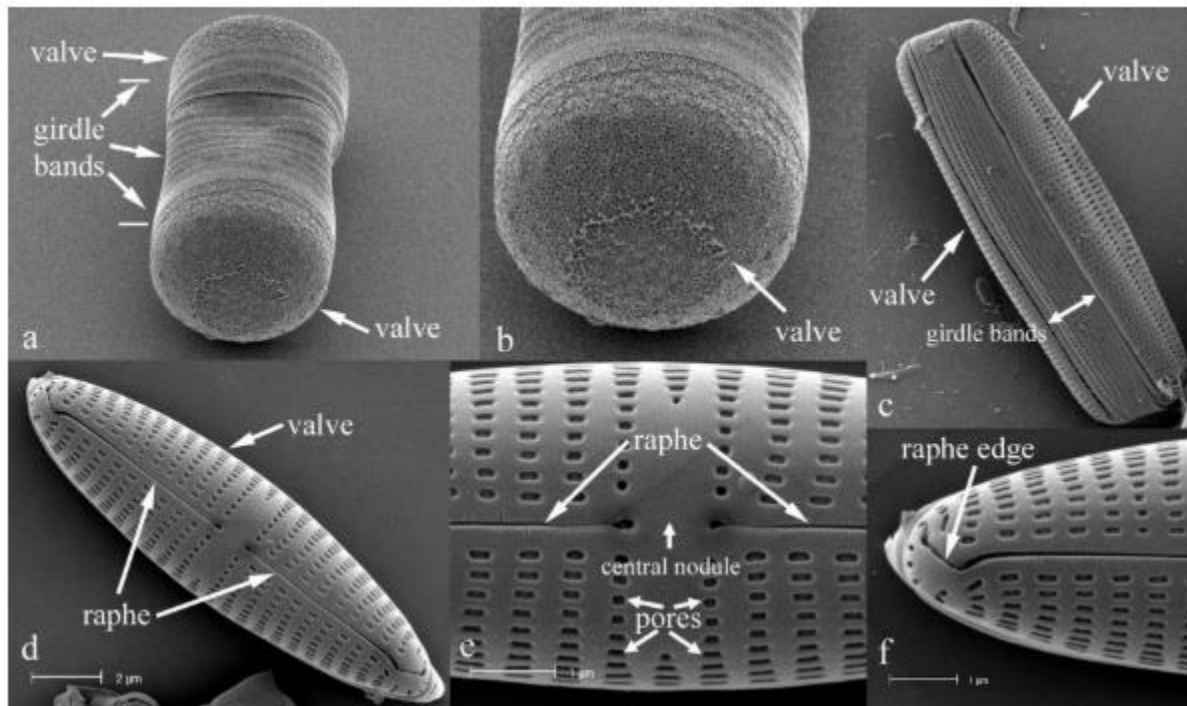


**Figure 1.3.** Electron micrographs of the diatom *Melosira* sp. (a) and *Navicula* sp. (c) – (f) (Taken from <sup>3</sup>)

As a diatom comes into contact with a surface, motile diatoms tend to move to an optimal area by a process known as gliding (i.e., cell motility), where suitable environmental conditions exist (i.e., light, nutrients, protection from tidal currents). Some species of diatoms (i.e., *Achnanthes*) may even produce other adhesive structures (pads or stalks), which allows for an accumulation of diatom species over large areas of a surface and is usually referred to as 'rafting' – allowing cells to adhere strongly to a surface for further proliferation <sup>3</sup>.

## 1.2 Development of antifouling (AF) systems

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The problem of biofouling is not a new concept, with some of the earliest reports dating back as far as the time of the early Phoenicians and Carthaginians, who were said to have used pitch and copper sheathing on ship's hulls. Other ancient cultures were also reported to have used wax, tar and asphaltum for antifouling <sup>30,31</sup>. By the 5<sup>th</sup> century B.C., arsenic and sulphur coatings mixed with oil were said to be used to prevent shipworms. With the arrival of the Vikings (10 A.D.), came the use of 'seal tar' however from the 13<sup>th</sup> to the 15<sup>th</sup> century, pitch (combined with oil, resin, or tallow) was used to protect ships from fouling. In the 16<sup>th</sup> century, wooden sheathing combined with a layer of hide and tar was used however, for the most part, lead sheathing was much more popular at the time as evidenced in its use by England, Spain and France <sup>31</sup>.

Copper had been used previously on Phoenician warships and on Greek and Roman boats as fastenings. The first reported case of copper in underwater systems occurred during the rule of Danish King Christian IV in 1618, with the use of a coppered keel. However, the use of copper as an antifouling system can be dated back to 1625, when Beale used a combination of cement, iron, and copper (most likely copper sulphide or copper arsenic ore). In 1758, came the use of copper sheathing on the *HMS Alarm* and by 1780, copper sheathing was widely used in the British Navy for vessel protection. Copper sheathing on wooden ships became so popular during this period that England banned the exportation of copper, stating its value as a war material. Yet, in the 17<sup>th</sup> century, it was discovered by Sir Humphrey Davy, that it was the dissolution of copper that prevented fouling in the marine environment <sup>31</sup>.

In the 18<sup>th</sup> century, with the introduction of iron ships, the use of copper sheathing declined due to its corrosive effects on iron. Numerous alternative technologies had been trialed including, but not limited to, zinc, lead, nickel, arsenic, galvanized iron, and coppered wooden sheathing. Non-metallic AF alternatives were also tested such as felt, canvas, and rubber sheathing – with attention falling on the development of AF compositions<sup>32</sup>. Historically, biocidal coatings containing heavy metals such as copper were used to prevent fouling. In the 1960's, came the use of self-polishing paints, made up of organotin(tributyl tin TBT and triphenyl tin TPT) compounds. The self-polishing copolymer coating was designed to erode at a continuous rate over the lifetime of the coating and was termed 'self-polishing' as it was claimed that the process constantly presented a smoother surface, with subsequent reductions in drag and increases in fuel economy for shipping. When SPCs were combined with TBT, an especially effective antifouling surface was created. These self-polishing copolymer organotin systems were highly effective in antifouling, with ships avoiding dry-docking for up to 5 years <sup>31,33–36</sup>. From the 1970's onwards, information began to emerge highlighting the toxic effects of these TBT-based antifouling paints on the marine environment, which resulted in the restriction of these types of paints in vessels less than 25 m in length. In 2001, the 'International Maritime Organization' decided to gradually phase out the use of TBT-based paints <sup>33,34,36,37</sup>.

Eventually, in 2008, regulations were put in place to insist upon a change from self-polishing copolymer organotin systems (due to toxicity in the environment) to silicone and copper-consisting antifouling technologies. However, the four decades of market domination by TBT coatings had meant that there was little development in alternative non-toxic antifouling solutions and total prohibition had meant that a race to develop alternative technologies was a must. In recent years, the attachment mechanisms of microfouling communities that contribute to fouling have become of interest and require in-depth understanding to develop the best antifouling alternatives for the marine industry including ships, sensors, AUVs and buoys to name a few <sup>38</sup>.

A search for new non-toxic marine coatings meant that the opportunity to explore 'green' methods of antifouling had arisen, with the consequence that developing non-biocidal methods of preventing fouling received much attention where natural fouling defense mechanisms have been mimicked through chemical, physical, and/or stimuli-responsive methodologies <sup>35</sup>.

In 2010 the final report of a 60-month European project: Advanced nanostructured surfaces for the control of biofouling (AMBIO) was published <sup>39</sup>. The team of scientists reported the study of 500 different nanostructured coatings, representing 64 generic coating chemistries that were prepared at laboratory-scale and evaluated for their antifouling and fouling-release performance. The study led to fifteen coatings selected for testing in a range of field and end-user scenarios. Several coatings showed promise, with some leading to commercialized products and others showing potential for further development <sup>40–48</sup>.

Figure 1.4 below shows the comparison of the growth trends looking at four key research subject areas from 2009 – 2019 indexed by Web of Science. Surprisingly, the subject area of biomimetic surfaces was among the least researched areas of interest during the past 10 years.

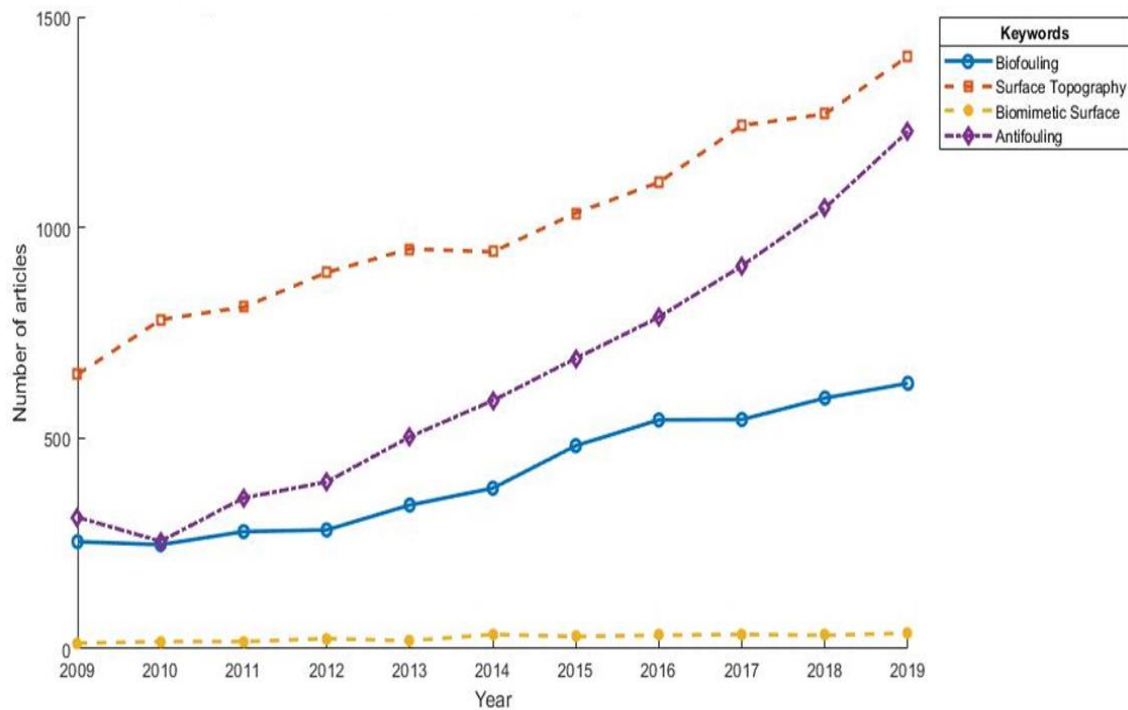


**Figure 1.4.** Comparison of the research trends of keyword selected articles from 2009 – 2019 indexed by Web of Science.

### 1.3 Biomimetics

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The imitation of natural systems to produce effective antifouling materials is often referred to as 'biomimetics'. The world of biomimetics is a multidisciplinary one, needing careful understanding of 'biological structures', processes and principles of various organisms found in nature and based on this, designing nanodevices and nanomaterials that are of commercial interest to industry. Looking to the marine environment for bioinspired surfaces offers researchers a wealth of topographies to explore. Particular attention has been given to the evaluation of textures based on marine organisms tested in either the laboratory or the field. The findings of this section relate to the numbers of studies on textured surfaces demonstrating antifouling potential which are significant. However, many of these are only tested in the laboratory, where it is acknowledged a very different response to fouling is observed.

Biofouling is a surface-based phenomenon therefore it is not surprising that the substrate-environment interface has a significant influence on the type, rate and extent of fouling that may occur <sup>42–46</sup>. A range of fabrication techniques is available to produce a wide variety of designed surface structures with high fidelity and relatively low-cost compared to previous decades <sup>49</sup>. There are extensive examples in the literature of sophisticated sub micrometer scale pattern fabrication <sup>48,50,51</sup>. Previously, surface features were categorized as either structural topography or chemical patterning, however at the nanoscale, the distinction between purely physical and purely chemical patterning of surfaces is now being eroded. Both 3-D physical and chemical nanoscale organization are now possible within a range of methods, including but not limited to; optical lithography, microcontact printing, electron beam lithography, ion beam lithography, soft lithography, direct laser interference patterning and 3-D printing. The knowledge of nanotechnology, and with it, the ability to manipulate surface nanostructure, offers the potential to both enhance the efficacy of existing materials and to produce a completely novel, and perhaps a non-toxic mechanism of antimicrobial activity <sup>46,52,53</sup>. The purpose of this chapter is to outline some of the earliest and latest knowledge, surrounding biological responses to marine inspired surface topography. It deals with the potential of surface modification in general, and techniques used, as a viable component of future aquatic antifouling strategies.

## 1.4 Production methods

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In order to be able to assess the organism-surface interaction, it is often necessary to replicate these surfaces for testing purposes. The work of Jinhong Fu and coworkers and Marin Steenackers and coworkers as part of the AMBIO project, has shown the development of hierarchical structures on surfaces <sup>54,55</sup>. Fu *et al.* <sup>56</sup> defined a controllable way to produce hierarchical micro- and nanostructured surfaces simultaneously by changing the pH. This enabled the tuning of the size range of the morphologies. The topography of the multilayer structure was fixed by thermal cross-linking and turned into a superhydrophobic surface by the chemical vapor deposition of (tridecafluorooctyl)-triethoxysilane. In a separate study by Steenackers *et al.* <sup>57</sup>, self-initiated photografting and photopolymerization (SIPGP) of styrene and acrylic monomers on structured  $\omega$ -functionalized biphenyl thiols self-assembled monolayers (SAMs) on gold was shown. This was a three-step approach allowing the preparation of defined structured polymer brushes without the need of a specific surface bonded photo initiator function. Polymer brushes were selectively formed on cross-linked SAM regions. The polymer layer thickness was controlled by the extent of electron-induced cross-linking and head group conversion of the SAM layer <sup>39,55</sup>.

In a recent study, picosecond (ps) laser texturing of stainless steel was carried out by Sun *et al.* <sup>58</sup>, generating micro-groove and micro-pit arrays which were tested in the laboratory in artificial seawater. The results were reported to be a fast, highly controllable picosecond laser patterning way for preparing hierarchical micro/nanostructures, combined with the chemical modification by silica sol, was proposed to fabricate the anti-biofouling stainless steel superhydrophobic surfaces (SHSSs). The results of five weeks seawater immersion

test showed that the specimens with SHS demonstrate significant anti-biofouling effect. It is not clear if the texture alone can provide valuable inhibition of biofouling – though the technique is worth considering due to its applicability to steel.

In addition to the elegant chemical methods of generating surface features and topographies, the growing interest in materials science, has led to a wide range of physical fabrication methods (Figure 1.5). These methods are used to replicate and/or produce textures that are inspired by either attachment point theory or by surface features of natural organisms. These are summarized in Table 1.2.

**Table 1.2.** Summary of manufacturing methods commonly used for the production of nano- and micro-scale textured surfaces.

| Method                                                                                   | Description                                                                                                                                                                                                                                                                                                                                                                                                                       | Reference |
|------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Photolithography *                                                                       | Formation of a pattern in a layer of photoresist which can be transferred by etching into an underlying film.                                                                                                                                                                                                                                                                                                                     | 4,59,60   |
| Electron beam lithography *                                                              | Produces surface patterning between 3–5 nm following exposure to electron beam.                                                                                                                                                                                                                                                                                                                                                   | 61–63     |
| Ion beam lithography *                                                                   | Produces surface patterning of <100 nm due to the nature of the ion.                                                                                                                                                                                                                                                                                                                                                              | 61,64,65  |
| Proximity rolling-exposure lithography (PREL) and electrochemical micromachining (EMM) * | Produces surface patterning over a large surface area, with the ability to produce texturing of various shapes that are otherwise impossible with some of the other techniques.                                                                                                                                                                                                                                                   | 66–69     |
| Two-photon lithography and atomic layer deposition (ALD) *                               | Two-photon lithography produces 3-D complex surface topographies with resolutions of around 150 nm, however, requires a photosensitive polymer resin, preventing its use with metallic materials. ALD produces accurate uniform films, offering controllability at atomic level, wafer-scale substrates, and high-aspect ratio models. The combination of the two offer a promising tribological solution in small-scale systems. | 70,71     |

|                                                            |                                                                                                                                                                                                                                                                                                                                                                                                                                   |            |
|------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Two-photon lithography and atomic layer deposition (ALD) * | Two-photon lithography produces 3-D complex surface topographies with resolutions of around 150 nm, however, requires a photosensitive polymer resin, preventing its use with metallic materials. ALD produces accurate uniform films, offering controllability at atomic level, wafer-scale substrates, and high-aspect ratio models. The combination of the two offer a promising tribological solution in small-scale systems. | 70,71      |
| Soft lithography                                           | Produces topographies at the micro- and nano-scale, using PDMS as a master template (Figure 1.3 (c)).                                                                                                                                                                                                                                                                                                                             | 4,72–74    |
| Micro-contact printing *                                   | Involves the fabrication of a “stamp” from PDMS by replica molding, the stamp is covered in ink, pressed and the solvent is left to evaporate, leaving the molecules to be transferred on to the substrate (Figure 1.3 (d), (e)).                                                                                                                                                                                                 | 4,75       |
| Hot embossing *                                            | Involves the use of thermoplastic polymers to create micro-patterned surfaces, involving softening the polymer, pressing the template onto the warm polymer, and revealing the micro-patterned surface after cooling (Figure 1.3 (f)).                                                                                                                                                                                            | 4,76–78    |
| 3-D printing *                                             | A relatively new technique offering low-cost, efficiency and fast prototyping—requires more in-depth examination.                                                                                                                                                                                                                                                                                                                 | 4,61,79,80 |
| Picosecond laser texturing *                               | Involves the texturing of stainless steel to create an AF superhydrophobic surface. Results indicated a 50% decrease in the mean microbial attachment area ratio—a significant effect in comparison to the untextured stainless steel.                                                                                                                                                                                            | 81,82      |

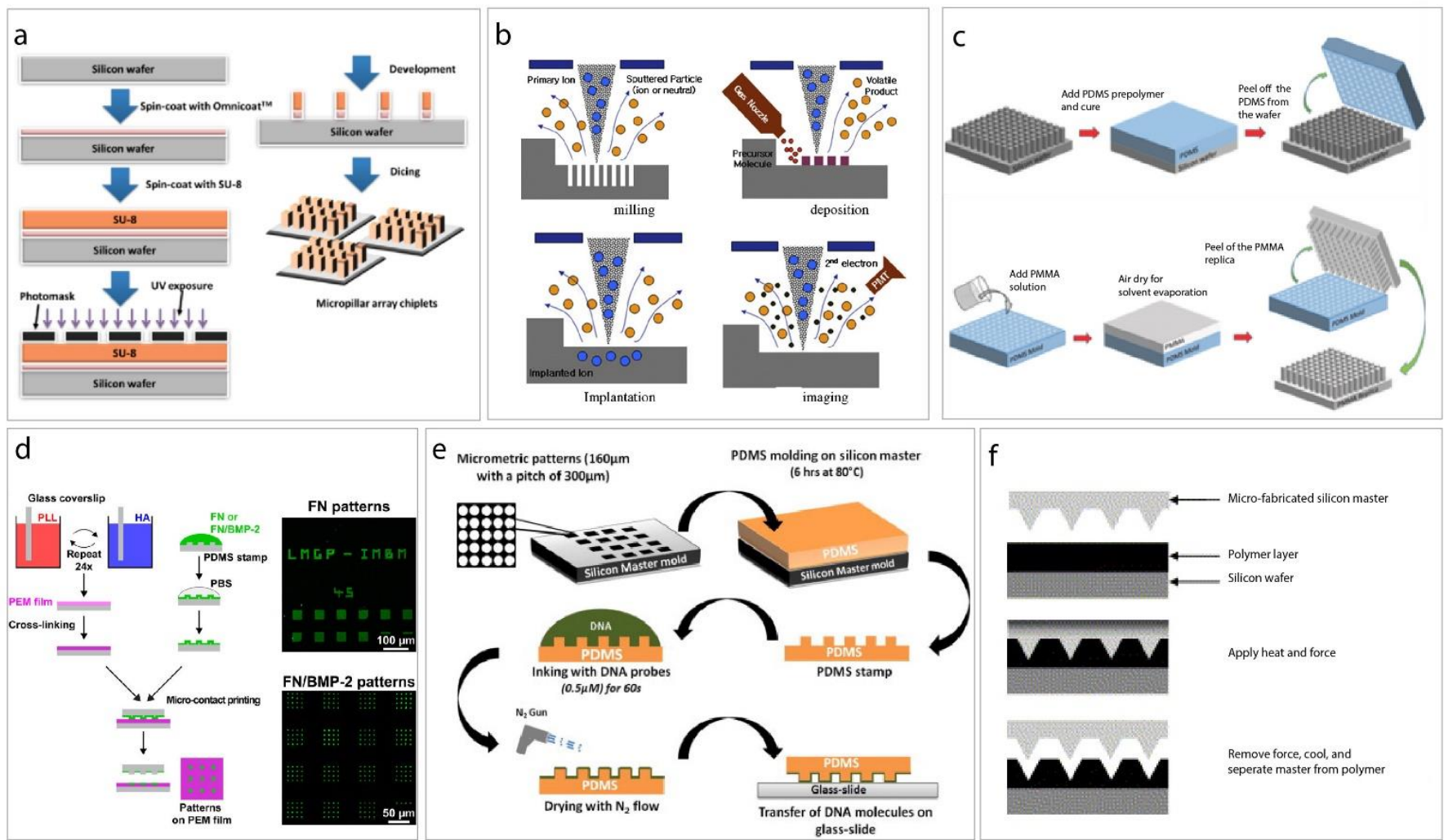


**Figure 1.5.** Schematic illustrations of micro- and nano-fabrication methods: (a) production of micro-scale surface topographies using photolithography. (b) electron beam lithography. (c) solvent casting (i.e., soft lithography). (d) micro-contact printing. (e) direct microcontact printing. (f) hot embossing. (available from keaipublishing under open access journal “Bioactive Materials 3”<sup>4</sup>).

## 1.5 Surface modification

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Controlling cellular interaction with a surface is often complex, demanding careful consideration of multiple factors such as roughness, wettability, hydrodynamics, mechanical properties, and topography. Many fouling organisms (i.e., bacteria, diatoms) exist in the micrometer size range with nanometer size ranges of surface attributes. Learning to control surface topography in these micro- and nano-scale levels plays a crucial role in understanding and thus, controlling bacterial attachment and biofilm formation <sup>4</sup>.

Currently, the explanation behind biofilm attachment on submerged surfaces is not well understood. A number of theoretical models have been proposed over the years to explain this attachment, which are summarized below.

One of these models is the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (Figure 1.6) <sup>83–85</sup>. The DLVO theory is expressed as shown in Equation (1):

$$\Delta G^{adh}(d) = \Delta G^{vdW}(d) + \Delta G^{dl}(d) \quad (1)$$

where  $\Delta G^{vdW}$  refers to the attractive van der Waals forces,  $\Delta G^{dl}$  to the electrostatic repulsion forces and  $\Delta G^{adh}$  to the sum of the interaction of bacteria with a substrate <sup>54,55,86</sup>.

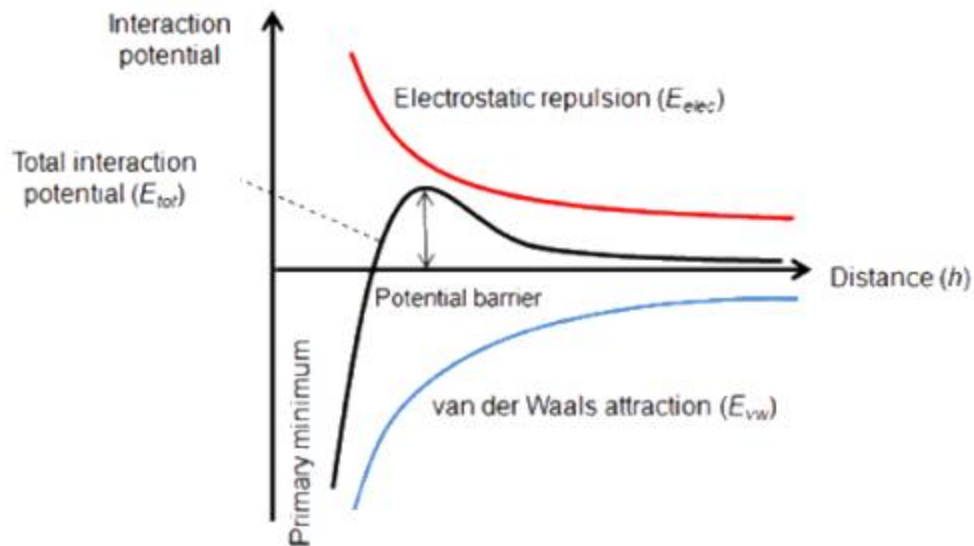


**Figure 1.6** 'Interaction Energy for stable dispersions' in accordance with the DLVO theory <sup>5</sup>.

The foundation of the DLVO theory is to differentiate interactions between colloidal particles or a colloidal particle and a substrate. This theory also offers an explanation behind the adhesion of algal cells to a surface. Bacteria cells range in size from 0.5 – 2  $\mu\text{m}$ , similar to the size of colloidal particles; this theory has been extensively used in material science to explain the interactions that occur between bacteria and a substrate. Equation (1) above shows that both the van der Waals forces,  $\Delta G^{\text{vdW}}$ , and the electrostatic repulsion forces,  $\Delta G^{\text{el}}$ , are dependent on the distance between a cell and a surface. A later extension of this theory led to a theoretical model called the extended DLVO theory. This model accounts for hydrophobic, hydrophilic and osmotic interactions (although osmotic interactions were later said to have little effect in bacterial adhesion) <sup>54,55,86</sup>. The extended DLVO theory can be summarized as follows:

$$\Delta G^{adh}(d) = \Delta G^{vdW}(d) + \Delta G^{dl} + \Delta G^{ab} \quad (2)$$

where  $\Delta G^{ab}$  refers to acid and base interactions <sup>55</sup>.

Another theoretical model proposed for the explanation behind cell adhesion is that of thermodynamic theory. Thermodynamic theory expresses forces (i.e., van der Waals, electrostatic and dipole) on the basis of free energy <sup>55</sup>. Thermodynamic theory can be summarized by Equation (3):

$$\Delta G^{adh} = \gamma_{sm} - \gamma_{sl} - \gamma_{ml} \quad (3)$$

where  $\gamma_{sm}$  refers to the solid-microorganism,  $\gamma_{sl}$  to the solid-liquid and  $\gamma_{ml}$  to the microorganism-liquid free energies <sup>55</sup>.

The basis of the thermodynamic theory relies on free energy; essentially, adhesion will occur on a surface if the free energy is negative. Unlike the conventional DLVO theory, thermodynamic theory does not care about the distance between the cell and its substrate. This theory assumes that bacterial interaction with a substrate is reversible, which may not always be the case — it does not explain the behavior observed in bacterial systems. However, it does state common observations in relation to wettability; hydrophilic surfaces will attract bacteria with hydrophilic properties and hydrophobic surfaces will attract bacteria with hydrophobic properties <sup>83,84</sup>.

A popular mechanism used to explain the adhesion of cells to a substrate is attachment point theory <sup>87,88</sup>. Here, the fouling organism experiences increased attachment where there are multiple attachment points (Figure 1.7 (a)) and reduced attachment when the number of attachment points are decreased (Figure 1.7 (b) – (d)). This can often be related to microtexture in the sense that highly complex topographies (i.e., whereby the microtexture is smaller than that of the organism) will not be favorable for attachment. On the other hand, where the microtexture is larger than the organism, settlement is reported to occur <sup>6</sup>.

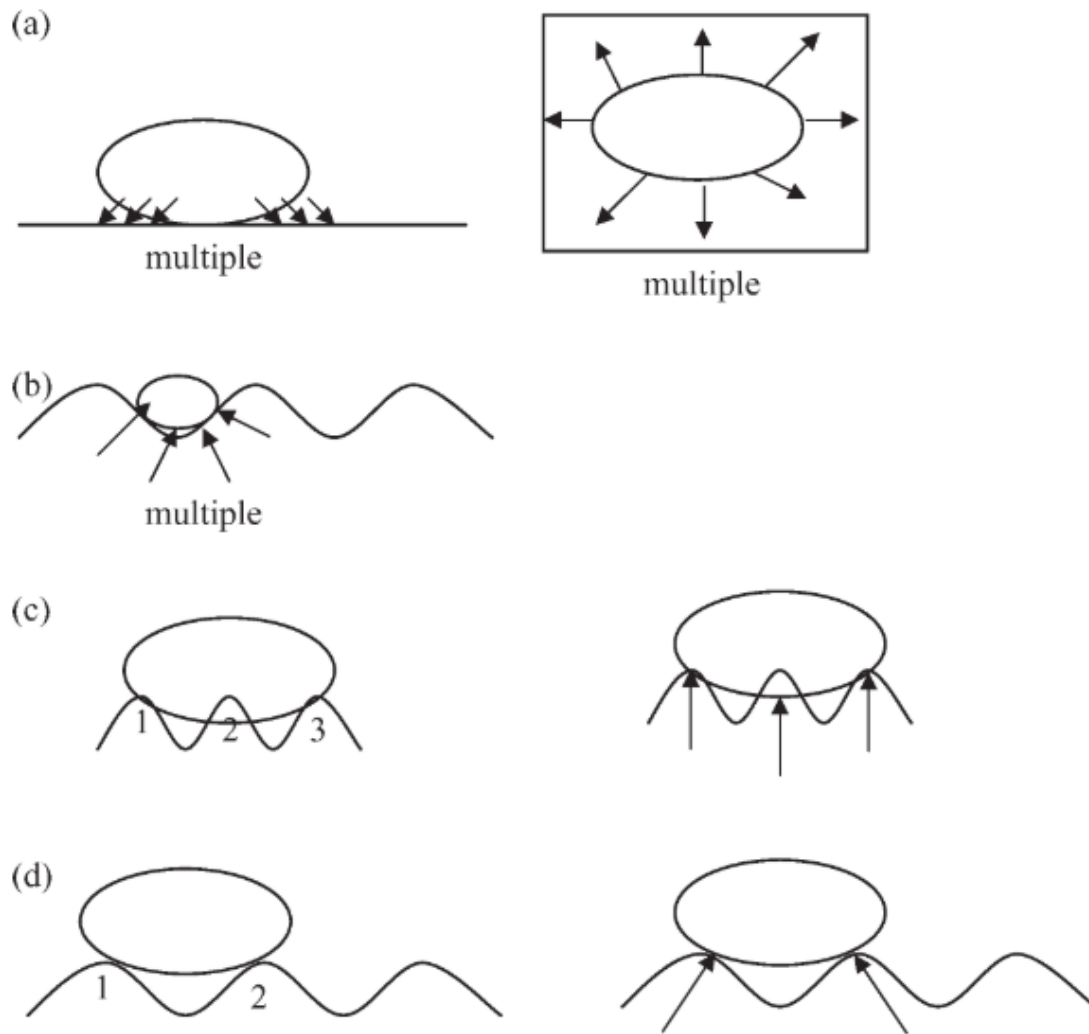


**Figure 1.7.** Schematic illustration of the theory of attachment of cells to a substrate according to attachment point theory; (a) diatom attachment on a smooth surface; (b) – (d) diatom attachment on textured surfaces of varying topographical patterning <sup>6</sup>.

### 1.5.1 *Surface roughness*

---

Early indications show that substrate roughness and topography increase the adhesion of most common fouling groups, and this is attributed to features allowing protection from hydrodynamic shear forces of removal and predation, or by increasing the surface area available for attachment. Most early studies that considered the influence of substrate roughness on fouling accumulation typically did not report any attempts to characterize the roughness scales <sup>82</sup>. However, the later re-evaluation of surface roughness has indicated that rather than a function of the purely passive mechanisms, active exploration of suitable surfaces for settlement leads to increased settlement on “preferred” surfaces <sup>89</sup>. Table 1.3 details the scale lengths of surface topographies commonly found on developed antifouling materials.

**Table 1.3.** Description of the different scale length topographies observed in common antifouling materials<sup>2</sup>.

| Scale                              | Description                                                                                                                                     |
|------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|
| Macrotopography; Ra >10 µm         | Surface finishes from cutting tools (i.e., grinding, turning, or milling)                                                                       |
| Microtopography; Ra ~1 µm          | Important in hygienic surfaces                                                                                                                  |
| Nanotopography; Ra <1 µm           | A shiny surface that appears smooth to the eye yet retains nanoscale features on the surface                                                    |
| Angstrom-scale topography; 1–10 nm | Functional groups on the surface affecting the ability of a cell to sense the surface (i.e., polymer brushes, self-assembled monolayers (SAMs)) |
| Molecular topography; molecules    | Influential in surface charge and affects cell-surface binding.                                                                                 |

### 1.5.2 Surface wettability

---

Surface wettability and surface energy are important characteristics of a material in both nature and in technology development. How the nature of the topography of a given surface influences the wettability of that surface, particularly in terms of establishing (super)hydrophobic surfaces, is now well established. It is now accepted that super hydrophobicity can only be obtained by introducing a certain degree of surface roughness, that is, a low surface energy is not enough <sup>90–93</sup>.

A surface's wettability is defined by its water contact angle (WCA), described by Young in 1804 <sup>93</sup>. It is accepted that when the contact angle is  $< 90^\circ$  the surface is hydrophilic, when the contact angle  $\geq 90^\circ$  the surface is hydrophobic. A surface having a water contact angle  $\geq 150^\circ$  is usually classified as superhydrophobic, i.e., water repellent. Young determined that the equilibrium contact angle,  $\theta_0$  of a liquid droplet on a flat substrate is determined by the interfacial energies, between the substrate, the liquid, and its vapor as shown below:

$$\cos \theta_0 = \left( \frac{\gamma_{sv} - \gamma_{sl}}{\gamma_{lv}} \right)$$

The hydrophobicity of a smooth surface is limited by the surface's chemistry; however the wetting behavior of a surface is also dependent on a surface's topography <sup>94,95</sup>. Surface roughness can have a dramatic impact on the materials hydrophobicity/hydrophilicity. This effect of roughness on the contact angle was first considered by Wenzel <sup>95</sup>. He recognized the importance of surface roughness and proposed a modification to Young's equation, which included a roughness factor,  $r$ , defined as the ratio between the actual rough surface area and the geometric projected area. According to Wenzel's equation, a solid substrate with wetting tendency ( $\theta < 90^\circ$ ) will wet more easily if its surface is rough, but, on the other hand a solid substrate with water repelling tendency ( $\theta > 90^\circ$ ) will repel more when having a rough surface:

$$\cos \theta_r^w = r \cos \theta_0$$

However Young and Wenzel only considered chemically homogeneous surfaces. Cassie and Baxter <sup>94</sup> extended Wenzel's work to non-homogeneous and porous surfaces. Cassie and Baxter equations can be also applied to rough hydrophobic surfaces. Equation 3 shows that as the surface is considered as a composite of solid and air, with a contact angle of  $\theta_r^c$ :

$$\cos \theta_r^c = f(\cos \theta_0 + 1) - 1$$

where  $f$  is the fraction of liquid—solid contact, the composite contact is established when  $\theta_0 > \theta_c$  and the threshold contact angle is defined by:  $\cos \theta_c = (f - 1)/(r - f)$ . So, for a hydrophobic rough surface, the liquid repellency prevents the liquid from fully penetrating into the depressions of the roughness morphology. Penetration of pores will occur spontaneously only for  $\theta < 90^\circ$  <sup>96,97</sup>.

From a self-cleaning perspective, the contact angle is not the only significant parameter for defining hydrophobicity. For self-cleaning surfaces, a low level of water drop adhesion to the surface is also important. This is the product of the WCA and the contact angle hysteresis (CAH), the difference between advancing and receding contact angles. A combination of high WCA and low CAH results in a decreased force being required to set a droplet in motion <sup>98</sup>.

$\Delta\theta$  is small on a chemically homogeneous and hydrophobic surface, this means that a liquid droplet will be unstable and will slide off the substrate if the substrate is tilted (conversely, if the surface chemistry is non-homogenous  $\Delta\theta$  will be large and the droplet will be effectively ‘pinned’ to the substrate’s surface.

### 1.5.3 Hydrodynamics

---

Hydrodynamic stresses play an essential role in most if not all physiological processes. In particular, cellular processes (i.e., cell morphology, intracellular processes, kinetics and cell to cell signaling) can be easily influenced by hydrodynamics<sup>99</sup>. In designing a material with an antifouling (AF) effect, a deeper understanding of the fluid mechanics at play in the micrometer to nanometer scale is essential<sup>99</sup>. The intertidal zone is an area in the marine environment exposed to air at low tide, and covered in seawater at high tide, leading to a huge diversity of plant and marine life<sup>7</sup>. It is an extremely harsh environment where the effect of a number of stresses (i.e., drag, lift acceleration) on plant and animal life are evident. In the intertidal zone, water velocities can reach between 10 and 15 ms<sup>-1</sup><sup>100</sup>, and storm waves can reach 25 ms<sup>-1</sup> in addition to accelerations of more than 400 ms<sup>-2</sup><sup>101</sup>. Hydrodynamic forces are said to have a huge effect on the ability of fouling organisms to settle. In this area of the marine environment, marine organisms are not prone to fouling, even though they are subjected to the same fouling pressures as found elsewhere. These non-fouling organisms with enhanced surface topography and optimal hydrodynamics offer an excellent opportunity to develop a non-toxic antifouling solution<sup>7</sup>. Reynolds number is defined as ‘the ratio of inertial forces to viscous forces in fluid flow’. It essentially expresses the influence of size and shape of organisms moving in a fluid<sup>7,102</sup>. Reynolds number can also be an indicator of the scale separation in fluid flow<sup>102</sup>. Microorganisms (i.e., bacteria, , ciliates) experiencing Reynolds numbers of around 10<sup>-5</sup> are said to be in an environment in which viscous forces dominate over inertial forces. As a result, bacteria, plankton and ciliate function at low Reynolds number<sup>102</sup>. These hydrodynamic interactions significantly influence the ability of an organism to settle on a

surface, allowing an organism to identify a suitable surface. In contrast to this, organisms experiencing Reynolds numbers between  $10^3$  to  $10^9$  are said to be in an environment in which inertial forces dominate over viscous forces. Therefore, larger organisms and underwater surfaces operate at high Reynolds number <sup>102</sup>. One of the challenges in creating an effective antifouling bioinspired solution is adapting systems that are both effective at low Reynolds number (i.e., over small surfaces) as well as systems that are effective at high Reynolds number (i.e., over large surfaces). Figure 1.8 outlines the variation of Reynolds number experienced by marine organisms with respect to speed <sup>7,102</sup>.

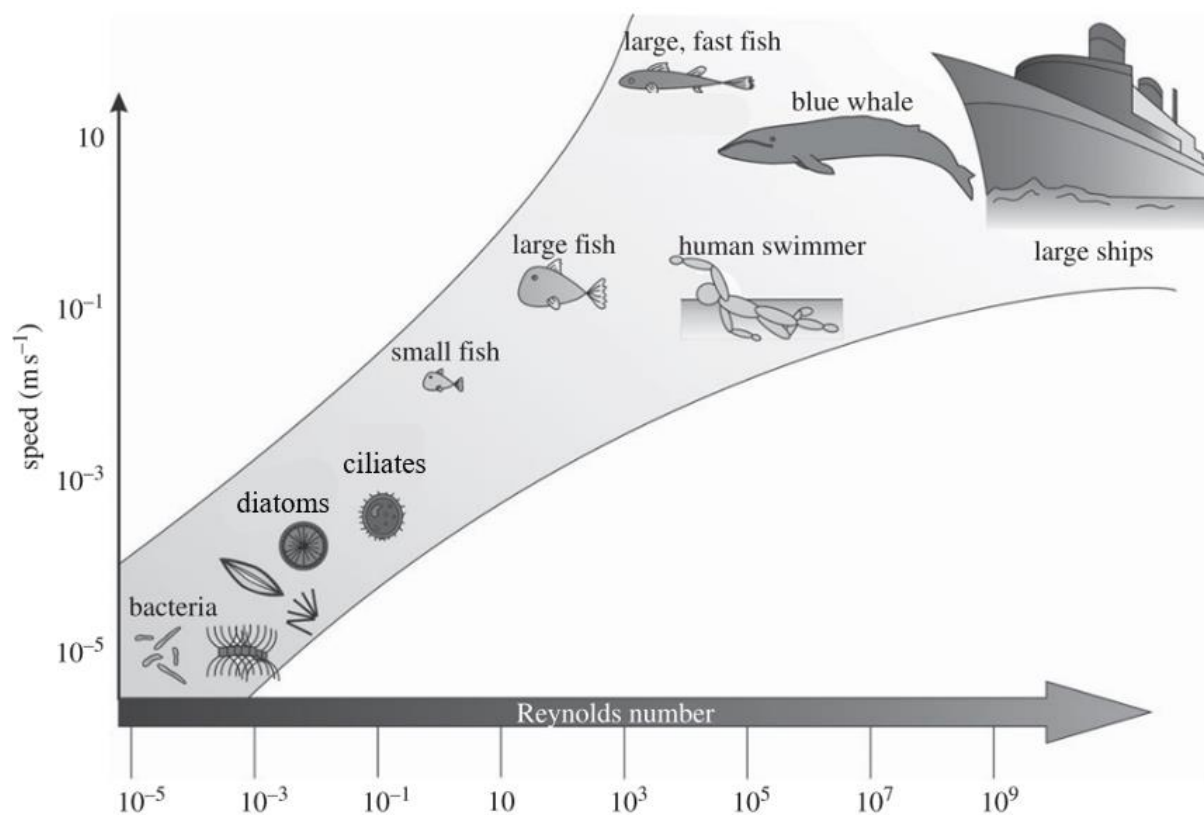


**Figure 1.8.** The variation of Reynolds number in marine organisms with respect to speed (Taken from <sup>7</sup>).

## 1.6 Surface topography

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Surfaces from marine organisms capable of reducing or preventing biofilm formation are of interest in engineering and materials sciences <sup>103–105</sup>. Biomimetic surface modification has been considered in antifouling material development and a number of studies have examined antifouling potential of topographic patterns, textures and roughness scales found on organisms <sup>35,105,106</sup>. Many marine organisms, as a result of living in the ocean (i.e., shark, dolphin, and whale), have evolved characteristics which are understood to prevent the attachment of biofouling organisms on their skin. Table 1.4 summarizes a range of studied marine organisms and their reported surface characteristics which have been tested for antifouling potential.

One of the earliest known studies of marine-inspired biomimetic AF models investigated was that of the sea fan, *Pseudopterogorgia acerosa* (Pallas) <sup>107</sup>. Vrolijk *et al.* first presented the research in 1990 with the characterization of two gorgonian octocorals, *Pseudopterogorgia americana* (Gmelin) and *Pseudopterogorgia acerosa* (Pallas). The surface of *P. americana* (Gmelin) was generally smooth, with a dense mucus layer, impeding the observation of surface topography. On the other hand, the surface of *P. acerosa* (Pallas) was discovered as having a surface topography consisting of spicules, with mean surface roughness around 2–4  $\mu\text{m}$ . Contact angle measurements completed on the gorgonian species showed low surface energies of 23–27 mN/m, equating to the region of the Baier curve closely linked with minimal bioadhesion <sup>32,107</sup>. Baier observed this phenomenon in 1973, concluding that surfaces with low critical surface tensions (20–30 mN/m) were ‘minimally bioadhesive’ <sup>32</sup>. It was concluded that the gorgonian species,

*P. acerosa*, may use this passive AF mechanism against biofilm formation in the marine environment <sup>107</sup>.

Marine invertebrates from the phylum *Echinodermata*, including the starfish, sea urchin or sea cucumber are well-documented as being excellent antifouling models as they have been found to remain largely free from epibiont colonization <sup>49,100</sup>. A study by Bers and Wahl, 2004, investigated brittle sea star, *Ophiura texturata* <sup>87</sup>. Replicas were fabricated, using Devcon 2-TON epoxy resin and Coelan resin pigment following exposure to the field for 28 days and examination by weekly cell counting <sup>87</sup>. Characterizations on the surface of *Ophiura texturata* revealed knob-like structures with diameters of around 10  $\mu\text{m}$ . Results of the study concluded that the sessile organism, *Z. commune*, was repelled by the brittle star in week three whereas *Polydora sp.* was attracted to the brittle star during the last week of the study <sup>87</sup>. This study shows promising results for such textures and offers potential for further exploration if the textures can be replicated in a robust fashion. In a study by Guenther *et al.* (2007) investigating the AF potential of four tropical sea star species, *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagonia* and *Archaster typicus* <sup>108</sup>, field studies revealed that during the dry season, the topographies had no real effect on the general percentage cover and community composition. These tropical stars consist of a unique topography of paxillae ranging in size from 50–379  $\mu\text{m}$  (height), 108–204  $\mu\text{m}$  (diameter) and 17–108  $\mu\text{m}$  (spacing). These features were replicated using epoxy resin, Devcon 2-TON. However, for two of the stars, *Cryptasterina pentagonia* and *Archaster typicus*, surface topography demonstrated a small effect on the fouling community composition and percentage cover <sup>108</sup>. In 2002, Callow *et al.* highlighted the effect of topography shape and size on fouling composition and cover <sup>88</sup>.

It was observed that the microtopographies of the tropical stars provided favorable attachment points for larvae to settle. This offers some explanation into the higher percentage cover of fouling organisms on the stars in comparison to smooth and rough control surfaces <sup>88</sup>. Again, a species worth considering for further investigation.

*Mytilus edulis*, through biofouling-facilitated invasion, is a model organism for comparison of fouling communities across a wide range of geographical locations <sup>87</sup>. Field studies of the blue mussel, *Mytilus edulis*, with micro-ripples of 1–1.5  $\mu\text{m}$ , was investigated in a study by Bers and Wahl, 2004 <sup>87</sup> revealing an initial AF response but lesser effects as time progressed. Replicas of the surface were made using epoxy resin, Devcon 2-TON and colored using Coelan resin pigment, followed by exposure for 28 days in the marine environment. An initial reduction of barnacle settlement was observed in the first week of the study on the replica, however there was little effect on the inhibition of other fouling organisms in the second and third week, with the final week receiving increased barnacle attachment. Another study by Bers *et al.* in 2006 <sup>109</sup> investigated the microtopography of *M. edulis* and *Perna perna*. It was reported that the mytilid species with intact microtopographies and periostracum were much less fouled in comparison to 'roughened anisotropic surfaces' <sup>109</sup>. Following on from this study, in 2010, Bers *et al.* <sup>110</sup> conducted a global investigation into the fouling defense mechanisms of mytilid shell microtopographies; *M. edulis*, *M. galloprovincialis*, *Perna perna* and *P. viridis*. These four different mytilid shell species were acquired from eight different regions of the world and the microtopographies were replicated with high resolution resin, Devcon 2-TON. While the results of this study showed a deterrence effect on fouling organisms in the early stages of fouling (weeks 3 and 6), the microtopographies failed to present an antifouling

effect in the later stages <sup>110</sup>. In 2003, Scardino *et al.* studied the microtopography and AF properties of the shell of two bivalve mollusk species, *Mytilus galloprovincialis* and *Pinctada imbricata*. The shells were characterized through AFM and SEM, which revealed ridged topography of 1–2  $\mu\text{m}$  distance and a depth of 1.5  $\mu\text{m}$  for *Mytilus galloprovincialis*. *Pinctada imbricata* characterization revealed a non-repeating topographical pattern <sup>111</sup>. Unlike other studies, the natural shells were exposed in field experiments to the marine environment for 14 weeks. Results of this study concluded that bivalve species, *M. galloprovincialis* was rarely fouled – less than 10% fouled across all size classes of the species whilst *P. imbricata* had much higher levels of fouling <sup>111</sup>. Scardino also characterized blue mussel, *Tellina plicata* in 2006 to reveal a surface topography of around 2–4  $\mu\text{m}$  <sup>6</sup>. Biofouling assessment was completed using a designed 4 h cell settlement assay. It was concluded inhibition of fouling occurs for microfouling species (i.e., diatom) by limiting the number of favorable attachment points using highly ornate surface topography <sup>6</sup>. However, selective laboratory studies have their limitations. A study by Aldred *et al.* relates adhesion strength to site selection of common macrofouling organisms such as barnacle cypris larvae. In general, these organisms settle on surfaces that contain a large number of attachment points, leading to high attachment strength. This mechanism is similar to the popular Sharklet topography. Using this mechanism, it was reported that fouling can be significantly reduced, using topographies that present the lowest number of attachment points <sup>6</sup>. In 2006, Guenther documented the diversity of fouling organisms on pearl oyster species, *Pinctada fucata*, *Pteria penguin* and *Pteria chinensis*. *P. fucata* lacked regular microtopography and no significant difference in the diversity of fouling species was observed after 12 weeks and during the 16-week

sampling period. However, the fouling communities found on pearl oyster species *Pteria penguin* and *Pteria chinensis* showed significant differences during this time <sup>112</sup>. A study on bivalve species', *Dosinia japonica* and *Mimachlamys nobilis* was carried out in 2013 <sup>113</sup>. Replication of the texture, carried out in E44 epoxy resin and polyurethane (PU), revealed that topography can prevent the adhesion of *N. closterium* cells with E44 epoxy resin replicated *D. japonica* displaying the best antifouling capability <sup>113</sup>.

Replication of a texture in elastomer, poly(dimethyl)siloxane (PDMS) was conducted by Wang *et al.* who took inspiration from the scales of the yellowfin leatherjacket, *Triacanthus blochii*. A soft lithography process, called nanocasting, was used to replicate the surface microtopography topographical features of the scales consisting of needle-like patterns of height, 300 µm, spacing, 100 µm and diameters between 10–40 µm. Although there were no biofouling experiments carried out during this study, this is the first reported case of fish scale replication and serves as an example of creating highly accurate surface replications, without the added cost <sup>114</sup>. This replication procedure is widely used in areas of study such as microfluidics, and it shows potential, also, for replication of test structures that can be field tested due to the robustness of the material. A recent study carried out by Richards *et al.* reported the replication of the marine Brill fish, *Scophthalmus rhombus*, using a 3-D printing process (Nanoscribe 2-Photon 3-D printing) and soft lithography. The microtexture consisted of micro-ridge features of height, 75 µm, spacing, 10 – 7 µm and diameter, 10 µm. It was observed that cells attached in greater numbers to the microtexture replicated using soft lithography methods (PDMS casting), however further exploration is required. This is the first reported replication of the scales of the Brill fish, *Scophthalmus rhombus* <sup>80</sup>.

Of the lesser groups of organism's studies, Crustaceans (or 'crustacea') come from a large family of both marine and land arthropods. This family is defined by the presence of a hard shell or exoskeleton. The colonization of the crab shell surface presents huge advantages for epibionts as the activities of the host (i.e., movement, feeding) may result in feeding chances for other species. However, epibiotic colonization can also cause huge problems in terms of affecting the host's survival. As a result of this, crustaceans have evolved characteristics to prevent their colonization by epibionts (i.e., burrowing, cleaning) <sup>87,105</sup>. Sullivan *et al.* investigated the carapace of the crustacean, *Cancer pagurus* for its potential as an antifouling surface. The study reported the chemical composition, spatial distribution, size and shape descriptors of the microscale surface features of *C. pagurus* for the first time <sup>105</sup>.

Reported studies that are very promising for marine application suggest that dogfish egg cases have the ability to resist macrofouling up to 6 months (although microfouling still occurs). The dogfish egg case, *Scyliorhinus canicula*, although non-living, provides another biomimetic model for antifouling technologies. On examination of the micro-topographical features present on the surface, ridge-like patterns were revealed of dimensions between 30–50  $\mu\text{m}$ , with a mean surface roughness of approximately 3.7  $\mu\text{m}$ . From this work, surface topography seemed to play a role in the deterring of marine foulers, with most other reported AF surfaces having similar dimension and roughness <sup>115</sup>. Bers also investigated this phenomenon. High resolution replications were made using Devcon 2-TON epoxy resin and colored using Coelan resin pigment. Experiments were conducted in the field for 28 days in the Western Baltic, followed by weekly cell counts and statistical analysis using one-way analysis of variance (ANOVA) and Levene's

Test. Results of this study indicated deterrent effects on microfoulers and initial barnacle reduction <sup>87</sup>. The reproduction of the dogfish egg case, *Scyliorhinus canicula*, paved the way for the development of foul-release (FR) bivalves, providing a first report on the bioinspired production of 'natural AF surface' <sup>116</sup>.

Shark skin is perhaps the most widely reported bioinspired texture, which has unique topographical patterns with specific characteristics to help the shark in its natural habitat [98–101]. The skin has microscale sized ridges which are said to prevent the reproduction of eddies in the turbulent boundary layer (i.e., reducing drag) <sup>7</sup>. The shark skin diamond shaped topography has been found to prevent microorganisms from attaching to the surface. It was also found successful in reducing *Escherichia coli* and *Staphylococcus A* proliferation in hospitals <sup>106</sup>.

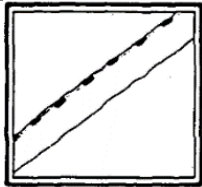
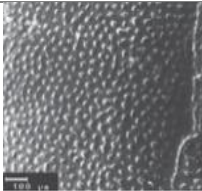
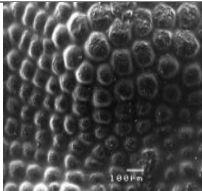
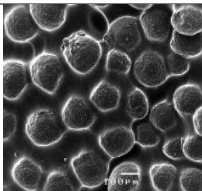
Other reported bioinspired textures are that of dolphin and whale skin. Early work in this area took place in 1983, by Baier *et al.* who investigated the antifouling potential of the skin of porpoises and killer whales <sup>116,120</sup>. Characterization of the bottlenose dolphin, *Tursiops truncatus* and killer whale, *Orcinus orca*, exposed micro-topographical ridge features, 0.41–2.35 mm (width) and 7–114 mm (height), reducing drag and fouling significantly. Surface tension measurements were observed to fall within the range for minimal bioadhesion (20–30 mN/m) <sup>116</sup>. The reproduction of porpoise skin, *Tursiops truncatus*, was attempted using tethered polymer chains however the antifouling potential of these replications were never documented <sup>120</sup>.

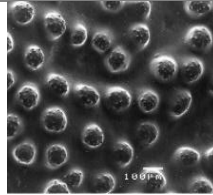
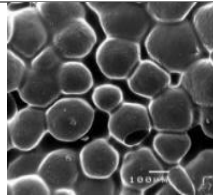
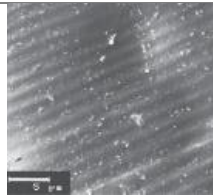
Many cetaceans remain relatively free from fouling organisms, presenting a very clean skin surface. This realization was investigated in 2002, by Baum *et al.* with the study of pilot whale skin, *Globicephala melas*. The epibiont-free surface of *Globicephala melas*

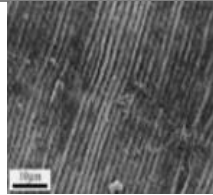
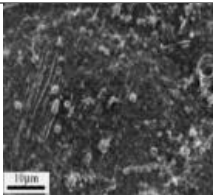
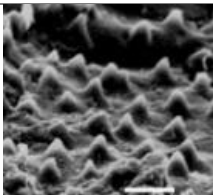
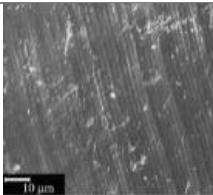
can be credited to the topographical structures on the skin with an average pore size of  $0.20\ \mu\text{m}^2$ . It has been suggested that organisms larger than the scale of microtopography present on the surface will not settle due to reduced attachment points<sup>6</sup>. In this case, the pores present on the surface of *Globicephala melas* were significantly smaller than those of fouling organisms present in the marine environment. This property, along with the speed of the marine mammal (i.e., jumping, surfacing) allows for the removal of weakly bound epibionts, providing fewer favorable attachment points – this has paved the way for the development of nano-structured and nano-pored surfaces <sup>116</sup>. Cao investigated a range of nano-structured and nano-pored surfaces for their AF potential <sup>121</sup>. The surface morphology of *G. melas* was essentially copied using 'spray coated multi-layered polyelectrolytes. This topography succeeded in the prevention of zoospore species, *Ulva*, from settling on the surface – mainly due to the microtopography (600 nm) of the surface under flow. It was also noted that the skin of the pilot whale was in fact, an amphiphilic surface – one which contains dual-wettability, both polar and nonpolar groups <sup>121</sup>.

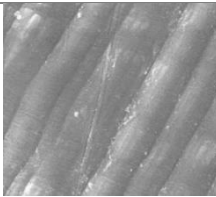
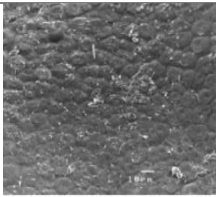
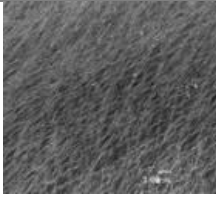
It was reported that the response to morphology was retained, irrespective of chemistry. The lowest level of settlement was observed for structures of the order of 2 mm. The strength of adhesion of settled spores was found to be lowest on the surface with the sub-micrometer-sized features. The authors recommended that in attempts to explore the potential of morphology to deter settlement, hierarchical surfaces are needed to deal with the different preferences of the target organisms. From the wide range of surface textures developed and shown in Table 1.4., many have been tested in the field.

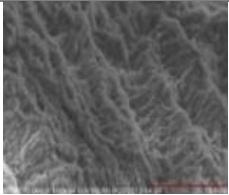
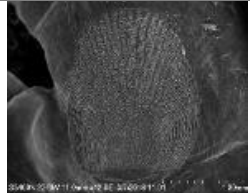
**Table 1.4** Summary of bioinspired micro-topographies reported from marine organisms.

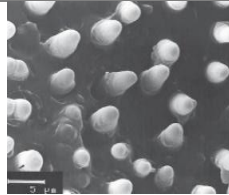
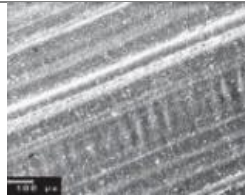
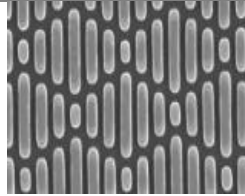
| Species                                                                                                                                                                                              | Type of Study    | Performance                                                                                                                                                                                                                                                   | Visual                                                                                | Ref |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|-----|
| <p>Sea fan:<br/><i>Pseudopterogorgia</i><br/><i>acerosa</i></p> <p><b>Dimension:</b><br/>Spicules, 2–4 <math>\mu\text{m}</math></p>                                                                  | Characterization | <p><b>Antifouling effect:</b><br/>“Release of fouling” at an ideal surface energy range of 20–30 <math>\text{dyn cm}^{-1}</math>.</p> <p><b>AF mechanism:</b> Surface chemistry</p>                                                                           |    | 107 |
| <p>Brittle star: <i>Ophiura</i><br/><i>texturata</i></p> <p><b>Dimension:</b> Knobs,<br/>10 <math>\mu\text{m}</math> in diameter</p>                                                                 | Field            | <p><b>Antifouling effect:</b><br/>Deterrent effects on microfoulers.</p> <p><b>AF mechanism:</b> Surface topography</p>                                                                                                                                       |    | 87  |
| <p>Sea star:<br/><i>Linckia laevigata</i></p> <p><b>Dimension:</b> Paxillae<br/>100<math>\mu\text{m}</math> (h), 116 <math>\mu\text{m}</math><br/>(d),<br/>17 <math>\mu\text{m}</math> (spacing)</p> | Field            | <p><b>Antifouling effect:</b> No effect on the fouling composition, community, and percentage cover during dry season.</p> <p><b>AF mechanism:</b> Surface topography</p>                                                                                     |    | 108 |
| <p>Sea star:<br/><i>Fromia indica</i></p> <p><b>Dimension:</b> Paxillae<br/>52 <math>\mu\text{m}</math> (h), 172 <math>\mu\text{m}</math><br/>(d),<br/>108 <math>\mu\text{m}</math> (spacing)</p>    | Field            | <p><b>Antifouling effect:</b> No effect on the fouling composition, community, and percentage cover during dry season. <b>AF mechanism:</b> Surface topography (requires a combination of behavioral, mechanical and/or chemical antifouling mechanisms).</p> |  | 108 |

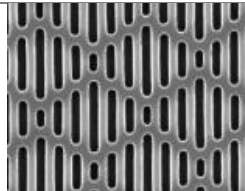
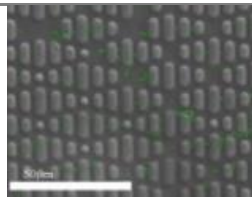
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|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|-----------|
| <p>Sea star:<br/><i>Cryptasterina</i><br/><i>pentagonia</i></p> <p><b>Dimension:</b> Paxillae<br/>50 <math>\mu\text{m}</math> (h), 108 <math>\mu\text{m}</math><br/>(d),<br/>103 <math>\mu\text{m}</math> (spacing)</p> | Field | <p><b>Antifouling effect:</b> No effect during the dry season. Transitory effects on the fouling community composition during wet season. <b>AF mechanism:</b> Surface topography (requires a combination of behavioral, mechanical and/or chemical antifouling mechanisms).</p> |    | 108       |
| <p>Sea star:<br/><i>Archaster typicus</i></p> <p><b>Dimension:</b> Paxillae<br/>379 <math>\mu\text{m}</math> (h), 204 <math>\mu\text{m}</math><br/>(d),<br/>98 <math>\mu\text{m}</math> (spacing)</p>                   | Field | <p><b>Antifouling effect:</b> No effect during the dry season. Transitory effects on the fouling community composition during wet season. <b>AF mechanism:</b> Surface topography (requires a combination of behavioral, mechanical and/or chemical antifouling mechanisms).</p> |    | 108       |
| <p>Blue mussel: <i>Mytilus</i><br/><i>edulis</i></p> <p><b>Dimension:</b> Micro-ripples,<br/>1–1.5 <math>\mu\text{m}</math></p>                                                                                         | Field | <p><b>Antifouling effect:</b> Initial reduction of barnacle settlement. Some deterrent effects observed in weeks 3 and 6. However, the microtopographies were not able to prevent fouling in later stages.</p> <p><b>AF mechanism:</b> Surface topography</p>                    |  | 87<br>110 |

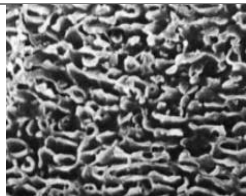
|                                                                                                                                    |            |                                                                                                                                                                                                                                                                                                                                                  |                                                                                       |            |
|------------------------------------------------------------------------------------------------------------------------------------|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|------------|
| Blue mussel: <i>Mytilus galloprovincialis</i><br><b>Dimension:</b> Ridges, 1–2 $\mu\text{m}$ (width) and 1.5 $\mu\text{m}$ (depth) | Field      | <p><b>Antifouling effect:</b> Less than 10% across all size classes were fouled. Some deterrent effects observed in weeks 3 and 6. However, the microtopographies were not able to prevent fouling in later stages.</p> <p><b>AF mechanism:</b> Surface chemistry, microtopography and Attachment Point Theory</p>                               |    | 111<br>110 |
| Pearl oyster: <i>Pinctada imbricata</i><br><b>Dimension:</b> Non-repeating pattern                                                 | Field      | <p><b>Antifouling effect:</b> High levels of fouling. <b>AF mechanism:</b> Surface chemistry and Attachment Point Theory</p>                                                                                                                                                                                                                     |    | 111        |
| Bivalve: <i>Tellina plicata</i><br><b>Dimension:</b> Projections, 2–4 $\mu\text{m}$                                                | Laboratory | <p><b>Antifouling effect:</b> Reduced number of attachment points results in reduced adhesion of diatom species. <b>AF mechanism:</b> Attachment Point Theory</p>                                                                                                                                                                                |  | 6          |
| Mussel: <i>Perna perna</i><br><b>Dimension:</b> Ripples, 1.5–2 $\mu\text{m}$                                                       | Field      | <p><b>Antifouling effect:</b> Replicas with intact isotropic topographies and smooth controls were much less fouled than roughened anisotropic surfaces. Deterrent effects observed in weeks 3 and 6. However, the microtopographies were not able to prevent fouling in later stages. <b>AF mechanism:</b> Surface chemistry and topography</p> |  | 109<br>110 |

|                                                                                             |       |                                                                                                                                                                                                                          |                                                                                       |     |
|---------------------------------------------------------------------------------------------|-------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|-----|
| Mussel:<br><i>P. viridis</i><br><b>Dimension:</b><br>Not disclosed in study                 | Field | <b>Antifouling effect:</b> Some deterrent effects observed in weeks 3 and 6. However, the microtopographies were not able to prevent fouling in later stages.<br><b>AF mechanism:</b> Surface topography                 |    | 110 |
| Pearl oyster:<br><i>Pinctada fucata</i><br><b>Dimension:</b><br>Non-regular                 | Field | <b>Antifouling effect:</b> No significant difference in fouling communities after 12 weeks and during the 16-week sampling period.<br><b>AF mechanism:</b> Combination; physical, chemical and/or environmental.         |    | 112 |
| Pearl oyster:<br><i>Pteria penguin</i><br><b>Dimension:</b> Ripples,<br>0.8 $\mu\text{m}$   | Field | <b>Antifouling effect:</b> Fouling communities found were significantly different both after 12 weeks and during the 16-week sampling period. <b>AF mechanism:</b> Combination; physical, chemical and/or environmental. |  | 112 |
| Pearl oyster:<br><i>Pteria chinensis</i><br><b>Dimension:</b> Ripples,<br>0.6 $\mu\text{m}$ | Field | <b>Antifouling effect:</b> Fouling communities found were significantly different both after 12 weeks and during the 16-week sampling period. <b>AF mechanism:</b> Combination; physical, chemical and/or environmental. |  | 112 |

|                                                                                                                                                                                                                                   |            |                                                                                                                                                                                              |                                                                                      |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|-----|
| <p>Bivalve:<br/><i>Dosinia japonica</i></p> <p><b>Dimension:</b><br/>Ribs, 300–800 nm</p>                                                                                                                                         | Laboratory | <p><b>Antifouling effect:</b><br/>Topography can prevent the attachment of <i>N. closterium</i> cells. <b>AF mechanism:</b> Surface topography</p>                                           |   | 113 |
| <p>Yellowfish leatherjacket:<br/><i>Triacanthus blochii</i></p> <p><b>Dimension:</b><br/>Needles, 100 <math>\mu\text{m}</math> (spacing), 300 <math>\mu\text{m}</math> (height) and 10–40 <math>\mu\text{m}</math> (diameter)</p> | Laboratory | <p><b>Antifouling effect:</b> First reported replication of <i>Triacanthus blochii</i> (yellowfin leatherjacket) using PDMS nanocasting. <b>AF mechanism:</b> Not tested in this study.</p>  |   | 114 |
| <p>Brill:<br/><i>Scophthalmus rhombus</i></p> <p><b>Dimension:</b><br/>Micro-ridges, 74.84 <math>\mu\text{m}</math> (length), 11.7 <math>\mu\text{m}</math> (slope), 16.6 <math>\mu\text{m}</math> (spacing)</p>                  | Laboratory | <p><b>Antifouling effect:</b> First reported replication of <i>Scophthalmus rhombus</i> using 3-D printing. <b>AF mechanism:</b> Attachment Point Theory (requires further exploration).</p> |  | 80  |

|                                                                                                                 |                                |                                                                                                                                                                                                                                                                            |                                                                                       |                   |
|-----------------------------------------------------------------------------------------------------------------|--------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|-------------------|
| Crab:<br><i>Cancer pagurus</i><br><b>Dimension:</b><br>Circular elevations, 200<br>µm and spicules, 2–2.5<br>µm | Field + Laboratory             | <b>Antifouling effect:</b><br>Repellent to<br>macrofoulers<br>(barnacles).<br>Settlement of fouling<br>organisms was affected<br>in different ways from<br>the surface<br>microtopographies. <b>AF<br/>mechanism:</b><br>Attachment Point Theory                           |    | 87<br>105         |
| Dogfish egg case:<br><i>Scyliorhinus canicula</i><br><b>Dimension:</b><br>Ridges, 30–50 µm                      | Field                          | <b>Antifouling effect:</b><br>Deterrent effects on<br>microfoulers. Initial<br>reduction of barnacle<br>settlement. No effects of<br>the surface structure of<br>the egg case. <b>AF<br/>mechanism:</b> Surface<br>topography                                              |    | 87                |
| Shark:<br>Sharklet AF<br><b>Dimension:</b><br>Ribs, 2 µm, 2 µm,<br>4–16 µm (width,<br>spacing, length)          | Laboratory<br>(Commercialized) | <b>Antifouling effect:</b><br>Reduced spore<br>settlement density by 86<br>%. <i>S. aureus</i> biofilm<br>percentage cover on<br>Sharklet AF covered<br>surface was 7 %<br>compared to 54 % for<br>smooth PDMS control.<br><b>AF mechanism:</b><br>Attachment Point Theory |  | 117<br>118<br>119 |

|                                                                                                                                                                                                  |                                        |                                                                                                                                                                                                                                                                                                          |                                                                                     |            |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|------------|
| <p>Shark:<br/>Recessed Sharklet AF</p> <p><b>Dimension:</b><br/>Ribs, 2 <math>\mu\text{m}</math>, 2 <math>\mu\text{m}</math>,<br/>4–16 <math>\mu\text{m}</math> (width,<br/>spacing, length)</p> | <p>Laboratory<br/>(Commercialized)</p> | <p><b>Antifouling effect:</b> <i>Ulva</i><br/>spore attachment<br/>independent of the area<br/>fraction of feature tops<br/>and number of<br/>features—spores<br/>attached in lower<br/>numbers here.</p> <p><b>AF mechanism:</b><br/>Attachment Point Theory</p>                                        |  | <p>119</p> |
| <p>Shark:<br/>Placoid scale</p> <p><b>Dimension:</b><br/>2 <math>\mu\text{m}</math>, 1.5 <math>\mu\text{m}</math>, 2 <math>\mu\text{m}</math><br/>(width, height,<br/>spacing)</p>               | <p>Laboratory</p>                      | <p><b>Antifouling effect:</b><br/>Decrease in <i>E. coli</i><br/>attachment by 75%<br/>when measuring pristine<br/>patterns and up to 56%<br/>when measuring<br/>patterns undergoing<br/>extreme mechanical<br/>wear.</p> <p><b>AF mechanism:</b><br/>Attachment Point Theory</p>                        |  | <p>104</p> |
| <p>Bottlenose dolphin:<br/><i>Tursiops truncatus</i></p> <p><b>Dimension:</b><br/>Ridges, 0.41–2.35 mm<br/>(width), 7–114 mm<br/>(height)</p>                                                    | <p>Laboratory</p>                      | <p><b>Antifouling effect:</b><br/>Surface tensions in the<br/>range for minimal<br/>biofouling attachment<br/>(20–30 <math>\text{mN m}^{-1}</math>), low<br/>drag, micro-<br/>topographical features<br/>contributing to a fouling-<br/>free surface.</p> <p><b>AF mechanism:</b><br/>Surface energy</p> | <p>–</p>                                                                            | <p>116</p> |

|                                                                                                                          |                  |                                                                                                                                                                                                                                                                      |                                                                                     |     |
|--------------------------------------------------------------------------------------------------------------------------|------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|-----|
| <p>Killer whale: <i>Orcinus orca</i></p> <p><b>Dimension:</b></p> <p>Ridges, 0.41–2.35 mm (width), 7–114 mm (height)</p> | Laboratory       | <p><b>Antifouling effect:</b></p> <p>Surface tensions in the range for minimal biofouling attachment (20–30 mN m<sup>-1</sup>), low drag, micro-topographical features contributing to a fouling-free surface.</p> <p><b>AF mechanism:</b></p> <p>Surface energy</p> | –                                                                                   | 116 |
| <p>Pilot whale: <i>Globicephala melas</i></p> <p><b>Dimension:</b></p> <p>Ridges, 2 µm and pores, 0.20 µm</p>            | Characterization | <p><b>Antifouling effect:</b></p> <p>Average pore size (0.20 µm<sup>2</sup>) below that of most biofouling organisms—low numbers of organisms and salt crystals. <b>AF mechanism:</b></p> <p>Attachment Point Theory</p>                                             |  | 85  |

## 1.7 Conclusion

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Inspiration from marine organisms has provided surface textures that have been replicated using a variety of fabrication techniques. These textures have been tested in the lab and field for their antifouling potential, with varied success. Many biofouling studies are lab-based using testing with only one species of organisms which are easier to statistically analyze and quantify. This is one of the reasons why many successful lab-based antifouling (AF) solutions fail when applied to field trials. However, the success of these AF technologies is likely to be very different when applied under environmental and field conditions. Of the surveyed papers, many of the marine organism texture features are in the micron range. These vary from 1 – 10  $\mu\text{m}$ , 100 – 500  $\mu\text{m}$  with few studies in the nanometer range. Few innovative techniques have been adopted for replication of surface features. The challenge is in meeting the required dimensions as these are limited by the capability of the replication technique, and also the ease of replicating from a small-scale surface to a larger scale. This has been shown to be a challenge using the techniques described in Table 1.1. However, innovations in roll-to-roll manufacturing can potentially realize the delivery of larger scale replicas of the structure. Micro-contact printing for example or 3-D printing are offering greater flexibility in material development. An effective AF solution will need to consider combining both surface chemistry, like the very elegant technique by Rosenhahn <sup>122</sup> with suitable topography. While textured surfaces alone have not demonstrated complete antifouling success, evidence suggests that texture plays a significant role. Existing studies discussed in this literature review, principally focus on the applicability of the topographies inspired by shark, dolphin, and crustacean, for example.

However, there are very few novel biomimetic natural surfaces that have demonstrated significant antifouling potential. These textures typically are very complex with hierarchical structures – varying in dimensions. Development and evaluation of fabrication methods to create or replicate patterned surfaces at both micro- and nano-scale levels is required. The replication of effective surface topographies for large scale applications remains a challenge and choice of texture is critical in achieving success. Further research on marine inspired textures with potential antifouling capability, is required to understand the mechanisms involved and the potential for larger scale application.

## 1.8 Aims and objectives

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Biofouling is a major problem in the marine environment whereby submerged surfaces undergo fouling to some degree due to the build-up of organic matter. The problems associated with this include increase in fuel consumption, increase in drag, decrease in ship speeds as well as an increase in general maintenance and cleaning costs, causing a huge economic burden for the marine industry. Historically, TBT-based compounds would have been used in the prevention of biofouling. However, its ban in 2008 (due to its toxic effect on marine species), means that the development of environmentally benign AF systems is becoming increasingly important. The aim of this thesis is to investigate both chemical (through the use of sol-gel chemistry) and physical (through the modification of topography) mechanisms of preventing biofouling in the marine environment. Particular attention will be paid to the examining the effectiveness of surface topographical features on the adhesion of microfouling communities to a substrate. As mentioned, with the total prohibition of the use of TBT in 2008, a huge drive to develop a non-toxic antifouling solution has been made. Many species of marine organisms are not prone to fouling by other species<sup>17</sup>. The surfaces of many marine animals feature intricate topography<sup>123</sup>. Recent publications have indicated that the influence of topography prevents the settlement of micro-organisms to a substrate, with many of these topographical features derived from marine animals<sup>6,87,88</sup>. Therefore, a main objective of this thesis is to evaluate the potential of the marine Brill fish, *Scophthalmus rhombus*, as inspiration to develop an antifouling texture for the first time. In preparation of this, both traditional AF solutions and novel engineered surface topographical features will be studied for AF potential.

It is anticipated that advances will be made in understanding the basic principles of the biofouling process and cellular behaviour through surface modification will be made, which will help develop current knowledge surrounding novel AF solutions.

Chapter 2 discusses the methods that have been used to assess the antifouling effectiveness of novel materials and coatings.

Chapter 3 deals with the accumulation of biofouling on developed transparent coatings and commercial paints in the marine environment.

Chapter 4 aims to investigate the biofouling process over a 12-month period in the marine environment.

Chapter 5 aims to characterize the topographical features of the Brill fish scale, *Scophthalmus rhombus*, for inspiration as an antifouling material, and evaluates available microfabrication methods for the production of this texture.

Chapter 6 focuses on testing the selected bioinspired surface textures developed in Chapter 5 in a series of static and dynamic laboratory assays using two model diatom species, *Amphora coffeaeformis* and *Nitzschia ovalis*.

Chapter 7 discusses the overall conclusions drawn from the work presented in the thesis, as well as providing strategies for the further development of these findings in subsequent work.

# Chapter 2:

## Materials and Methods

## 2.1 Introduction

---

This thesis looks at the development of novel materials and coatings for the inhibition of the early stages of biofouling in the marine environment, which in turn, can help prevent the development of a conditioned layer to prevent larger biofouling organisms (i.e., barnacles, mussels) from adhering to a substrate. This thesis examines both the chemical and physical modification of surfaces for prevention of biofouling in the marine environment. Chapter 4 outlines the antifouling effectiveness of novel transparent sol-gel coatings and three commercial AF paints, Trilux, Cruiser Uno, and Micron Extra. Chapter 5 and 6 looks at the physical modification of a surface through the use of microtextured 3-D printed surfaces inspired by the Brill fish scale, *S. rhombus*. The processes discussed in this chapter outline the various methods carried out throughout the thesis for the development of novel antifouling systems.

## 2.2 Chemicals

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IP-S (photoresist) and dill glass substrates (25 x 25 mm<sup>2</sup>; 0.7 mm thickness; soda lime, ITO-coated) were purchased from Nanoscribe, Karlsruhe, Germany. Propylene glycol monomethyl ether acetate (PGMEA, >99.5 %), novex 7000 Engineering Fluid (methyl perfluoro propyl ether), glutaraldehyde 25% in H<sub>2</sub>O solution, methanol (≥99.9 %), acridine orange base 5 g (dye content 75 %), heptane (95 %), OTS (octadecyltrichlorosilane, >90 %), isopropanol (99.7 %), Total Protein Kit, Micro Lowry (Peterson's modification), bovine serum albumin (BSA) (400 µg mL<sup>-1</sup>), anthrone (ACS reagent, 97 %), phenol solution (5 %), sulphuric acid (98 %), D-(+)-glucose (≥ 99.5 %), tetraethyl orthosilicate (TEOS), diethoxydimethylsilane (DMDEOS), ethanol, triethoxy(ethyl) silane (ETEOS), ethoxytrimethyl silane (TMES), chlorotrimethylsilane (CTMS) and hydrochloric acid (HCl) (37%) were all purchased from Sigma-Aldrich, Arklow, Wicklow, Ireland. 11` Royal Nature Reef Salt 10 kg was obtained from Marine Aquatics, Walsall, West Midlands, UK. Whatman membrane filters nylon (0.45 µm pore size, 47 mm diameter) was provided by VWR, Blanchardstown, Dublin, Ireland. *Amphora coffeaeformis* (CCAP 1001/2) marine diatom, *Nitzschia ovalis* (CCAP 1052/12) marine diatom and f/2 + sodium metasilicate medium (f/2 + Si) (Guillard's medium) were obtained from the Scottish Marine Institute, SAMS Limited, Scotland, UK. Polydimethylsiloxane (PDMS, Sylgard 184k) was provided by Dow Corning, Cheadle, UK. RepliSet-F-5 was purchased from Struers, Zomerdijk, Netherlands. Antifouling paints, Trilux, Cruiser Uno and Micron were obtained from International Yacht Paint, Marine Suppliers, Howth, Ireland. The DNeasy PowerSoil Pro Kit (Qiagen, # 47014, Manchester, UK) was used to extract DNA from biofilm samples in

the field. All chemicals purchased were of analytical grade and aqueous solutions were prepared with deionised water (DI) ( $18.2 \text{ M}\Omega \text{ cm}^{-1}$ ).

## 2.3 Instrumentation

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The Nanoscribe Photonic Professional GT 3-D printing system was used to fabricate surface micro-textures (Nanoscribe, Karlsruhe, Germany). Digital light microscopy images were taken on a Keyence VHX2000E 3D digital microscope (Keyence, Milton Keynes, Buckinghamshire, UK). A Laurell WS-650MZ-23 spin coater (Laurell Technologies Corporation, North Wales, Pennsylvania, USA) was used to apply sol gel coatings to glass slides and to conduct spinning tests of micro-textures. A WD-180R VEDA airbrush gun was used to apply coatings to glass slides by spray coating (VEDA, Amazon, UK). Cool White LEDVANCE 4000K 30W/33 LED-lamps (Osram, Ledvance Amazon, UK) were used as a light source for growing diatom cultures under laboratory conditions. A UV-1800 and UV mini-1240 (Shimadzu, Duisburg, Germany) was used to measure the absorbance of reaction solutions in the quantification of protein (Lowry) and carbohydrate (Anthrone-sulphuric acid) in biofilm. An Optika B-500BPH light microscope (Optika, Ponteranica, Italy) and BRAND counting chamber (Blaubrand Neubauer improved, Sigma, Arklow, Wicklow, Ireland) was used to count cell numbers of laboratory grown diatom cultures. Water contact angle (WCA) measurements were carried out on a FTA200 Dynamic Contact Angle Analyzer (FTA, Newark, CA, USA). Surface dimension and roughness measurements were carried out on a Bruker Contour GT Profilometer (Bruker, Massachusetts, USA). Scanning electron microscopy images and Energy Dispersive X-Ray (EDX) analysis were taken on a Hitachi S3400-N SEM (Hitachi, Buckinghamshire, UK) and the Jeol JSM-IT 100 InTouchScope (JEOL, Hertfordshire,

UK). Samples were gold sputter coated using an agar sputter coater (Agar Scientific, Essex, UK). 3D modelling software, SolidWorks (2017 Edition, SolidWorks, Waltham, MA, USA) was used to design and create STL files for micro-textures. An Epilog Laser (Epilog, Clevedon, UK) and Graphtec knife cutter (Graphtec, Wrexham, UK) was used to fabricate sample holders for field and dynamic testing of micro-textures. Fiji (Image J) image processing software (<https://imagej.net/>) was used for image analysis of textured surfaces. Origin Pro 2021b (<http://silverdalescientific.co.uk>), SigmaPlot 11.0 (<http://sigmaplot.co.uk/>) and BioRender (<http://biorender.com>) was used to plot obtained data and figures.

## 2.4 Site description

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### **Site 1:** Galway Bay (53.2000° N, 9.2333° W)

Figure 2.2 illustrates the test bed where the materials were deployed. The location selected for this experiment was Galway Bay, County Galway, Ireland (53.2000° N, 9.2333° W) (Figure 2.1), depth tidal range was approximately 2.5 m – 3 m for the microfouling study (panels attached to buoy) and 24 m – 28 m for the macrofouling study (panels attached to underwater observatory). Figure 2.2 illustrates the test bed where the materials were deployed. Microscope slides were coated with commercial paint (Trilux, Micron and Cruiser Uno as control) and novel transparent antifouling coatings were deployed on a polymethyl methacrylate (PMMA) panel (57.5 cm × 25 cm) for durations of nine and thirteen months to assess the durability and performance of novel transparent coatings biofilm growth at different depths and time periods under different conditions. The deployment device containing samples was retrieved after 9-mo and 13-mo from Galway Bay by the diving team at SmartBay Ireland. The panel containing the glass slides was fixed in Styrofoam to minimise movement and damage during transportation to the laboratory for analysis. Samples were analysed immediately on delivery.

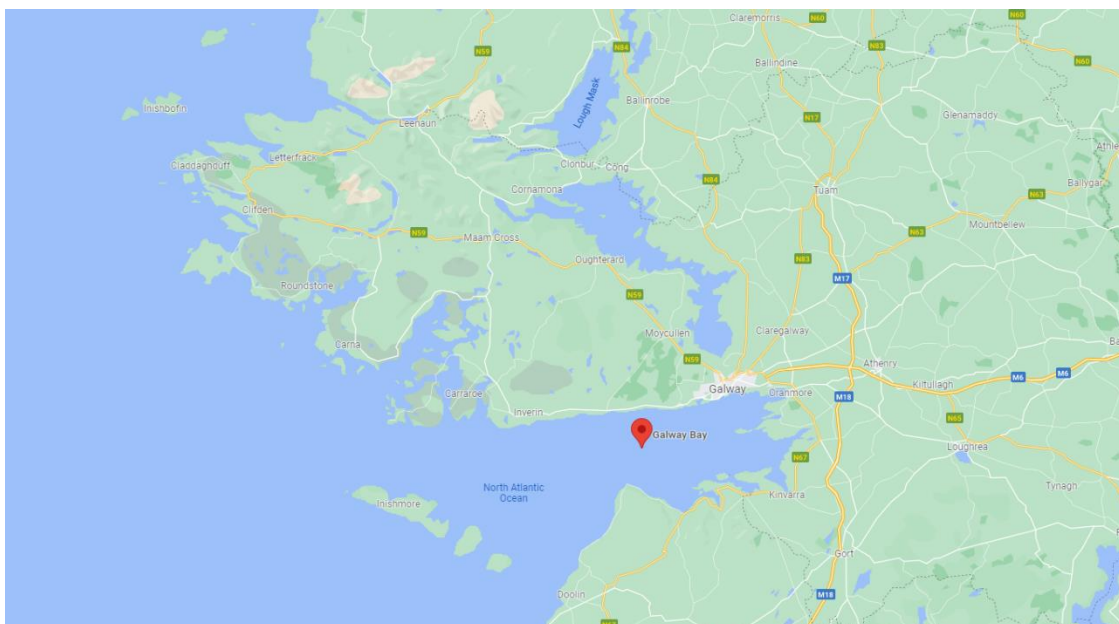


**Figure 2.1.** Site of deployment location at Galway Bay, Ireland (53.2000° N, 9.2333° W).

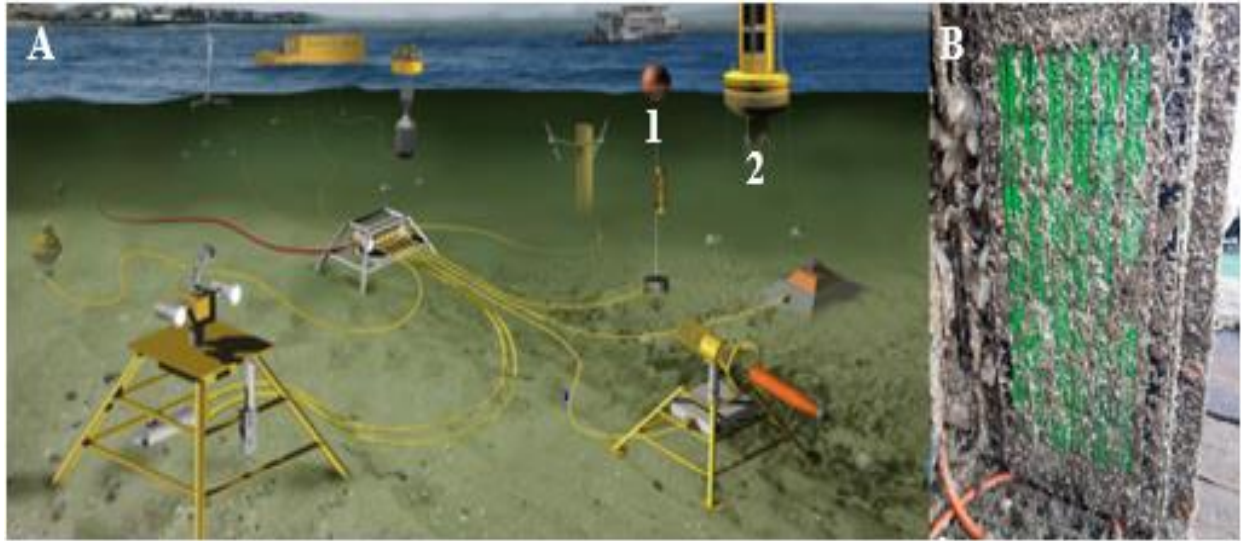


**Figure 2.2.** (a) Renewable test site in Galway Bay where panels were deployed; 1; panels attached to buoy for microfouling study; 2; panels attached to underwater observatory for macrofouling study (b) panels deployed (Image source: SmartBay Ireland).

**Site 2:** Dublin Bay (53.3421°N, 6.1513°W)

The site chosen for this study was Poolbeg Yacht & Boat Club, Dublin Bay, Ireland (53.3421°N, 6.1513°W) (Figure 2.3). Poolbeg Yacht & Boat Club lies in the centre of Dublin, close to Dublin Port, on the southern side of the River Liffey. Slides were deployed on three faces of a 3 mm polymethyl methacrylate (PMMA) laser cut holder (54 x 30 cm) (i.e., to allow for rotation around its axis to achieve homogenous biofilm formation) using 3-D printed brackets fabricated by Mr. Sean Power (Figure 2.4). Samples were deployed off a pontoon at the site. A sample holder, constructed to house glass slides, was fabricated from 3 mm Polymethyl methacrylate (PMMA) using an Epilog laser. Slides were fixed to the base of the holder using 3-D printed rods and 6 mm plastic nuts and bolts (Radionics, Ireland).



**Figure 2.3.** Site of deployment location at Poolbeg Yacht & Boat Club, Dublin Bay, Ireland (53.3421°N, 6.1513°W).

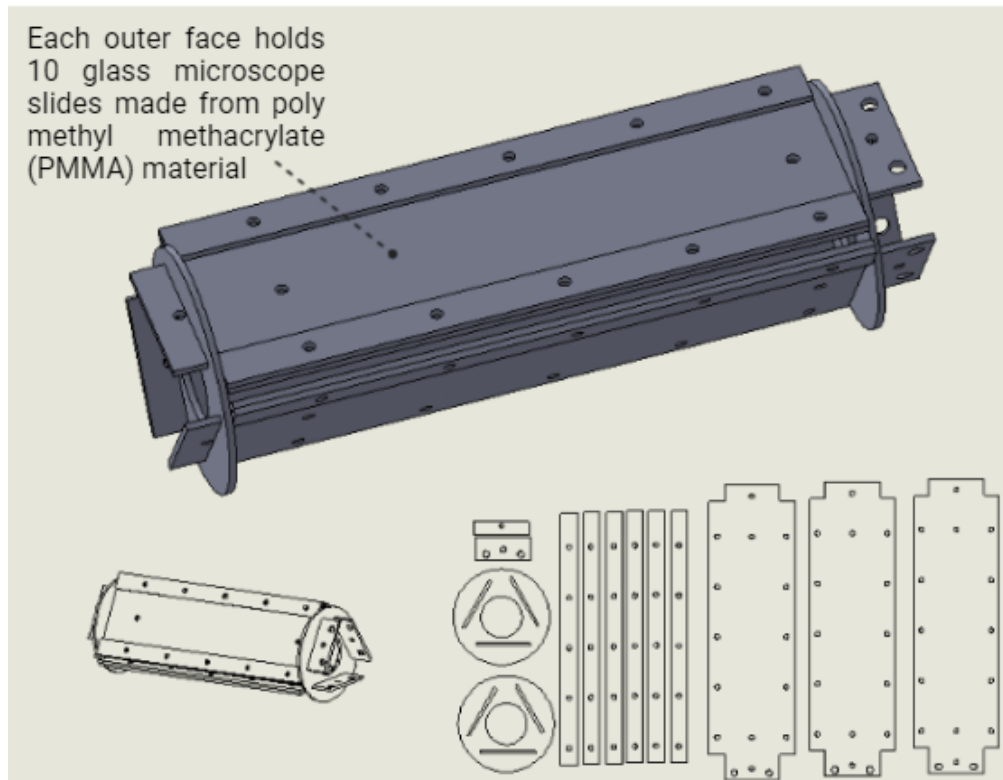


**Figure 2.4.** Rendered CAD drawing showing the initial design of the test frame for use in the Dublin Bay study (Chapter 4) The frame is laser cut from poly methyl methacrylate (PMMA) material and holds 30 glass microscope slides (10 slides per outer face) secured using 5 mm nylon machine screws (Inset: individual components of the test frame).

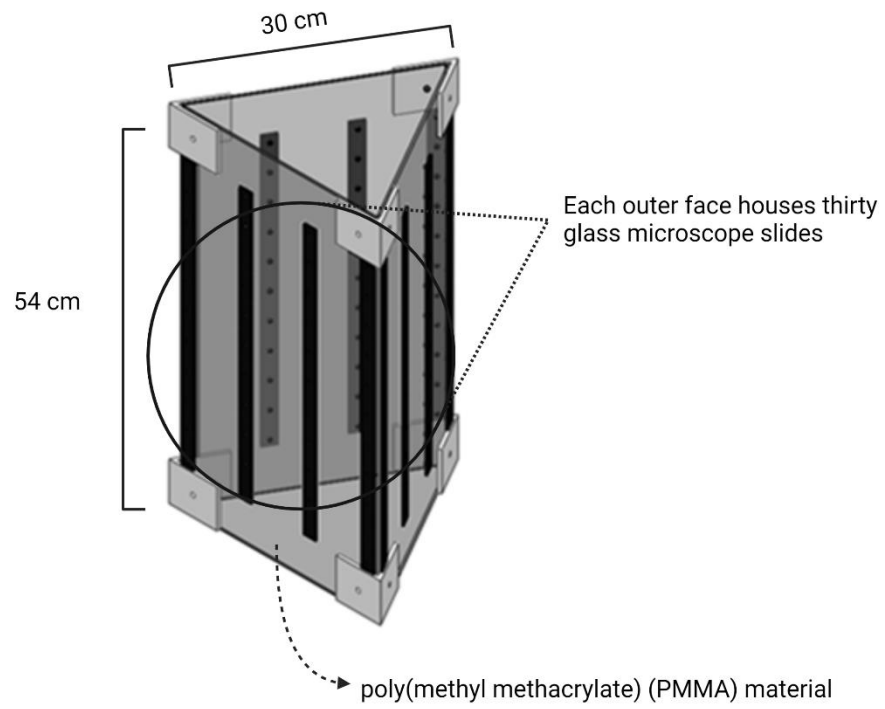


**Figure 2.5.** Rendered image showing final design of test frame made from polymethyl methacrylate (PMMA) material to incorporate coupons of standard microscope glass slide dimensions (75 mm x 25 mm).

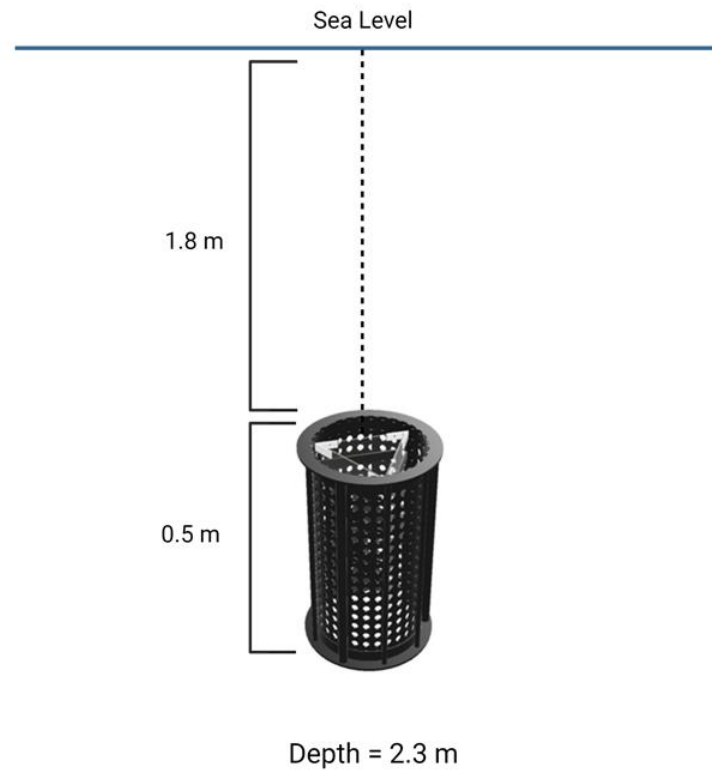


**Figure 2.6.** Schematic of mooring used for the deployment of ninety standard glass microscope slides at a depth of 2.3 m.

## 2.5 Culture conditions

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Marine diatom species, *Amphora coffeaeformis* and *Nitzschia ovalis* were selected for biofouling laboratory studies. Organisms were grown in water with 0.45 µm filtered sea salt and supplemented with nutrients for Guillard's F/2 + Si medium. Guillard's medium is prepared as outlined below in Table 2.1. The pH is adjusted to 8.0 with 1 M NaOH or 1 M HCl before autoclaving. The medium is then autoclaved at 15 psi for 15 mins. The cultures were grown in a 'batch-type' growth system in 5L Pyrex bottles, in a temperature range of 15°C to 23 °C and with a 12:12 light: dark cycle, with continuous light of 65 µmoles/m<sup>2</sup>/s. The cultures were also homogenized using filtered-air pumps and soft bubblers. Cell concentration was monitored on a regular basis by optical density at a wavelength of 750 nm using a UV-Vis spectrophotometer, keeping the cells in the exponential growth phase in the range of 0.3 – 0.5 AAU.

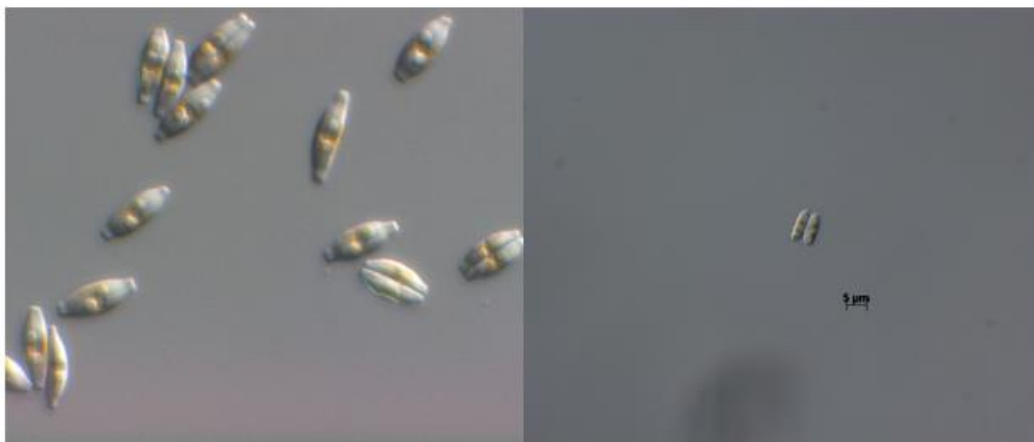


**Figure 2.7.** Light microscopy (LM) images taken on the Keyence 3-D digital light microscopy of laboratory grown diatom species, *A. coffeaeformis* (left) and *N. ovalis* (right) (Image sourced from the Culture Collection of Algae and Protozoa, SAMS, Scotland, U.K.).

**Table 2.1.** The components of f/2 + sodium metasilicate medium (f/2 + Si) (Guillard's medium) used to grow laboratory diatoms, *Amphora coffeaeformis* and *Nitzschia ovalis*.

|   | <b>Solution</b>     | <b>Component</b>                                    | <b>mL per L</b> |
|---|---------------------|-----------------------------------------------------|-----------------|
| 1 | Sodium nitrate      | NaNO <sub>3</sub>                                   | 1               |
|   | Sodium phosphate    |                                                     | 1               |
| 2 | monobasic dihydrate | NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O |                 |
|   |                     | NA <sub>2</sub> EDTA                                | 0.1             |
|   |                     | FeCl <sub>3</sub> ·6H <sub>2</sub> O                |                 |
|   |                     | CuSO <sub>4</sub> ·5H <sub>2</sub> O                |                 |
| 3 | Trace elements      | ZnSO <sub>4</sub> ·7H <sub>2</sub> O                |                 |
|   |                     | CoCl <sub>2</sub> ·6H <sub>2</sub> O                |                 |
|   |                     | MnCl <sub>2</sub> ·4H <sub>2</sub> O                |                 |
|   |                     | Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O |                 |
| 4 | Vitamin mix         | Cyanocobalamin                                      | 1               |
|   |                     | Biotin                                              |                 |
| 5 | Sodium metasilicate | Na <sub>2</sub> SiO <sub>3</sub> ·9H <sub>2</sub> O | 1               |

## 2.6 Chlorophyll $\alpha$ estimation

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The concentration of chlorophyll  $\alpha$  was determined in 25 mL aliquots of diatom cultures of *Amphora coffeaeformis* and *Nitzschia ovalis* that were filtered in low vacuum pressure (700 mb) through GF/F glass fibre filters (VWR, Ireland) with a pore size of approximately 0.7  $\mu\text{m}$  kept in the dark at  $-20\text{ }^{\circ}\text{C}$  for 12 h. Subsequently, 8 mL of 90 % (v/v) acetone was added to each 25 mL filtered aliquot, followed by sonication in an ultrasound bath for 5 min, a liquid-solid extraction in the dark at  $4\text{ }^{\circ}\text{C}$  for at least 3 h with the cellular material representing the solid, and finally, centrifugation for 10 min at 5000 rpm and recovery of the supernatant. Absorbances were read at 665 nm and 750 nm in a UV-Vis double beam spectrophotometer, allowing the calculation of the chlorophyll a concentration by means of the equation of Marker *et al.* (1980). Cells were kept at a chlorophyll a concentration of approximately  $0.3 - 0.4\text{ }\mu\text{g mL}^{-1}$  for cell settlement assays. Cell numbers were estimated by counting using a light microscope and an improved Neubauer bright-line hemocytometer (0.1 mm depth) to achieve a cell density of approximately  $3 \times 10^6$  cells per mL ( $n = 3$ ).

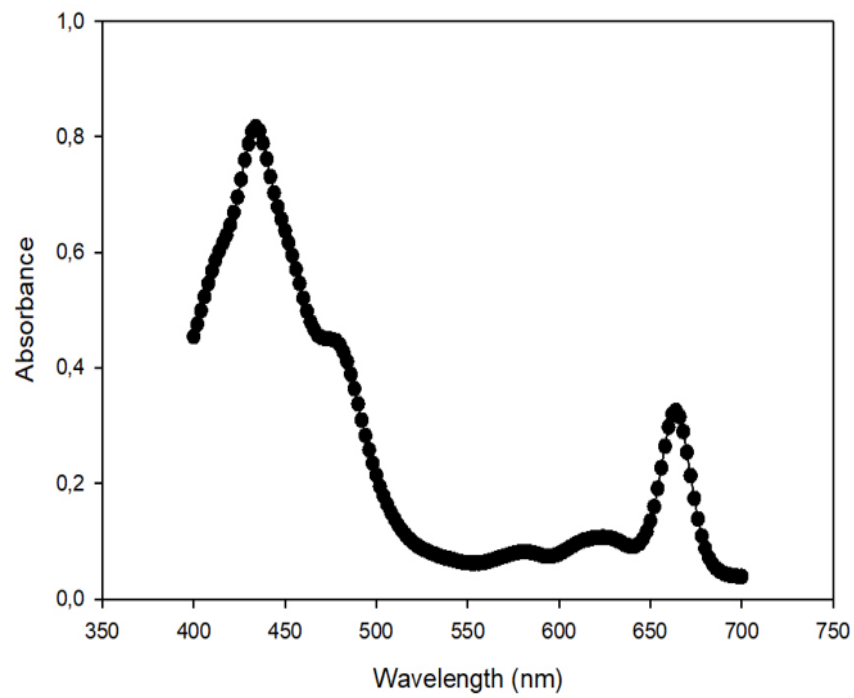


**Figure 2.8.** An example of characteristic absorption peaks for chlorophyll a (465 nm; 665 nm) measured on extracted chlorophyll from diatom species, *Nitzschia ovalis*, on a UV mini-1240 spectrophotometer.

## 2.7 Biological characterization

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A series of biochemical assays were used to assess the composition of biofouled slides and are described below.

### 2.7.1 Lowry protein assay

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The Lowry assay was chosen to quantify protein because it has high precision, low error and low limit of detection. A series of protein standard solutions in the range of 0 to 300  $\mu\text{g mL}^{-1}$  were prepared from a BSA stock solution (400  $\mu\text{g mL}^{-1}$ ). The contents of the Total Protein Kit (Micro Lowry, Peterson's Modification, Sigma-Aldrich, Ireland) were used to prepare solutions as recommended in the technical bulletin provided. The Lowry reagent was prepared right before analysis by dissolving 2 g of Lowry in 40 mL (5 % w/v) of deionized water (18.2  $\text{M}\Omega\text{ cm}^{-1}$ ). The solution was dissolved by inverting the flask several times, avoiding agitation of the flask to prevent foaming. The Folin & Ciocalteu's Phenol Reagent Working Solution was prepared in the following way: Folin & Ciocalteu's Phenol Reagent (18 mL) was transferred to the amber glass bottle labelled working solution. A 10 mL volume of deionized water (18.2  $\text{M}\Omega\text{ cm}^{-1}$ ) was used to rinse the remaining contents of the Folin & Ciocalteu's Phenol Reagent bottle, and this rinse solution was then added to the working solution bottle. An additional 80 mL volume of deionized water (18.2  $\text{M}\Omega\text{ cm}^{-1}$ ) was added, the solution was mixed well and stored at room temperature before analysis). The protein stock standard solution (BSA, 400  $\mu\text{g mL}^{-1}$ ) was prepared by adding an appropriate volume of deionized water (approx. 5 mL, see vial for exact volume, in this case, 4.83 mL) to the vial (2 mg BSA).

The protein standard solutions were made up in the ranges of 0  $\mu\text{g mL}^{-1}$  to 300  $\mu\text{g mL}^{-1}$  by appropriately diluting BSA stock solution (400  $\mu\text{g mL}^{-1}$ ) and preparing to 10 mL with deionized water (18.2  $\text{M}\Omega\text{ cm}^{-1}$ ).

A 1 mL quantity of each BSA standard solution and a blank were taken, and 1 mL of Lowry reagent was added to each. The solution was mixed using a vortex before being left to react at room temperature for 20 min. A 0.5 mL aliquot of Folin & Ciocalteu's Phenol Reagent Working Solution was added to each before the solution was thoroughly mixed using the vortex. The solutions were allowed stand for 30 min at room temperature for the reduction of phosphomolybdotungstate. Absorbance was measured at 750 nm, and deionized water was used to zero the instrument. Subsequently, the absorbance of the standard solutions, control and unknown samples was measured using a Shimadzu UV 1800 spectrophotometer.

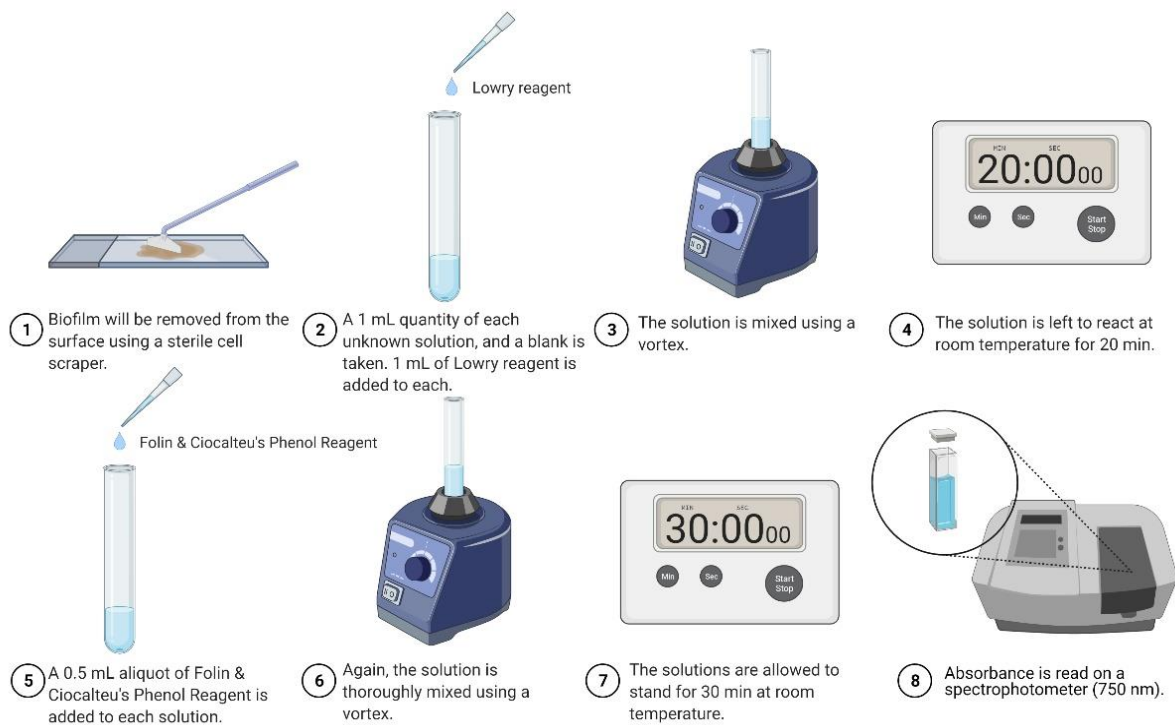


**Figure 2.9.** Illustration of the process used in the quantification of protein using the Lowry Protein Assay (Peterson's Modification) (created using BioRender.com).

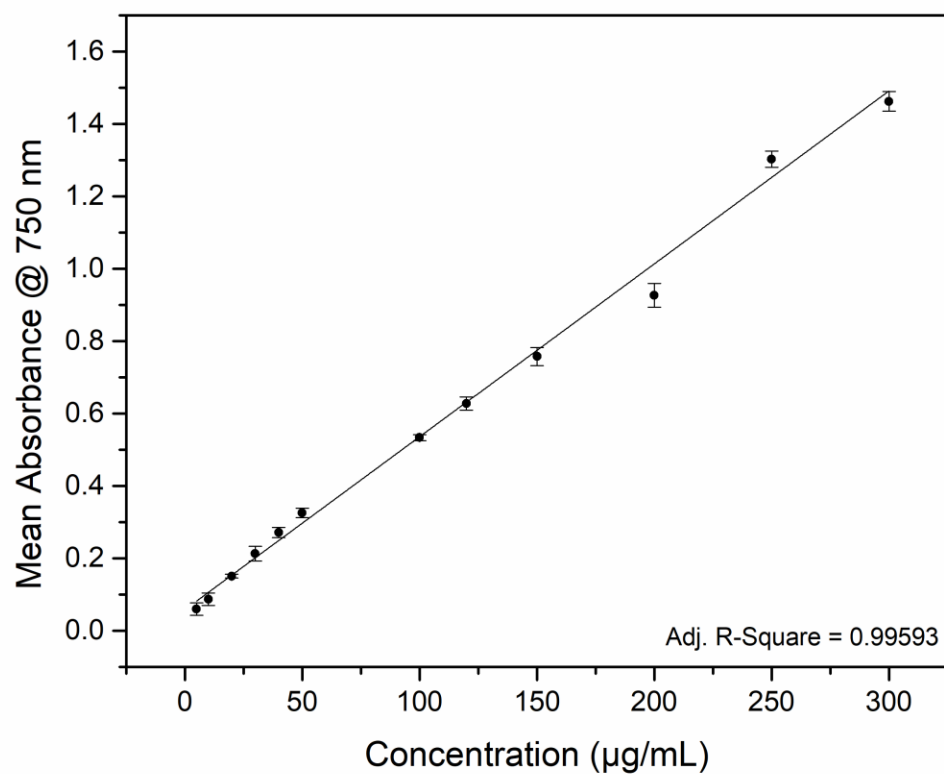


**Figure 2.10.** Calibration curve of the concentration of Bovine Serum Albumin (BSA) standard solutions using the Lowry Protein Assay at a wavelength of 750 nm (n = 3).

### 2.7.2 Anthrone-sulphuric acid assay

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Anthrone-sulphuric acid solution was prepared right before analysis by chilling 100 mL of concentrated sulphuric acid (98 %) in an ice bath for 20 min before adding 0.2 g of anthrone to make a 0.2 % w/v solution. A stock solution of 1000  $\mu\text{g mL}^{-1}$  D-(+)-glucose was made by dissolving 0.1 g of glucose in 100 mL of deionized water ( $18.2 \text{ M}\Omega \text{ cm}^{-1}$ ). Carbohydrate standards in the ranges of 0  $\mu\text{g mL}^{-1}$  to 70  $\mu\text{g mL}^{-1}$  were prepared by appropriately diluting the glucose stock solution and preparing to 25 mL with deionized water. A 0.5 mL volume of each standard D-(+)-glucose solution and unknown samples were added to labelled test tubes. A 2 mL volume of anthrone-sulphuric acid solution was added using a 2 mL glass pipette. Solutions were placed in a dry block incubator once the initial temperature of the incubator reached 80 °C. The solutions were left to incubate on the dry block until the temperature reached 95 °C. At this temperature, the solutions were left for another 15 min before removal. It is important to note that the solutions were removed earlier if the temperature rose to 100 °C as overheating of the solutions should be avoided. The solutions were allowed to cool to room temperature for 30 min while the absorbance was measured at 625 nm.

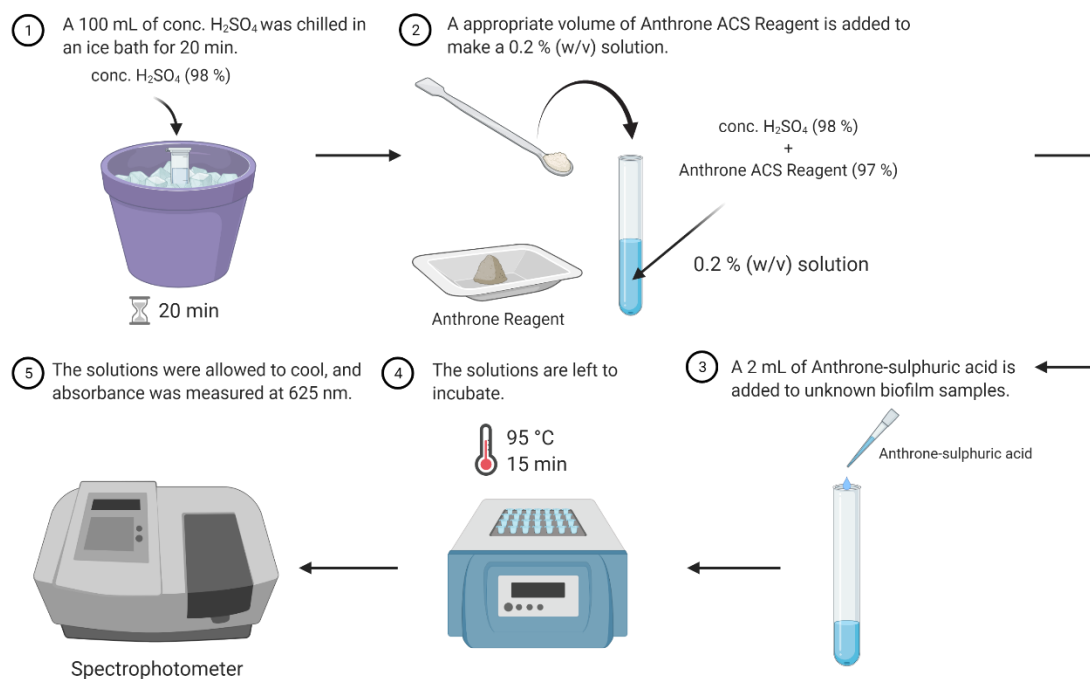


**Figure 2.11.** Schematic illustrating the quantification of carbohydrate using the Anthrone-sulphuric acid assay (created using BioRender.com).

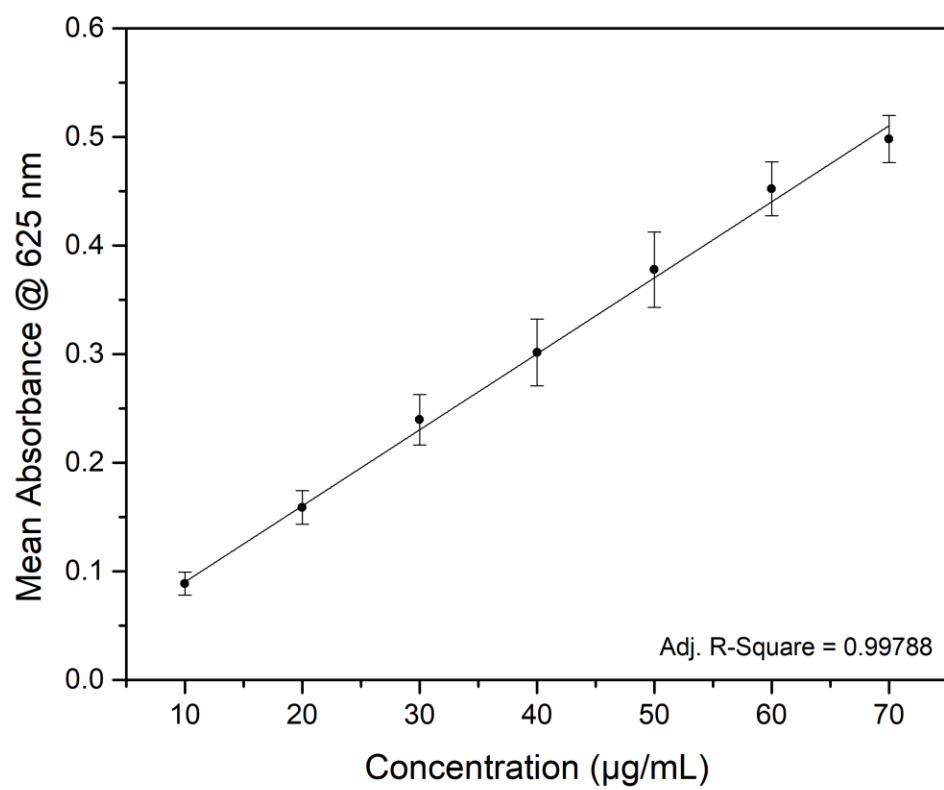


**Figure 2.12.** Calibration curve of the concentration of D-(+)-glucose standard solutions using the anthrone-sulphuric acid assay at a wavelength of 625 nm (n = 3).

### 2.7.3 DNA sequencing

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#### 2.7.3.1 Sample preparation

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Biofilm was dried and removed from the glass slides (i.e., deployed at Dublin Bay) by gently scraping the cells using a single-use rubber cell scraper, and broken up into a fine powder using a pestle and mortar (Figure 2.13). Samples were then placed into 2 mL Eppendorf tubes for extraction using the PowerSoil Pro Kit (Qiagen, Ireland # 47014).



**Figure 2.13.** Sample preparation procedure used for biofilm samples taken from Poolbeg Marina for eDNA extraction using the DNeasy PowerSoil Kit (Created using BioRender.com).

### *2.7.3.2 Sample extraction*

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DNA extraction was performed by Dr Molly Williams of the School of Biotechnology at Dublin City University. eDNA was extracted from the biofilm samples using the PowerSoil Pro Kit (Qiagen, Ireland). The PowerBead Pro Tube was spun briefly to ensure that the beads had settled to the bottom. 800  $\mu$ L of the solution labelled CD1 was added to the PowerBead tubes containing the biofilm samples, before being vortexed briefly to mix. The PowerBead Pro Tube was secured horizontally, and vortexed at maximum speed for 10 min. The PowerBead Pro Tube was then centrifuged at 15,000 x g for 1 min, before the supernatant was transferred to a clean 2 mL microcentrifuge tube. 200  $\mu$ L of the solution labelled CD2 was then added to the tube and vortexed for 5 s. Again, the samples were centrifuged at 15,000 x g for 1 min. Avoiding the pellet, 700  $\mu$ L of supernatant was then transferred to a clean 2 mL microcentrifuge tube. 600  $\mu$ L of solution CD3 was added and vortexed for 5 s. 650  $\mu$ L of lysate was loaded to an MB Spin Column. The sample was centrifuged at 15,000 x g for 1 min. The flow-through was then discarded, and the step was repeated to ensure all of the lysate has passed through the MB Spin Column. The MB Spin Column was then placed into a clean 2 mL Collection Tube. 500  $\mu$ L of Solution EA was added to the MB Spin Column, and centrifuged at 15,000 x g for 1 min. The flow-through was discarded and the MB Spin Column was placed back into the same 2 mL Collection Tube. 500  $\mu$ L of Solution C5 was added to the MB Spin Column, before centrifugation at 15,000 x g for 1 min. The flow-through was once again discarded and the MB Spin Column placed into a new 2 mL Collection Tube, before centrifugation at 16,000 x g for 2 min.

The MB Spin Column was then placed into a new 1.5 mL Elution Tube, before 50 µL of Solution C6 was added to the centre of the white filter membrane. Centrifugation was carried out at 15,000 x g for 1 min. The MB Spin Column was then discarded, and the DNA extraction process was complete.

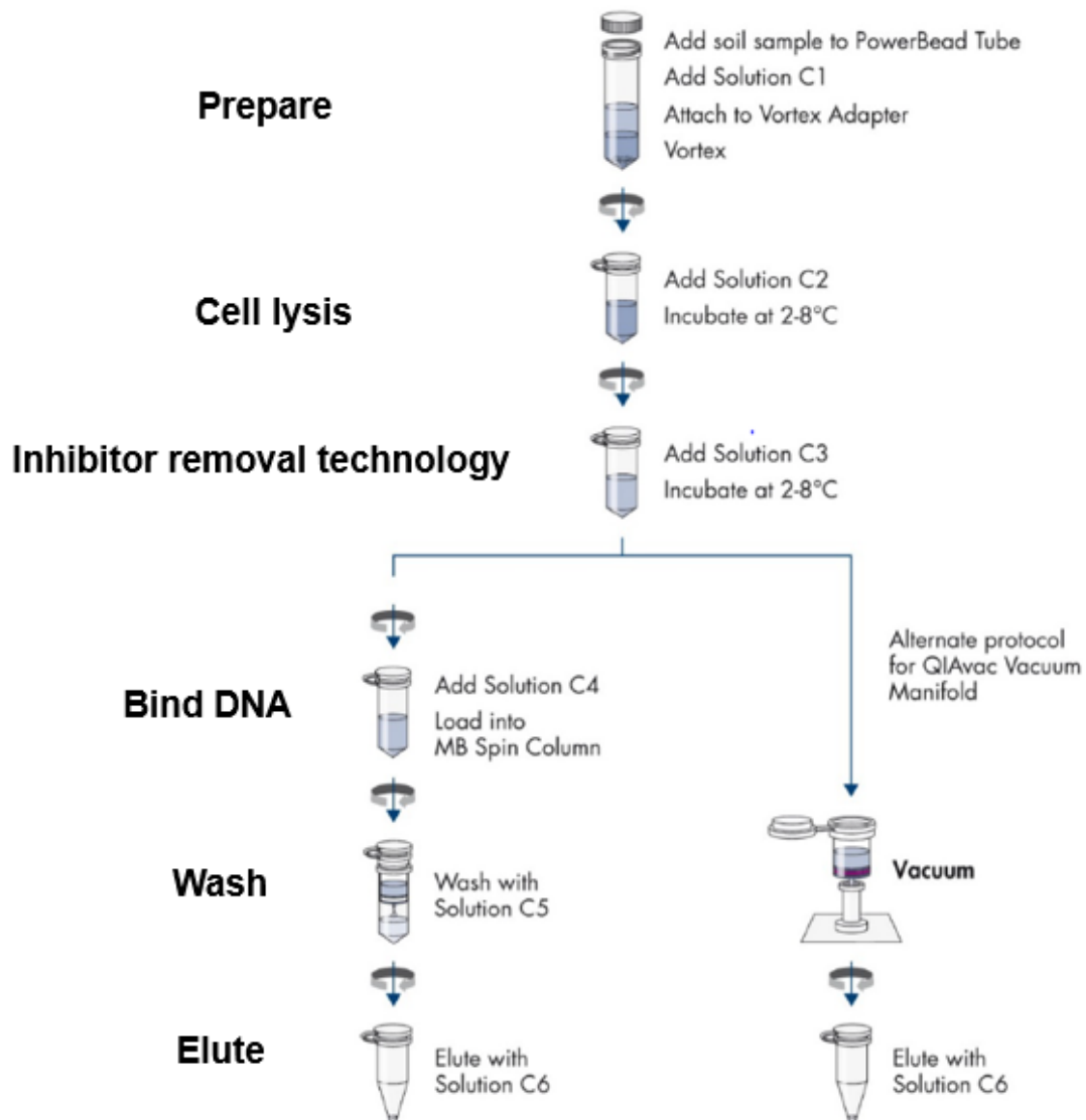


**Figure 2.14.** DNeasy PowerSoil Kit procedure (image source: DNeasy PowerSoil Kit Handbook 05/2017).

### 2.7.3.3 Sequencing

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Illumina sequencing was performed on seasonal data by Source Bioscience (Cambridge, UK). Source Bioscience is a company that performs genomic sequencing from provided RNA and DNA samples. Ribosomal RNA from the 16S v3-v4 regions was amplified and processed using a customized bioinformatics pipeline based on the QIIME2 package (v 2021.8.0). The analysis was composed of 3 key steps: (a) quality filtering, deionizing and merging of reads, (b) taxonomic assignment and (c) alpha rarefaction, with data provided to DCU from each step. In this study, we are most concerned with taxonomic assignment. For this, amplicon sequence variants (ASVs) (or Operational Taxonomic Units (OTUs)) were evaluated to a reference database of known organisms at seven different levels. In this study, we looked specifically at Level 2 (Phylum). Taxonomy assignment was performed using a Naïve Bayes classifier that was pre-trained on the 16S v3-v4 rRNA SILVA (97% OTU) reference database (version 138), using the q2-classify-skylearn plugin. Ribosomal RNA from the 18S gene was amplified and processed using a customized bioinformatics pipeline based on the QIIME2 package (v 2021.8.0). The analysis was composed of 3 key steps, with output files provided at each step as described previously. Taxonomic assignment was performed to genus level as described above.

## 2.8 Physical characterization

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The physical characterization of materials was performed in order to characterize the physical properties of the individual fabricated micro-textures. The measurements performed include scanning electron microscopy (SEM), 3-D digital light microscopy, water contact angle (WCA), optical profilometry.

### 2.8.1 *Scanning electron microscopy*

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Surface morphological measurements were taken on a Hitachi S3400-N scanning electron microscope (Hitachi, UK) and the Jeol JSM-IT 100 InTouchScope (Jeol, UK). Samples were imaged at a WD of approximately 8 mm – 9 mm and an accelerating voltage of 5 kV – 20 kV. In Chapter 4, biofilm samples collected from Dublin Bay were imaged at 5 kV over multiple random fields of field (FOV) and in triplicate. Using a diatom identification key, diatom species were identified to genus level. For characterization of engineered surface micro-textures discussed in Chapter 5, ten images of the fabricated surface micro-textures were taken, and Image J (Fiji) image processing software was used to calculate the dimensions of each of the micro-textures after manufacture by 3-D printing, polydimethylsiloxane (PDMS) casting and Repliset-F5 casting (n = 3). In Chapter 6, cell settlement was observed on each of the produced micro-textures (n = 3) using the Jeol JSM-IT SEM.

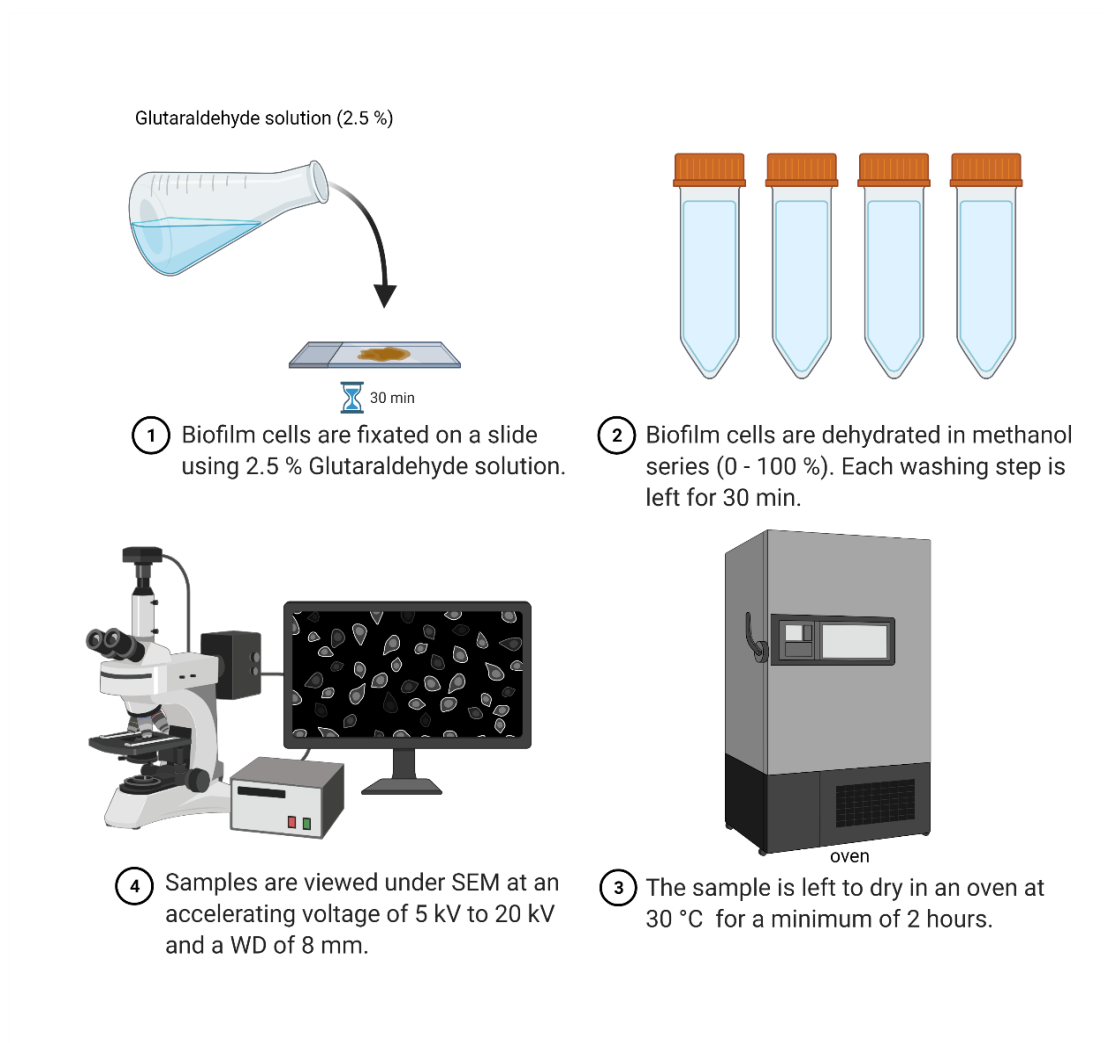


**Figure 2.15.** Schematic of the protocol used for the preparation of field samples for scanning electron microscopy (SEM) (created using BioRender.com).

### 2.8.2 *Energy dispersive x-ray analysis*

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Energy dispersive x-ray analysis (EDX) was carried out on the Jeol JSM-IT 100 InTouchScope (Jeol, UK). Measurements were taken at an accelerating voltage of 20 kV and 30 kV. A higher energy beam was used due to a low x-ray yield. For each sample, a spectrum was obtained which averaged an area. An elemental map was obtained using a map scan and the results were fitted on a pixel by pixel basis. A peak on the spectra is evidence that an element is present.

### 2.8.3 *Light microscopy*

---

Light microscopy (LM) images were taken on the Keyence 3-D digital microscope (Keyence, UK) with objective VH-Z500R/W/T. A series of four images were taken per micro-texture replicate ( $n = 3$ ) using the 3D composite image function to give a total of twelve images per sample.

### 2.8.4 *Water contact angle*

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Contact angle measurements were taken on an FTA200 Dynamic Contact Angle Analyzer (First Ten Angstroms (FTA), USA). Samples were cleaned prior to analysis with  $N_2$  as in the fume hood. UltraPure DNase/RNase-free distilled water was used to fill the syringe prior to analysis. The software program, FTA32 was used to measure the wettability of samples. Firstly, the sample was positioned on the sample stage. The 'pump out' function was used to allow the droplet to move from the syringe onto the sample surface (enlarging the size of the droplet). The 'pump in' function was used to draw the UltraPure distilled water back from the sample surface into the syringe (decreasing the size of the droplet). The flow rate and droplet size were controlled by the control panel at the side of the FTA32 software. The most commonly used droplet size is 15  $\mu$ L. The 'snapshot' function was then used to capture an image of the sample surface. The 'contact angle' function allows for an automatic calculation of the surface wettability of a sample. A blue baseline is presented at the bottom of the droplet with an orange/yellow tracing around the curvature of the water droplet. In Chapter 3, WCA measured the shape of ultrapure water droplets sitting on the surface of the sol gels.

Each coating was made in triplicate and WCA angle measured at three different points on each surface to calculate the mean WCA and standard deviation.

#### 2.8.5 *Surface roughness*

---

Surface roughness measurements were taken ( $n = 3$ ) on a Bruker Contour GT optical profilometer (Bruker, USA) instrument in order to calculate the height of designed surface features produced using 3-D printing. Samples were gold coated for 45 s prior to surface roughness measurement (i.e., due to the transparency of samples). Each sample was placed on the stage and the tilt, focus and auto intensity was set using the controls in the Live Video Display. The sample was raised until there was a small 'finger width' distance between the objective and the sample on the stage using the 'slow' button. The sample was levelled with the using the stage levelling controls until clear horizontal fringes were obtained. The light intensity and focus can then be readjusted to the desired level. A single measurement was then taken using the single acquisition button, with results appearing in the data visualization manager. Three measurements for each type of micro-texture were obtained and mean, standard deviation, and standard error were plotted using Microsoft Excel (2013).

## 2.9 Sol-gel coatings

A schematic representation of sol-gel synthesis and application is presented in Figure 2.16. Glass microscope slides (25 mm × 75 mm, Fisher Scientific, Ireland) were used as the substrate for the sol-gels and nanoparticles (NPs).

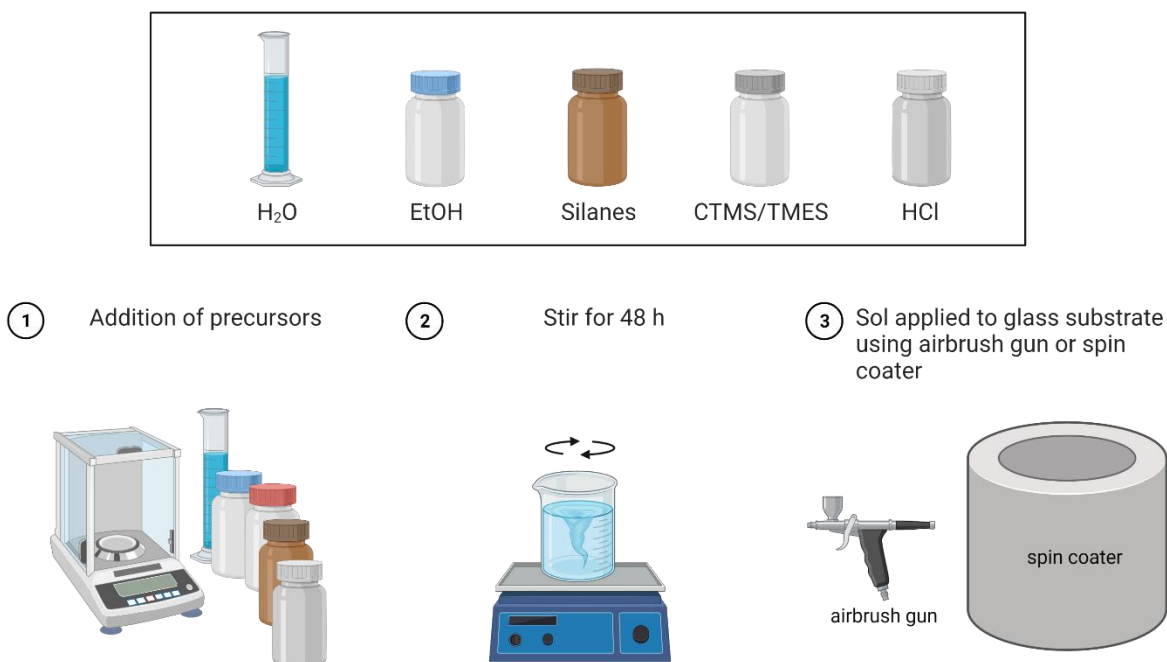


**Figure 2.16.** Schematic outlining the procedure for sol-gel synthesis and application; order of precursors: 1. H<sub>2</sub>O and EtOH; 2. Silanes; 3. CTMS/TMES; 4. HCl (37 %); 5. Stir for 48 h. (created using BioRender.com).

### 2.9.1 Sol gel synthesis

Sol-gels used for the generation of transparent, smooth coatings were prepared as outlined in Table 2.2 using a series of silane precursor mixtures hydrolysed with DI in the presence of ethanol (Table 2.2). The sol-gels were prepared using a molar ratio of 1 silane: 6.25 x C<sub>2</sub>H<sub>5</sub>OH: 4 x H<sub>2</sub>O: 0.007 HCl. Ageing of the sol-gels to achieve condensation was allowed to proceed for 48 h - for HCOO6, T<sub>2</sub> and B Sol and approx. 400 h - DMDEOS. Ageing was carried out at room temperature and under magnetic stirring (300 rpm).

**Table 2.2.** Sol-gel recipes: precursors used in the synthesis of the hydrophobic transparent sol-gel coatings used for deployment in Galway Bay. Volumes of precursors are given in milliliters (mL).

| Sol Name       | TEOS  | ETEOS | CTMS               | EtOH               | HCl   | H <sub>2</sub> O | DMDEOS | TMES |
|----------------|-------|-------|--------------------|--------------------|-------|------------------|--------|------|
| DMDEOS         | 2.679 | -     | -                  | 18.24              | 0.029 | 3.6              | 6.51   | -    |
| HCOO6          | 2.763 | 0.886 | 2.094              | 12.04              | 0.007 | 2.378            | -      | -    |
| T <sub>2</sub> | 4.06  | 0.84  | -                  | 11.67              | 0.007 | 2.252            | -      | 1.42 |
| B Sol          | 2.793 | 0.886 | 2.094 <sup>1</sup> | 12.04 <sup>2</sup> | 0.007 | 2.378            | -      | -    |

<sup>1</sup>Fumed silica methylated (FmS CH<sub>3</sub>) was used instead of ethanol (EtOH). <sup>2</sup>CTMS was added to sol after 24 h.

Topography modification of the coatings was realised using amorphous fumed silica (FmS) while surface chemistry of the coating was altered by methylation of FmS using CTMS. Briefly, a 3 g quantity of FmS (Aldrich, 0.007  $\mu\text{m}$ ) was weighed and transferred to a 100 mL glass bottle. A 100 mL volume of ethanol was added to the bottle and the solution was sonicated for 30 min. After sonication, the FmS was partially methylated by the addition of chlorotrimethylsilane (CTMS). The FmS and CTMS were mixed together to give a ratio of 10:1 (v/v) FmS: CTMS. This solution was then stirred using a magnetic stirrer for 24 h at room temperature. Using the FmS dispersion and the partially methylated FmS dispersion (FmS  $\text{CH}_3$ ) two-sol gels were prepared by mixing with  $\text{T}_2$ , i.e.,  $\text{T}_2/\text{FmS CH}_3$  (1:1) sol was prepared by mixing equal volumes of  $\text{T}_2$  and FmS  $\text{CH}_3$  (1:1, v/v) while  $\text{T}_2/\text{FmS}$  (5:3) was prepared by mixing  $\text{T}_2$  and FmS in a 5:3, v/v ratio. The newly formed solutions were allowed to age for another 24h, ambient temperature, 300 rpm.

### 2.9.2 Sol-gel application

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Glass slides ( $n = 10$ ) were prepared for coating via sonication in detergent and water for 30 min followed by sonication in IPA for 30 min. The glass slides were then left to dry at room temperature prior to use. Glass microscope slides (Fisher Scientific, Ireland, dimensions, 25 mm x 75 mm) were used as substrate for the deposition of sol-gel films by both spin coating and spray coating.

Spin Coating: The DMDEOS and HC006 sol-gels were spin coated onto clean glass microscope slides at 500 rpm for 15 s using a Laurell WS-650-23 spin coater.

Spray Coating:  $T_2/\text{FmS}$  (5:3),  $T_2/\text{FmS CH}_3$  (1:1) and B sol gels were spray coated using a WD-180R VEDA airbrush gun. Each glass substrate was placed 10 cm from the tip of the spray gun. The spray gun was then filled with the desired sol and the glass substrate was sprayed over a period of 10 s in a circular motion. Heat treatment at 110°C was used to liberate excess solvent and stabilize the coatings.

## 2.10 Texture fabrication

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A number of microfabrication methods for the production of synthetic bioinspired textures surfaces were investigated and are described below.

### 2.10.1 3-D printing

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A number of designs were constructed in SolidWorks was fabricated using the Nanoscribe Photonic Professional GT 3-D printing system (Nanoscribe, GmbH) (Figure 2.17). This 3D printing system has a 150mW femtosecond solid state laser which operates at a wavelength of 780 nm. The objective used in this process to focus the laser was a x25 Dip-in laser lithography (Dill) objective with a NA of 0.8. The negative-tone photoresist used in this process was IP-S. DeScribe software (Nanoscribe-provided) was used to transform the writing files from STL files created in SolidWorks 2017 to files that could be used with Nanowrite software (.gwl) (Nanoscribe-provided). This printer uses a direct laser writing process whereby polymer structures are formed by deflecting a laser beam into a photosensitive material using alternating X and Y laser scanning directions. Uncured photoresist was removed by placing structures in a solution of PGMEA (Propylene glycol monomethyl ether acetate, >99.5%) for 30 min followed by washing with Novec 7000 engineering fluid (methyl perfluoro propyl ether, Sigma, Ireland).

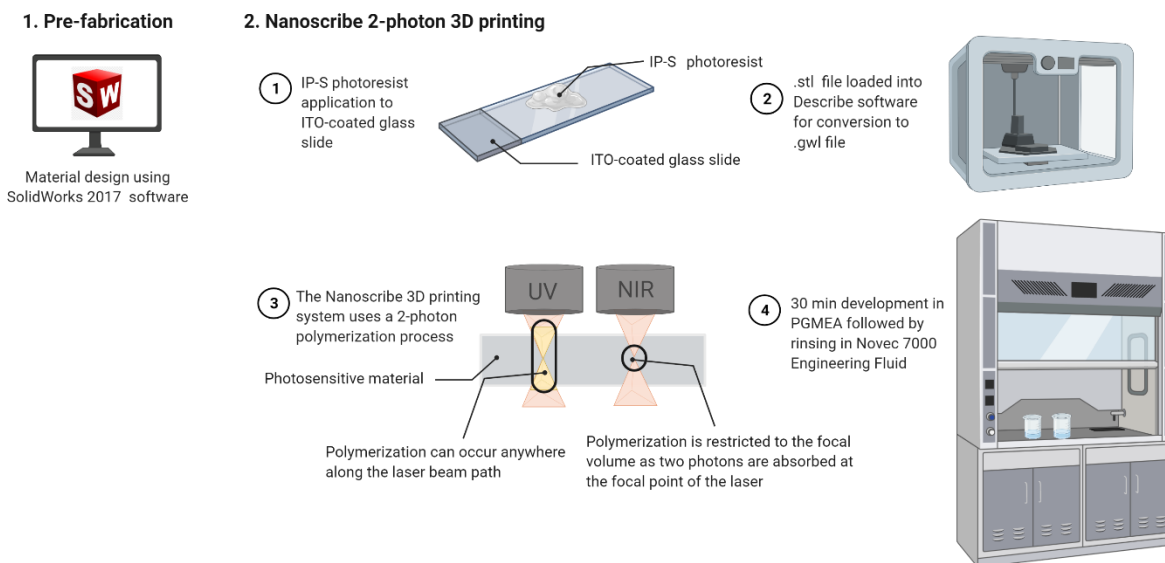


**Figure 2.17.** Schematic illustrating the 3-D printing process; 1. Pre-fabrication: A design file is created of the micro-texture using SolidWorks 2017 software; 2. Nanoscribe 2-photon 3-D printing: IP-S photoresist is applied to glass ITO-coated substrate. The design file (.stl) is loaded into the 3-D printer and converted to a file type which can be used with the 3-D printer (.gwl). A 2-photon polymerization process is used to fabricate the designed micro-structures followed by a development step involving the washing of designed micro-structures with PGMEA and Novec solutions for 30 mins (created using BioRender.com).

### 2.10.2 Polydimethylsiloxane casting

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The design used in this method was constructed in SolidWorks 2017 and fabricated using the Nanoscribe Photonic Professional GT 3-D printing system with negative-tone photoresist, IP-S as described previously. The 3-D printed texture was then vapour treated so that PDMS casting could be carried out. The part to be treated was placed in a vacuum chamber in a solution of 30 mL of hexane (95%, Sigma Aldrich) and 4.7  $\mu$ L of OTS (octadecyltrichlorosilane, >90%, Sigma Aldrich). The vacuum chamber was then pumped and left for 2 h. The part was baked at 100°C for 30 min. Mold casting was carried out by casting a liquid polydimethylsiloxane (PDMS, Sylgard 184k, 10:1) against the surface of the 3-D printed template after vapor treatment. The template and liquid PDMS was then solidified at 70 °C for 12 h. The PDMS layer was then peeled off resulting in a positive structure of the original printed template (Figure 2.18).

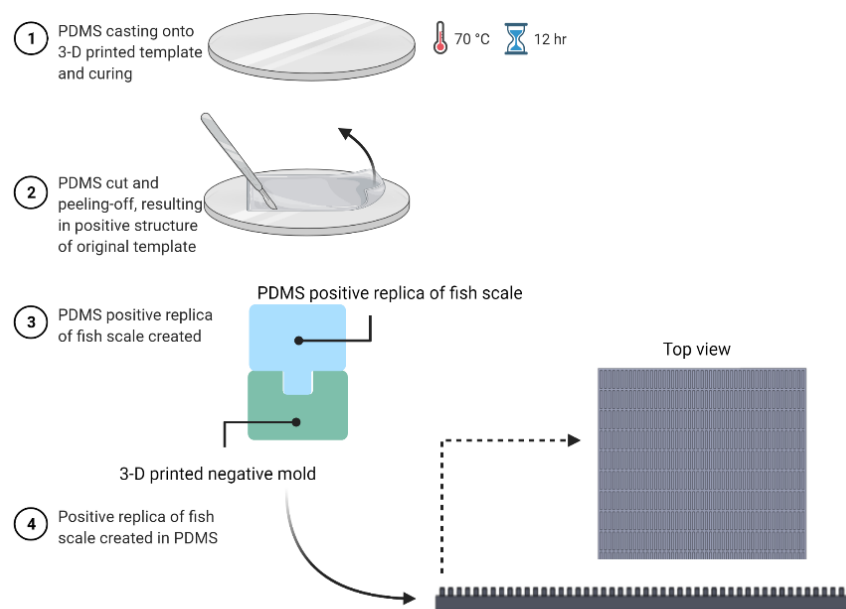


**Figure 2.18.** Schematic illustrating the process used in the fabrication of micro-textures using a soft lithography process, polydimethylsiloxane (PDMS) casting (created using BioRender.com).

### 2.10.3 Repliset-F5 casting

A dead sample of *Scophthalmus rhombus* was purchased from a fish shop in Howth, Co. Dublin, Ireland (6.066°W, 53.386°N). A 6 cm<sup>2</sup> skin sample was taken from the dorsal region of the fish along its lateral line using a scalpel to detach the skin surface from the underlying flesh. Repliset-F5 material is a two-part silicone rubber material that is often used for high resolution replicas. This material was applied to the fish and left to cure for 1 h. After this time, the material could be lifted off the skin sample to create a high-resolution replica. To this, PDMS (Sylgard 184k, 5:1) could be poured onto the RepliSet-F5 material to create positive replicas of fish scales (Figure 2.19).

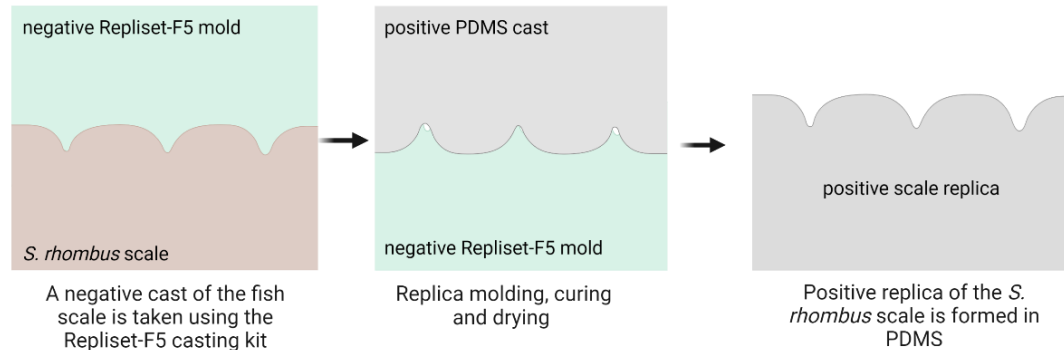


**Figure 2.19.** Schematic illustration of the casting process using the Repliset-F5 casting kit (created using BioRender.com).

### 2.11 Static adhesion studies

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Rapid biofouling assessment was performed using a 10 mL culture suspension of fouling organisms, *Amphora coffeaeformis* and *Nitzschia ovalis*. Cells were kept at a chlorophyll  $\alpha$  concentration of  $0.3 - 0.4 \mu\text{g mL}^{-1}$  and cell numbers were estimated by counting using an improved Neubauer bright-line hemocytometer to achieve a cell density of approximately  $3 \times 10^6$  cells per mL ( $n = 3$ )<sup>6</sup>. The textures were firstly purged with nitrogen ( $\text{N}_2$ ) gas to remove air pockets. Designed micro-textures (as described in Chapter 6) were immersed in the cell suspension for 3 h in individual petri dishes. Colonization of textures was observed by removing the micro-textures from the cell suspension and dipping twice sequentially in solutions of 50:50, artificial seawater: deionized water and deionized water. Samples were stained by immersion in  $10 \mu\text{g mL}^{-1}$  of acridine orange (AO) dye for 3 min at room temperature. Samples were rinsed three times sequentially in deionized water to remove excess stain and allowed to dry. Colonization of organisms on the candidate micro-textures was observed using the Keyence VHX2000E 3D digital light microscope with high resolution zoom lens VH-Z500R (500 x to 5000 x). Images were acquired at a magnification of 500 x from each candidate microtexture, control and ITO-coated glass sample ( $n = 3$ ) (Figure 2.20). Fiji (Image J) image processing software with plugin Weka Segmentation was used to calculate the percentage coverage of cells on the surface of the micro-textures.

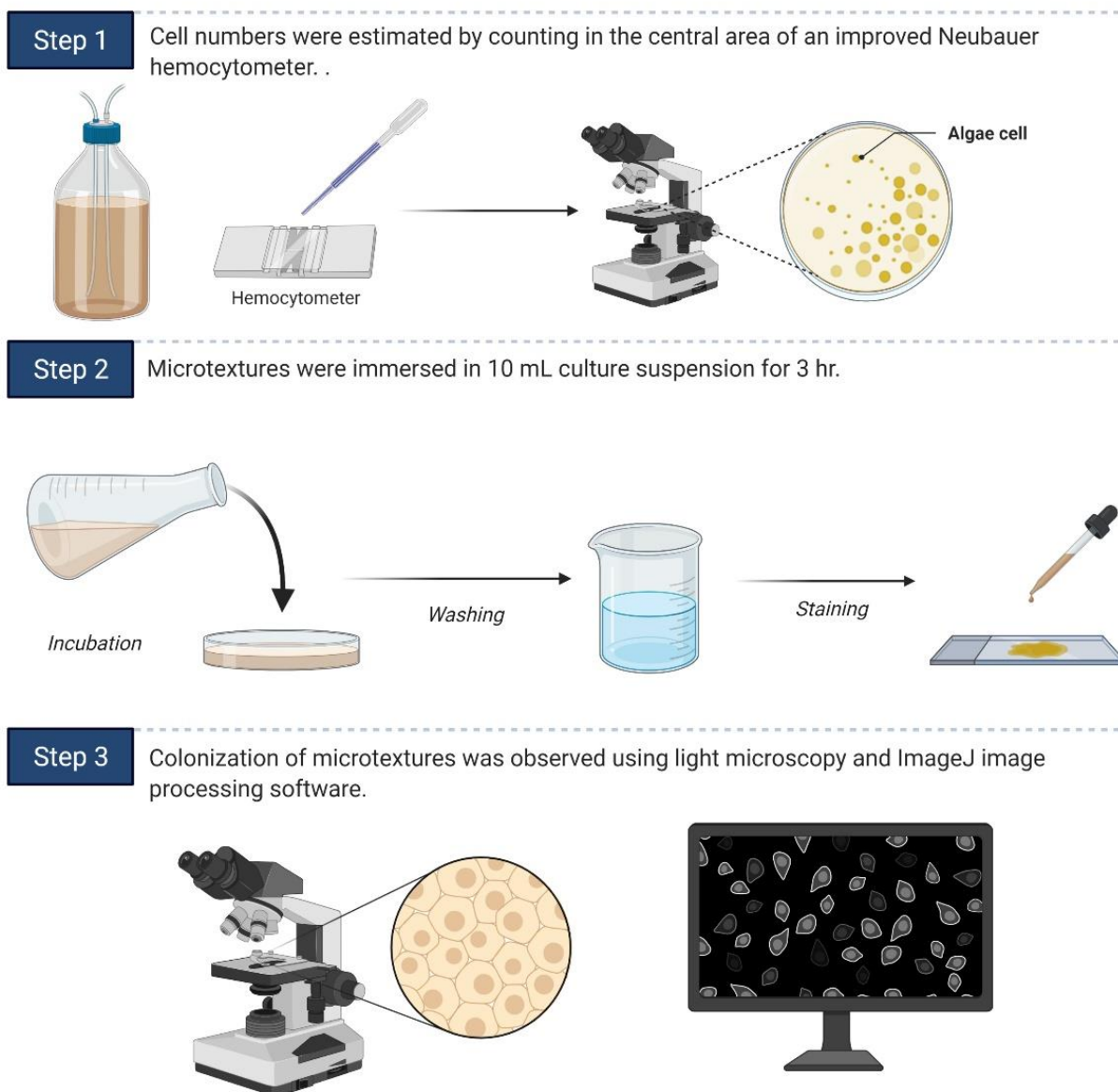


**Figure 2.20.** Schematic illustrating the described laboratory assay for *A. coffeaeformis* and *N. ovalis* settlement (created with BioRender.com).

## 2.12. Dynamic adhesion studies

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The best performing micro-textures observed from the laboratory assays described previously were selected for dynamic laboratory testing using diatom species, *N. ovalis*. Two different types of dynamic testing were performed as described below.

### 2.12.1 *Modified spin coater*

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A Laurell WS-650MZ-23 spin coater was modified to accommodate the desire for high-capacity spinning (up to 12,000 rpm) (Laurell Technologies Corporation, USA). Designed micro-textures were secured on the sample holder of the spin coater instrument using a glass microscope slide and a sticky carbon tab. Three speeds were chosen for the study: 3000 rpm, 6000 rpm and 12000 rpm. These speeds were chosen primarily on results obtained from FMEA studies performed by Professor Yan Delauré, trying to mimic stresses that the micro-textures would experience on tidal energy blade systems.

### 2.12.2 *Spin stand*

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Spin stand testing was performed by Dr. Eadaoin Carthy, DCU. A microfluidic-style disc (Figure 2.21) was fabricated using the Epilog laser to cut 3 mm PMMA material and the Graphtec knife cutter used to cut the PSA layers. The disc was attached to the spin stand motor spindle where it was secured. Firstly, the disc is spun at a low spin frequency for optimization of the strobe parameters (i.e., brightness and focus). Conditions can be modified on the charged coupled device (CCD) camera (PCO Pixelfly). LabView software was used to control the spin-frequency and acceleration. The acceleration of the disc was increased slowly to reach a maximum spin speed of 70 Hz, for which it was held for 5 min before removal.

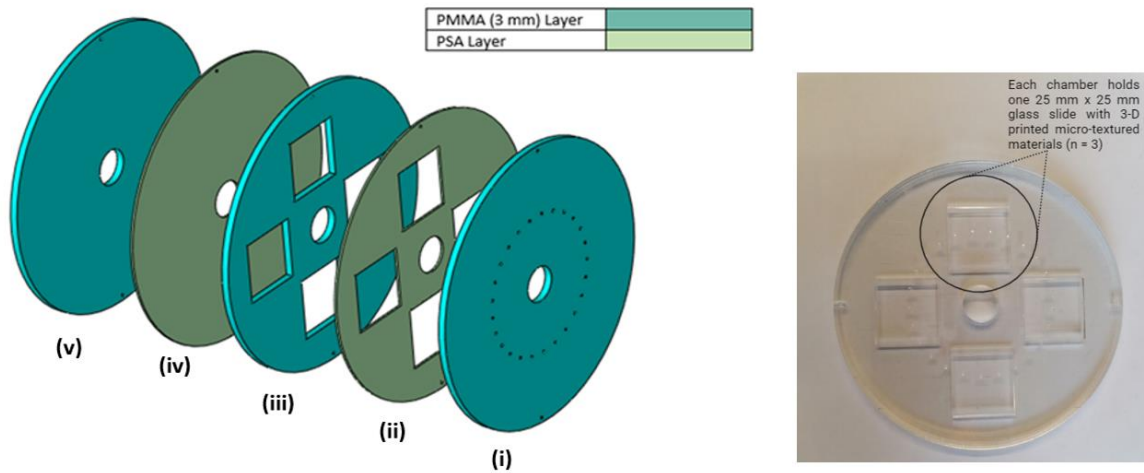


**Figure 2.21.** Left: Rendered image showing each layer of a microfluidic-style disc, where (i) is a PMMA layer with air vents; (ii) is a PSA layer with 25 mm x 25 mm chambers to house individual textured materials with adhered diatom cultures; (iii) is a PMMA layer with 25 mm x 25 mm chambers; (iv) is a base PSA layer; and (v) is a base PMMA layer; Right: Digital image showing fabricated microfluidic style disc used for dynamic testing on designed micro-textured surfaces.

### 2.13 Image analysis

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All image analysis was performed using Fiji (imageJ) image processing software. The images used in the analysis were scaled using the scale bar given in the images taken on the Keyence VHX2000E 3-D digital microscope. Images were cropped a to approximately 300 x 300  $\mu\text{m}$  to remove noise or imperfections such as unwanted particles, material/texture defects.

Image segmentation was performed using the TWS plugin which uses machine learning algorithms to classify a red-green-blue or greyscale image into different classes. The user trained the software by drawing strokes defined as a training set of pixels (STP) over the region of interest (ROI) for each class. An STP of two classes is necessary to start the segmentation. Two classes were used. Class 1 corresponds to the particles to be detected, in this case benthic diatom species, *A. coffeaeformis* and *N. ovalis*, appearing red. Class 2 corresponds to the background of the image, appearing green. These classes are comprised of sets of pixels that share similar visual characteristics such as colour, shape, or size. Different ROI were added to each class until a minimum of 50 measurements for each class was reached. The segmentation process was carried out using the Fast Random Forest algorithm which employs machine learning using decision trees to classify data based on user-defined patterns for each class. This algorithm was chosen because of its computational efficiency, probabilistic output, ability to handle a wide variety of image input characteristics and interactive enhancement based on error handling.

In addition, this algorithm avoids over-fitting the data by injecting randomness in the training trees and combining the output of multiple random trees into the final classifier; in our case 200 trees were performed using always in each analysis an out of bag error lower than 5% to arrive at the final classifier from which a probability map was obtained. The Random Forest algorithm proves to have robust performance when compared using eight evaluation metrics<sup>124</sup>. The probability map was cropped and processed using an ImageJ plugin to cluster colour pixel driven by the user input developed by Biomedical Imaging Group (<http://bigwww.epfl.ch/>). This ImageJ plugin allows to segment a colour image by pixel clustering. Five red patterns were used for class 1 (corresponding to diatom cells) and five green patterns for class 2 (corresponding to the background). The percentage area covered by each colour was calculated using the K-means clustering algorithm. The analyses were carried out on an individual sample basis. Subsequently, a mean value and a standard deviation were calculated in triplicate.

Chapter 3 focused on using ImageJ to capture macrofouling coverage and nearest neighbour distances. An image was taken of three replicate slides of each type of coating and loaded into Image J software. The scale of the image was set to those of a microscope glass slide (i.e., 25 mm x 75 mm). The image was converted to 8-bit and using the paintbrush tool, circles were drawn around the barnacles covering each slide. The threshold of the image was then adjusted to (0,0). The circles drawn around each of the barnacles could then be filled in using the paint bucket tool in a different colour (i.e., blue). The threshold of the image was adjusted again in the range of 160-180 (to yield an image of black circles (i.e., barnacles) and white outlines of the barnacles. The measurements were then set to include area and centroid. The analyse particles feature

was used to calculate number and area of barnacles present on each of the slides. Nearest neighbour distances were calculated using the plugin feature 'nnd' in the plugins tab of Image J. Chapter 6 focused on using Fiji (ImageJ) to calculate mean colony sizes on *A. coffeaeformis* and *N. ovalis* cells on each of the smooth and textured surfaces. The scale of the image was set to 300 x 300  $\mu\text{m}$ . The image was converted to 8-bit. The threshold of the image was then adjusted to yield an image of white circles (i.e., cells) and black background.

Each colony of cells could then be selected and from that, the measure tool was used to calculate approximate area ( $n = 30$ ). Mean, standard deviation and standard error was then calculated in Microsoft Excel (2016). A schematic of the image processing process using ImageJ (Fiji) that was adapted for this work is shown in Figure 2.22 below <sup>8</sup>.

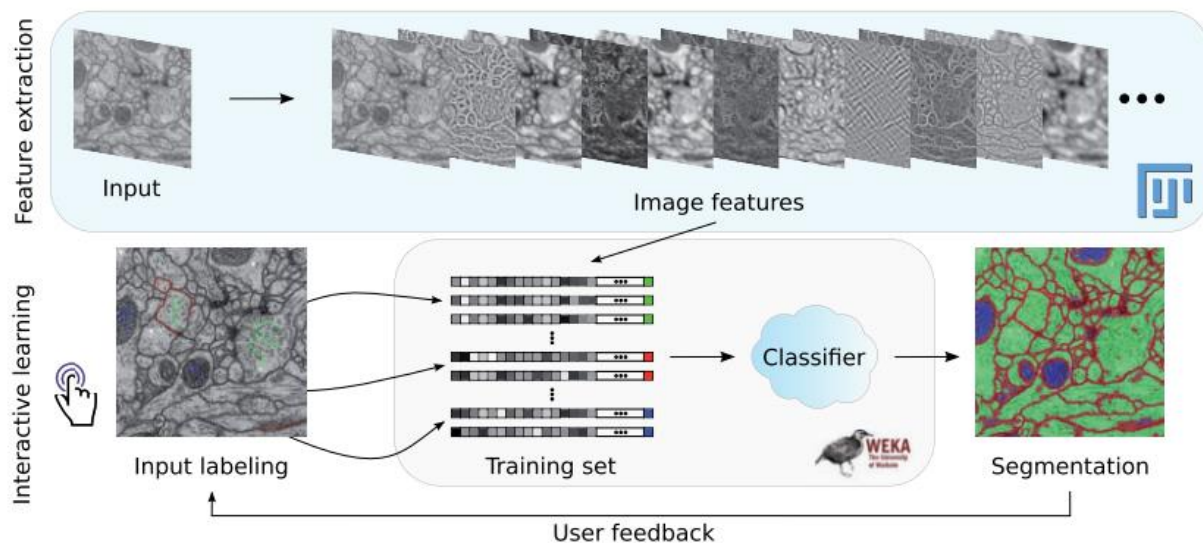


**Figure 2.22.** Schematic of the image processing procedure using Fiji software (Taken from <sup>8</sup>).

## 2.14 Statistical analysis

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Statistical analysis was performed using Microsoft Excel (2016) with the function T.TEST. In Chapter 3, t-testing was performed to understand which sol-gel coatings were most effective at preventing macrofoulers from attaching to glass microscope slides in comparison to commercial paints, Trilux, Cruiser Uno and Micron. A two-tailed distribution with unequal variance was performed. In Chapter 5, 1-way ANOVA followed by Tukey's *post-hoc* test was performed to check for significances in fouling cover between designed micro-textures using Sigma Plot 11.0 software.

## 2.15 Conclusion

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Unless otherwise stated, all methods used in the thesis have been described in this section. Any modifications to these methods have been dealt with in individual chapters.

# Chapter 3:

Temporal assessment of biofilm composition and diversity in a 12-month case study in Dublin Bay

### 3.1 Introduction

---

Artificial surfaces quickly become colonized by a conditioning film in an aquatic environment, allowing for the attachment of microfouling organisms (Figure 3.1). Marine biofilms are heavily dominated by diatoms, which are early autotrophic colonizers. The settlement of microfouling communities (i.e., bacteria, diatoms) eventually leads to macrofouling (i.e., larvae, barnacles, mussels), causing serious technical and economic issues for the marine industry as described previously (Chapter 1). Biofilm composition, structure and diversity of species can vary broadly due to a number of environmental factors (i.e., temperature, location, substratum) <sup>125</sup>. The marine environment is highly dynamic, both in terms of its biological and physio-chemical properties, but also depending on the region, there can be variability in temperature, precipitation, run-off and nutrient loading. Microbial biofilms are difficult to model in aquatic environments due to their heterogeneous nature. Therefore, it is the duty of researchers of AF technology to consider closely the ecosystem of the benthic environment for which their AF technology is designed <sup>125</sup>.

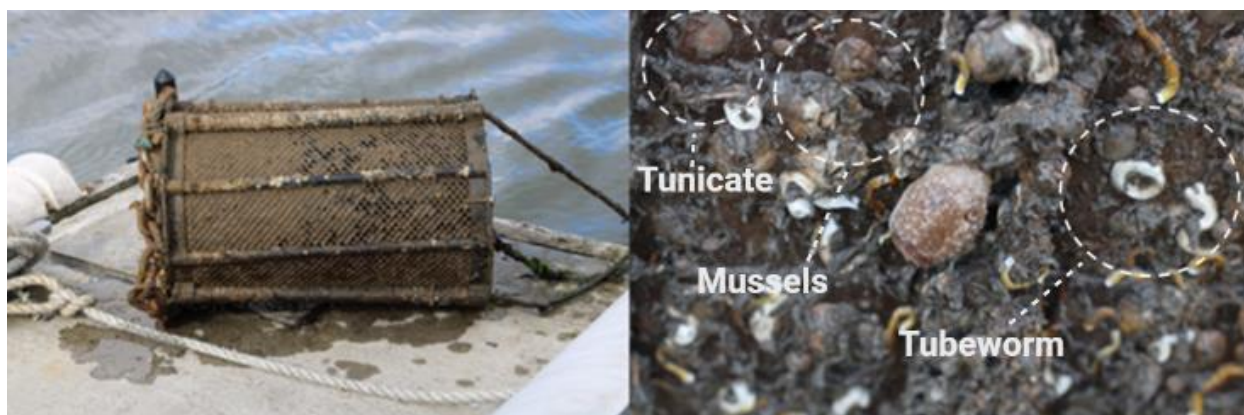


**Figure 3.1.** An example of biofouling in the marine environment: Left: lobster cage used to protect glass slide samples deployed in the field; Right: an example of macrofouling (i.e., tubeworms, mussels and tunicates), found adhering to a composite material deployed for 12-mo in Dublin Bay.

The work of Jean François Briand *et. al*, highlights the importance of studying the biofilm ecosystem for which testing of AF systems will take place. Briand has taken part in numerous studies on biofilm diversity along the Mediterranean Coast <sup>126–128</sup>. A two-week study carried out in 2012 by Briand examined marine biofilm communities on artificial surfaces at two contrasting (i.e., water quality) French Mediterranean coast sites; Toulon Bay (an enclosed military harbour) and ‘Calanque des salins’ (a small bay located in a natural protected area). Panels coated with four commercial antifouling (AF) paints, Polystyrene and Teflon were immersed for a period of 2 weeks. The study concluded that there were highly different densities of microorganisms (i.e., bacteria and diatoms) in terms of both sites and coatings. Lower rates of fouling were discovered at Porquerolles Island (Calanque des salins) than that observed in Toulon Bay. Two microalgal communities, *Licmophora gracilis* and *Cylindrotheca closterium*, were among the most commonly found species identified at both sites, regardless of the surface <sup>128</sup>. Following on from this, a 1-month summer immersion study in Toulon Bay was carried out in 2014 <sup>129</sup>, investigating biofilm colonization on seven different coatings (i.e., polyvinyl chloride

(PVC), a fouling release coating (FRC) and five self-polishing copolymer coatings (SPC), involving four commercial coatings). *Licmophora*, *Navicula* and *Nitzschia* were identified by microscopy to be the most dominant diatom species in Toulon Bay. In general, biocide-free coatings were observed to have a higher density of microfouling communities. Diatoms showed lower densities than heterotrophic bacteria. Other studies performed in the group, Bressy *et. al.* <sup>127</sup>, looked at spatial and temporal effects of biofouling communities at two contrasting French sites: (i) Lorient, Atlantic Coast (temperate, eutrophic) and (ii) Toulon Bay (mesotrophic, highly contaminated). Biofilm communities were affected by site-specific parameters. In Toulon Bay, microbial communities were affected by high temperature, salinity, lead. At the Atlantic coast site, communities were shaped by nutrients <sup>127</sup>. A similar study to the one reported in this chapter was carried out by Muthukrishnan *et al.*, (2014) <sup>130</sup>. This study investigated the composition of a biofilm on eleven commercial biocidal coatings for one year in the marine environment. The results of this study showed that depending on the type of coating used, distinct communities of bacteria were observed. There was also a clear indication of difference in biofilm composition between both Cu and Cu-free coatings. Another study by the same group (Muthukrishnan (2019)<sup>131</sup>). investigated the effects of substrate and location on the development of a biofilm on polyethylene terephthalate (PET), polyethylene (PE), wood and steel at two locations in the Sea of Oman. PET and PE surfaces revealed lower amounts of biomass build-up in comparison to steel and wood. PET had the highest abundance of bacterial communities at both locations. The findings of this study found that like Briand *et. al.*, microbial communities are influenced greatly from location to location and from substrate to substrate <sup>131</sup>.

Another study investigated the effect of commercial biocidal AF coatings on diatom communities in the marine environment for one year and in two locations in Muscat, Oman<sup>132</sup>. It was discovered that the growth and the amount of diatom communities were significantly affected by the type of biocidal AF coating chosen. Diatom genera, *Amphora*, *Cocconeis* and *Mastogloia* were among the most dominant diatoms found during the study. The findings of this study signify the importance of location, choice of AF solution on the composition of a biofilm as it can vary significantly from one location to the next. Biofouling remains one of the most problematic challenges for the marine industry in Ireland yet very few studies have examined marine biofouling from an Irish perspective. As a result of this, little information exists on species diversity and settlement strategies in Irish waters, which is essential knowledge for the design of effective AF materials. A study by Sullivan *et. al.*, investigated marine biofouling on different AF materials at an estuarine site, Poolbeg Marina in Dublin Bay (Dublin) and at Lough Hyne Marine Reserve (West Cork). It was concluded that the AF performance of coatings was significantly influenced by test site, where spatial and temporal variations exist<sup>12</sup>.

**Table 3.1** Summary of material deployed in each location in a study by Sullivan *et al.* (Adapted from <sup>12</sup>).

| Material/Coating     | Poolbeg Marina | Lough Hyne Reserve | AF Mechanism    | Material/Coating Thickness (µm) |
|----------------------|----------------|--------------------|-----------------|---------------------------------|
| Control glass slide  | ✓              | ✓                  | None            | 1000 ± 60                       |
| PDMSe-coated glass   | ✓              | ✓                  | Foul-release    | 48 ± 2                          |
| Unpolished SS 316L   | ✓              | ✓                  | None            | ~ 1000                          |
| Abraded SS 316L      | ✓              | —                  | None            | ~ 1000                          |
| PDMSe + Cu particles | ✓              | ✓                  | Cu <sup>+</sup> | Variable                        |
| <b>Commercial AF</b> |                |                    |                 |                                 |
| Waterways AF         | ✓              | —                  | Biocide         | 22.5 ± 0.85                     |
| Copper plate         | ✓              | ✓                  | Cu <sup>+</sup> | 350                             |
| Cruiser Uno AF       | ✓              | ✓                  | Biocide         | 20 ± 0.2                        |
| Trilux               | ✓              | ✓                  | Biocide         | 30.6 ± 0.9                      |
| Micron Extra         | ✓              | ✓                  | Biocide         | 17.2 ± 0.2                      |
| Interspeed Ultra     | ✓              | ✓                  | Biocide, SPC    | 27.8 ± 3.7                      |

This section intends to understand the process of biofilm formation at an Irish coastal site for a period of 12-months for the identification of common microfouling (both prokaryotic and eukaryotic) communities. This study will inform on species diversity in Irish waters as well as on cell-substrate interaction for the design of a novel bioinspired AF material.

### 3.2 Aims and objectives

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The aim of this chapter is to conduct an evaluation of biofilm development under real environment marine conditions over a 12-month period at an Irish coastal site, Poolbeg Marina, Dublin, Ireland.

The objectives of this work are to:

- 1) Characterize the evolution of the biofilm matrix over time by means of quantitative assays for protein and carbohydrate determination
- 2) Evaluate the diversity of attaching microfouling communities (i.e., diatoms) using scanning electron microscopy, with identification of diatoms to genus level
- 3) Investigate seasonal diversity of fouling organisms using molecular methods of 16S (i.e., prokaryotes) and 18S (i.e., eukaryotes) sequencing

### 3.3 Materials and methods

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The methods discussed here have been outlined in Chapter 2 previously. The determination of protein was carried out using the Lowry Protein Assay as described in Section 2.7.1. The determination of carbohydrate was carried out using the Anthrone-sulphuric acid assay as outlined in Section 2.7.2. Diversity of microfouling communities on a monthly basis was investigated using scanning electron microscopy (Section 2.8.1). Samples were Au sputter coated for 30 s (Agar Scientific) and examined at an accelerating voltage between 5 – 10 kV. A diatom identification key and a book, 'Diatoms: Biology and Morphology of the Genera' <sup>133</sup> was used to identify the diatoms to genus level. DNA extraction and sequencing was carried out as described in Section 3.4.5.

### 3.4 Results and Discussion

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#### 3.4.1 *Environmental sampling site*

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An evaluation of biofilm development on untreated glass microscope slides (25 x 75 mm) over a 12-month period was carried out at Dublin Bay. The site chosen for the deployment was that of Poolbeg Boat & Yacht Club (53.3421°N, 6.1513°W) (Figure 3.2 (a)). Poolbeg Boat & Yacht Club lies in the centre of Dublin, close to Dublin Port, on the southern side of the River Liffey. Dublin Port is the largest transport and passenger port on the island of Ireland (50 % of all trade), with private companies' operating all handling and shipping in the Port. As a result of this, lots of traffic passes through Dublin Port each day, making for a highly dynamic ecosystem.



**Figure 3.2.** Sampling process: (a) Site location – Poolbeg Marina, Dublin Bay (53.3421°N, 6.1513°W), (b) and (c) monthly sampling regime involving the collection of five glass slides per month for biological and microscopy characterization.

### 3.4.2 Environmental conditions

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Environmental conditions were monitored throughout the course of the investigation using a YSI EXO3 multiparameter sonde and data generated from the Environmental Protection Agency's sampling campaign monitoring water quality. The mean environmental parameters recorded for Dublin Bay from March 2020 to March 2021 are highlighted below in Table 3.2.

**Table 3.2.** Mean measurements of environmental parameters measured at Poolbeg Marina, Dublin Bay from March 2020 – 2021.

| Measured environmental variable | Summer | Winter |
|---------------------------------|--------|--------|
| Water temperature (°C)          | 14.69  | 7.63   |
| Salinity (%)                    | 24.89  | 22.14  |
| pH                              | 8.08   | 7.95   |
| <i>Chlorophyll a</i> (mg/mL)    | 1      | 1      |
| Depth (m) (of measurement)      | 8.25   | 7.4    |

### 3.4.3 Protein determination

---

The quantitation of protein concentration through Folin phenol reagent was first published by Wu *et. al.* in 1922 <sup>134</sup> leading to the Lowry method for protein quantitation which was published in 1951 <sup>135</sup>. The Lowry method <sup>135</sup> is a two-step analysis involving the oxidation of  $\text{Cu}^{2+}$  ions with a peptide bond of protein to produce  $\text{Cu}^{1+}$  ions. Once the  $\text{Cu}^{1+}$  ions are produced, they are available to catalyse the reduction of Folin & Ciocalteu's reagent. The reagent phosphomolybdotungstate is reduced to heteropolymolybdenum, changing the peak absorbance to 750 nm. The direct relation of initial protein concentration to the concentration of heteropolymolybdenum after the reaction allows the measurement of protein concentration based on the increase in absorbance of 750 nm light. The determination of protein in a biofilm matrix using the Lowry method has been widely reported <sup>136–138</sup>. The Lowry method allows for the quantification of proteins within the EPS matrix, however, biofilm biomass is not always correlated with EPS components <sup>139</sup>.

In this study, protein was quantified on a monthly basis using the Lowry protein assay (Peterson's Modification) ( $n = 3$ ). Figure 3.4 below shows results obtained from the investigation. Baier first noticed the presence of proteins in the conditioning layer of a biofilm <sup>12</sup>. Protein is considered one of the main constituents of the biofilm matrix, and as such, is a key factor in the attachment of biofouling organisms to the surface. Therefore, the prevention of protein adsorption in AF technology enables control over cellular attached on underwater surfaces <sup>12</sup>.

Significant differences (Table 3.3) by means of a 1-way ANOVA followed by Tukey's *post-hoc* test ( $P < 0.05$ ) were observed between protein concentrations observed in October and those observed in May, June, July, August, September, December, January, February and March. Significant differences were also observed between protein levels ( $\mu\text{g mL}^{-1}$ ) observed in November and those observed in May, June, July, August, September, December, January, February and March. Protein levels were expected to be higher in the summer months (i.e., due to the availability of increased light, temperature and nutrients) however, this was not the case. It is probable that the loading of macrofouling organisms during the Winter fouling season (i.e., barnacles, tubeworms) may have had an effect on the increase in protein concentrations observed but further study is required.

**Table 3.3.** Significance testing carried out using 1-way ANOVA followed by Tukey's post hoc test for Lowry protein assay carried out on biofilm samples extracted from Dublin Bay over a 12-month period (n = 3).

| Month            | Mean ( $\mu\text{g mL}^{-1}$ ) |
|------------------|--------------------------------|
| May (2020)       | 30.98                          |
| June (2020)      | 279.92                         |
| July (2020)      | 90.74                          |
| August (2020)    | 104.07                         |
| September (2020) | 219.92                         |
| October (2020)   | 1676.12                        |
| November (2020)  | 2329.41                        |
| December (2020)  | 372.08                         |
| January (2021)   | 107.13                         |
| February (2021)  | 176.75                         |
| March (2021)     | 146.49                         |

In a paper by Briand *et al.* <sup>127</sup>, it was noted that environmental parameters (i.e., salinity, temperature, location) have an effect on the diversity and density of fouling communities. Temperature in Dublin Bay was significantly lower ( $P = < 0.001$ ) in the Winter fouling season (Mean = 7.63 °C) (i.e., October 2020 – January 2021) compared with those observed in the Summer fouling season (14.69 °C) (May – September 2020) determined via t-testing using SigmaPlot 11.0 software. Biofouling organisms preferentially grow at temperatures of around 15 – 20 °C <sup>133</sup>. A paper by Admiraal <sup>140</sup> examined the effect of light and temperature on the growth rate of estuarine benthic diatoms in culture using four benthic diatom cultures. The four species were cultured at different temperatures, daylengths and quantum irradiances. One species, *Navicula arenaria*, achieved its highest growth rate at a temperature between 16 – 20°C. It was also observed that at lower temperatures, the growth rate across four species was similar. In this study, the influence of temperature and light during the Winter fouling season was observed to have an effect on the growth of biofouling organisms, which led to a decrease in protein absorption.

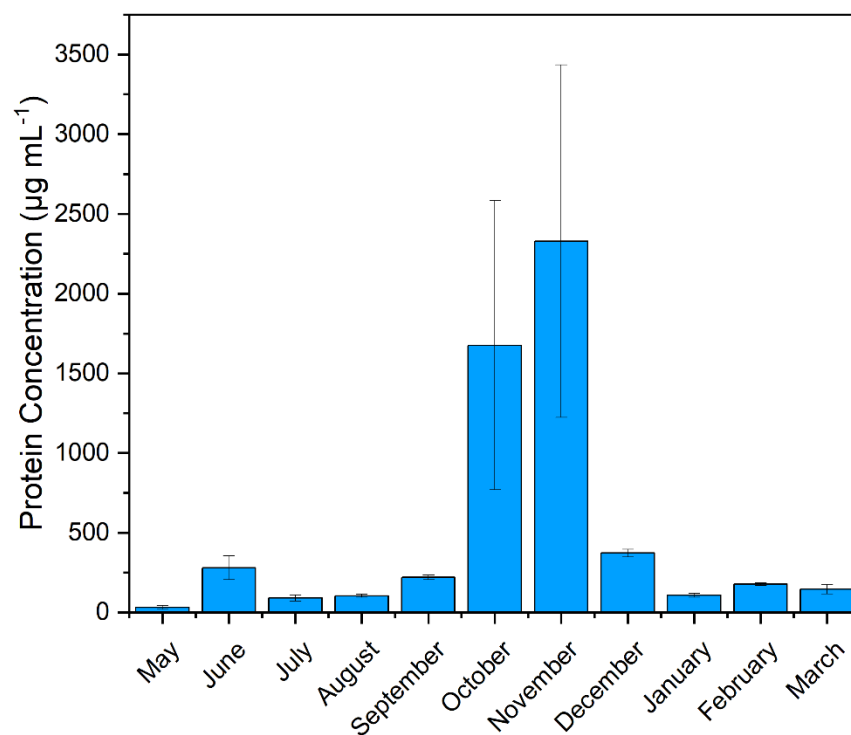


**Figure 3.3.** The concentration of protein on materials deployed in Dublin Bay over a 12-month period determined using the Lowry protein assay (n = 3).

#### 3.4.4 Carbohydrate determination

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The reaction involved in the anthrone method was first identified as a qualitative test for carbohydrate by Dreywood in 1946 and then adapted into a quantitative method by Morris in 1948 <sup>141</sup>. Since then, anthrone has been used as a convenient and specific reagent for the colorimetric estimation of carbohydrates. Carbohydrates undergo a dehydration reaction when dissolved in a sulfuric acid solution; the hydrolyzed carbohydrate molecules form furfural derivatives which are then available to form a complex with anthrone molecules. The resulting complex has a  $\lambda_{\text{max}}$  of 625 nm giving it a green colour and the change in absorbance at this wavelength can be directly linked to the initial carbohydrate concentration <sup>142</sup>.

Numerous studies have reported the use of the anthrone-sulphuric acid method to quantify biofilms<sup>138,141–143</sup>. Figure 3.5 below quantifies observed carbohydrate concentration in biofilm samples collected in Dublin Bay over a 12-month period. Significance testing was performed using 1-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test ( $n = 3$ ). Significant differences ( $P < 0.05$ ) (Table 3.4) occurred between carbohydrate levels observed in June and those observed in May, January, February and March. The same occurred between levels in October and those observed in May, January, February and March. Significance was also noted in comparison to May, July, August, September, January, February and March. Differences ( $P < 0.05$ ) were observed in July in comparison to May. Lastly, differences were observed in December (where the highest concentration of carbohydrates was seen) in comparison to all of the other months. As evidenced previously, the carbohydrate concentration found in extracted biofilm samples over a 12-month period fluctuates in response to seasonal

changes in temperature and available nutrients. The loading of macrofouling organisms (i.e., barnacles, tubeworms) during the Winter fouling period could explain the increase in protein (Figure 3.4) and carbohydrate (Figure 3.5) observed. The last stage in the biofilm process is the decrease of mechanical stability across the biofilm matrix <sup>2</sup>, causing some organisms to detach from the surface. This could relate to the fluctuation observed for carbohydrate levels in January, February and March.

**Table 3.4.** Significance testing carried out using 1-way ANOVA followed by Tukey's post hoc test for the anthrone-sulphuric acid assay carried out on biofilm samples extracted from Dublin Bay over a 12-month period (n = 3).

| Month            | Mean ( $\mu\text{g mL}^{-1}$ ) |
|------------------|--------------------------------|
| May (2020)       | 17.28                          |
| June (2020)      | 323.29                         |
| July (2020)      | 258.76                         |
| August (2020)    | 210.43                         |
| September (2020) | 185.14                         |
| October (2020)   | 347.52                         |
| November (2020)  | 490.38                         |
| December (2020)  | 808                            |
| January (2021)   | 68.6                           |
| February (2021)  | 101.38                         |
| March (2021)     | 57.53                          |

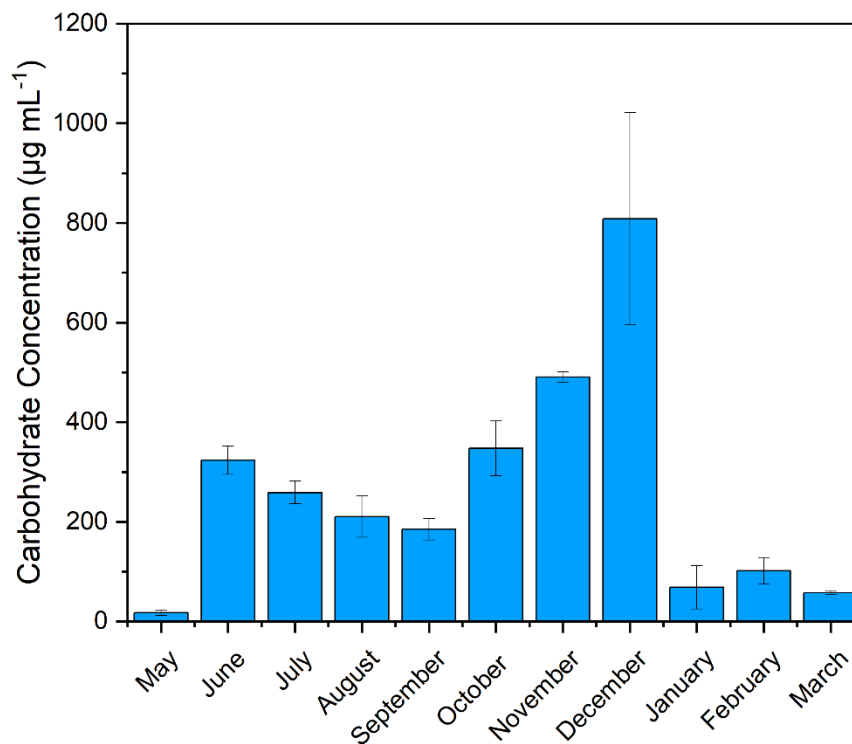


**Figure 3.4.** The concentration of carbohydrate on materials deployed in Dublin Bay over a 12-month period determined using the Anthrone-sulphuric acid assay (n = 3).

The diversity of fouling (both micro- and macro-fouling) communities is greatly influenced by environmental conditions as discussed previously <sup>127,144</sup>. In this study, both molecular and microscopy techniques were employed for the identification of the most dominant taxa in Poolbeg Marina over the 12-month period. DNA sequencing was employed to look at seasonal diversity of microfouling organisms (16S and 18S) as described in Chapter 2. Scanning electron microscopy (SEM) was used to look at diatom colonizers on a monthly basis as described in Chapter 2, Section 2.8.1 and is summarized in Table 3.5 below (n = 3).

### 3.4.5 DNA sequencing

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DNA sequencing was carried out by Source Bioscience (Section 2.7.3) (Cambridge, UK) to complement diversity studies completed with electron microscopy. Biofilm samples on glass microscope slides (75 x 25 mm) were retrieved from Poolbeg Marina each month in sterile sampling tubes and stored in the -20 °C freezer prior to use. The identification of biofilm communities is usually carried out using light or scanning electron microscopy to genus level. However, use of electron microscopy as standard practice in biofilm diversity studies has major disadvantages – including high cost and user compatibility<sup>129,144</sup>. In this study, 16S and 18S sequencing was used to explore the taxonomic composition of biofilm, with particular interest in looking at the seasonal diversity of fouling communities.

Figure 3.5 below illustrates the relative frequency (%) of the ten most abundant phyla present seasonally using 16S sequencing for the identification of prokaryotic communities. The V3 – V4 region of the 16S rRNA gene was successfully amplified and processed using a bioinformatics pipeline based on the QIIME2 package. Samples were taxonomically assigned using a reference database of known organisms at up to 7 levels: Level 1 (D0) = Kingdom, Level 2 (D1) = Phylum, Level 3 (D2) = Class, Level 4 (D3) = Order, Level 5 (D4) = Family, Level 6 (D5) = Genus and Level 7 (D6) = Species.

This study focused on the identification of bacterial (prokaryotic) and diatom (eukaryotic) communities to Level 2 (Phylum) as a screening tool for the identification of bacterial communities present in the initial development of a marine biofilm.

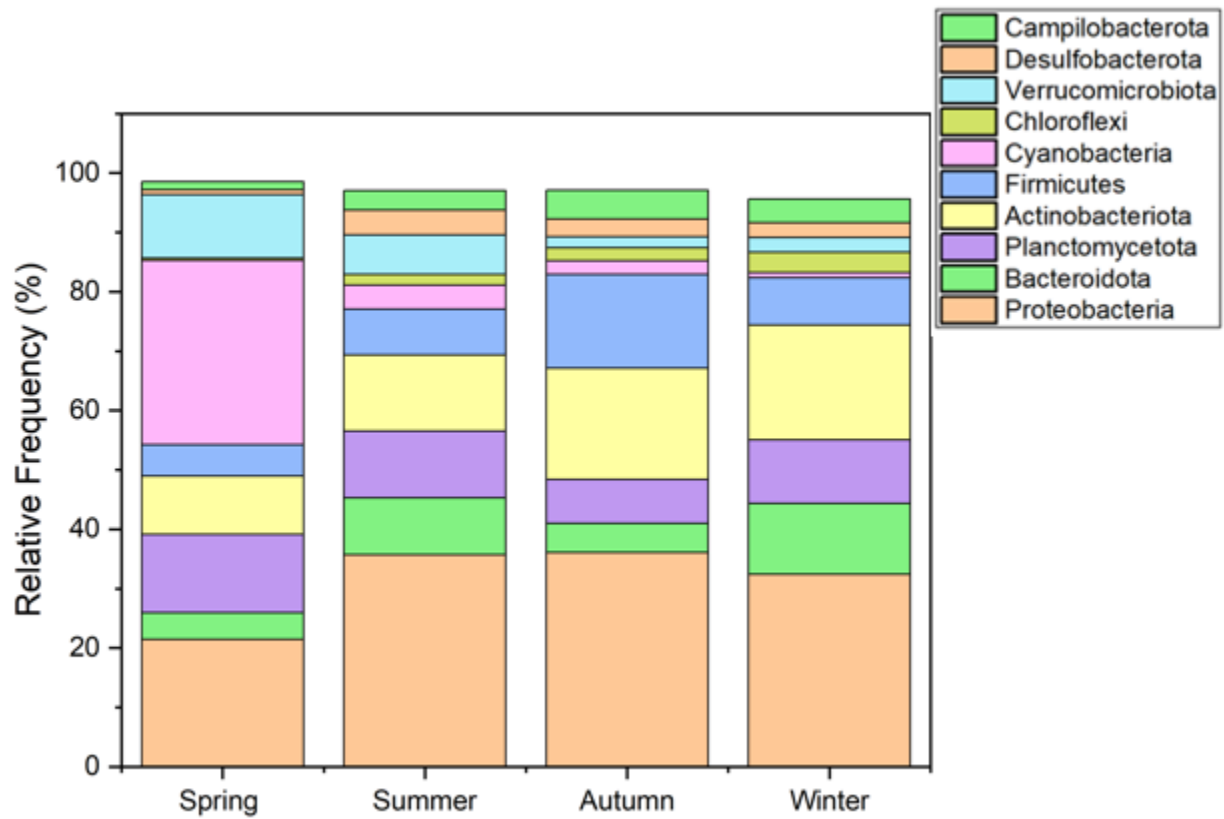


**Figure 3.5.** Relative frequency (%) of the ten most abundant phyla present amongst the four seasons using 16S sequencing.

The definition used to describe a bacterial biofilm is 'surface-associated bacterial communities enclosed in a polymeric matrix' <sup>145</sup>. The most dominant phyla observed among all four seasons was that of *Proteobacteria*, with very little shift in relative frequency (%) between seasons; Spring (21.43 %), Summer (35.69 %), Autumn (36.05 %) and Winter (32.37 %). A paper by Lee *et. al.*, <sup>145</sup> investigated biofilm communities in the initial stages of marine biofilm development on artificial surfaces, acryl, glass and steel. It was concluded that *Proteobacteria* were dominant among bacterial communities in the initial stages of development. Other papers by Lovell *et. al.*, <sup>146</sup> and Jones *et. al.*, <sup>147</sup> used molecular techniques which found that  $\alpha$ -*Proteobacteria* were dominant in marine biofilms. The most abundant phyla detected in Spring is that of *Cyanobacteria* (31 %) and there is a stark decrease in this organism in Summer (4.04 %), Autumn (2.30 %) and Winter (0.85 %). *Cyanobacteria* represents one of the initial colonizing organisms for the formation of a biofilm and are found in a wide range of habitats from soil, rocks, marine and freshwaters <sup>148</sup>, with a significant role in the synthesis of cyanobacterial EPS – providing a stable, hydrated and protected microenvironment for the developing film<sup>133,149</sup>. An increase in relative frequency in *Verrucomicrobiota* was observed in Spring (10.72 %), a gram negative bacteria indicative of Spring algae blooms <sup>150</sup>.

Figure 3.6 below illustrates the relative frequency (%) of the ten most abundant phyla present amongst the four seasons using 18S sequencing for determination of eukaryotic organisms. The 18S rRNA gene was successfully amplified and processed using a customized bioinformatics pipeline based on the QIIME2 package, and assignment to Level 2 (Phylum) level were determined. Generally, the relative frequency (%) of the most common phyla identified were diverse between seasons. During dry fouling season (Spring – Summer), *Arthropoda* were identified as the dominant phyla with a relative frequency of 32.82 % in Spring and a further increase to 57.91 % during the summer fouling months. Arthropods are invertebrate animals ranging in variety of forms such as lobsters, crabs, crayfish and barnacles <sup>151</sup>. It is worth noting that microscopy studies (SEM) did not identify visible Arthropods (i.e., barnacles) at this point in the study. Therefore, this could be an indicator of the early development (i.e., seven larval stages) of a marine barnacle previously described in Chapter 3 <sup>152</sup>. The phyla, *Chlorophyta*, is an indicator of green algae, a very diverse group of algae, found in most freshwater lakes, soil, marine, brackish and terrestrial surroundings. *Chlorophyta* are photosynthetic organisms, containing the green pigment chlorophyll, and can be anything from *Chlorophyceae* (mainly freshwater species) to sea lettuce <sup>153</sup>. Macrofouling organisms were evident in Autumn with an increase in the frequency of *Tunicata* (22.39 %), a marine invertebrate animal found in saltwater habitats<sup>149</sup>. This correlates well with visual inspection of the panels and under the microscope. The study did not identify any of the diatom genera identified through scanning electron microscopy studies detailed below in Table 3.5. This is due to the complexity of identifying the genes associated with some of the diatom genera, which means that they are not included in the sequencing database

used in this procedure. This provides a good reasoning for the use of scanning electron microscopy for the identification of the most commonly found diatom species in Irish waters.

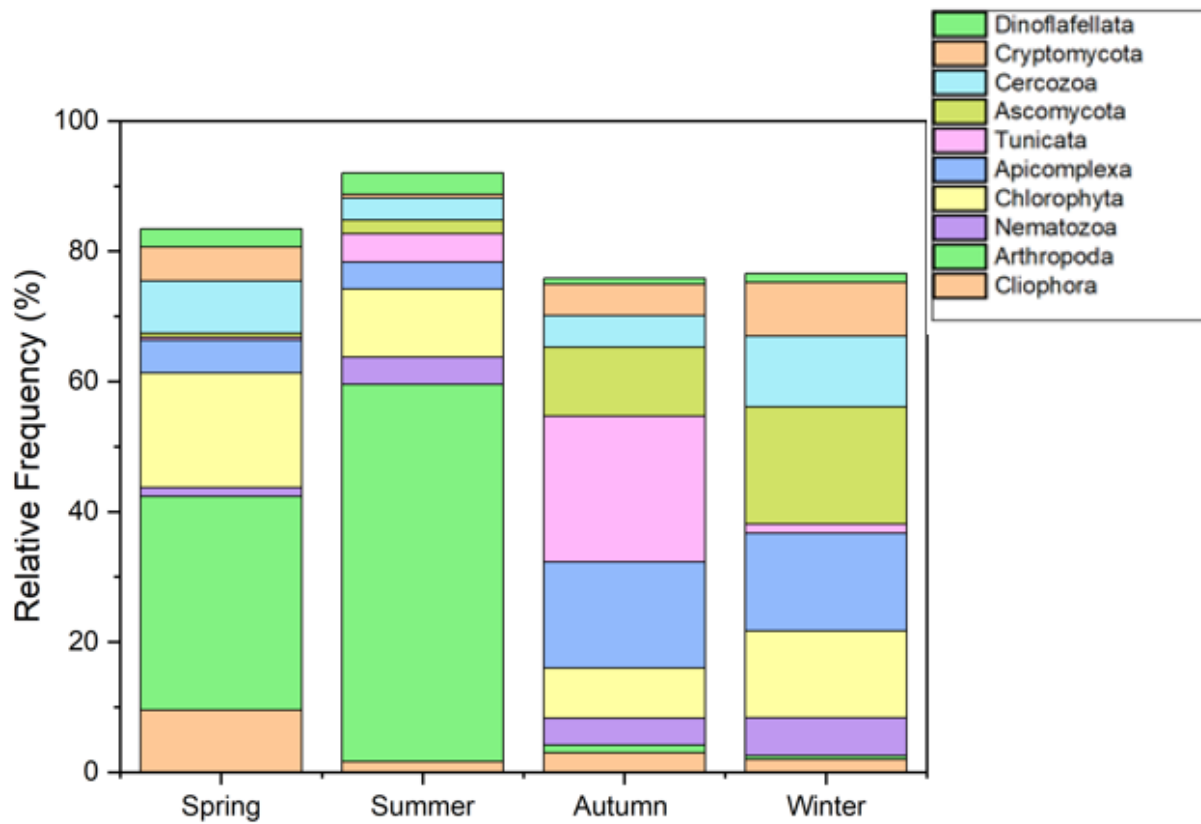


**Figure 3.6.** Relative frequency (%) of the ten most abundant phyla present amongst the four seasons using 18S sequencing.

Fouling organisms were identified to genus level using scanning electron microscopy and a diatom identification key<sup>133</sup>. The diatom genus, *Cocconeis* and *Navicula*, were identified as the most dominant appearing in the film from May to December 2020. In a paper by Regan *et. al.*, *Cocconeis* was among the most dominant genus identified settling on a series of novel transparent sol-gel coatings in Galway Bay <sup>154</sup>. It has been said that biofilm diversity can be a characteristic of the environment and time of year <sup>154</sup>. In this study, there was low species diversity overall, with most genera occurring on the slides repeatedly from month to month. The most commonly occurring diatoms were that of *Amphora*, *Cocconeis*, *Navicula* and *Minidiscus*, which is consistent with reports from those identified in Galway Bay <sup>154</sup>. However, with low species diversity, comes the advantage of designing AF materials to combat the adhesion strategy of specific biofouling organisms and its impact should not be overlooked.

Using molecular methods (18S), eukaryotic communities were identified to phylum level. The observation of *Arthropoda* and *Tunicata* through the seasons correlated well with what was observed using a combination of visual inspection and scanning electron microscopy (SEM). However, phyla of the class, Bacillariophyceae, responsible for diatoms, were not identified using 18S sequencing – which makes an argument for the use of microscopy techniques in the identification of a diatom genus. The identification of a common diatom found species found in Irish coastal areas for example *A. coffeaeformis*, also informs on the selection of a commonly found diatom for use in laboratory testing for AF materials.

**Table 3.5.** Observed possible diatom genus on glass slides deployed for 12-months in Dublin Bay (+ = Present) \*Samples could not be collected in M1 (April) due to COVID-19 restrictions.

| Label | Genus/Month            | May | June | July | Aug | Sept | Oct | Nov | Dec | Jan | Feb | Mar |
|-------|------------------------|-----|------|------|-----|------|-----|-----|-----|-----|-----|-----|
| 1     | <i>Amphora</i>         | +   | +    | +    |     |      |     |     |     |     |     |     |
| 2     | <i>Navicula</i>        | +   | +    | +    | +   | +    | +   | +   | +   |     |     | +   |
| 3     | <i>Cocconeis</i>       | +   | +    | +    | +   | +    | +   | +   | +   |     |     | +   |
| 4     | <i>Minidiscus</i>      | +   |      | +    | +   | +    | +   |     |     |     | +   | +   |
| 5     | <i>Melosira</i>        | +   | +    | +    | +   | +    | +   | +   |     | +   |     |     |
| 6     | <i>Thalassiosira</i>   | +   |      |      | +   | +    | +   | +   |     |     |     |     |
| 7     | <i>Fragilaria</i>      | +   |      |      | +   | +    |     |     |     |     |     |     |
| 8     | <i>Gonphonema</i>      |     |      |      |     |      | +   |     |     |     |     |     |
| 9     | <i>Coccolithophore</i> | +   |      |      |     |      |     |     |     |     |     |     |
| 10    | <i>Cymatopleura</i>    |     | +    |      |     |      |     |     |     |     |     |     |
| 11    | <i>Skeletonema</i>     |     |      |      |     |      | +   | +   |     |     | +   |     |
| 12    | <i>Gyrosigma</i>       |     |      |      |     |      |     | +   |     |     |     |     |

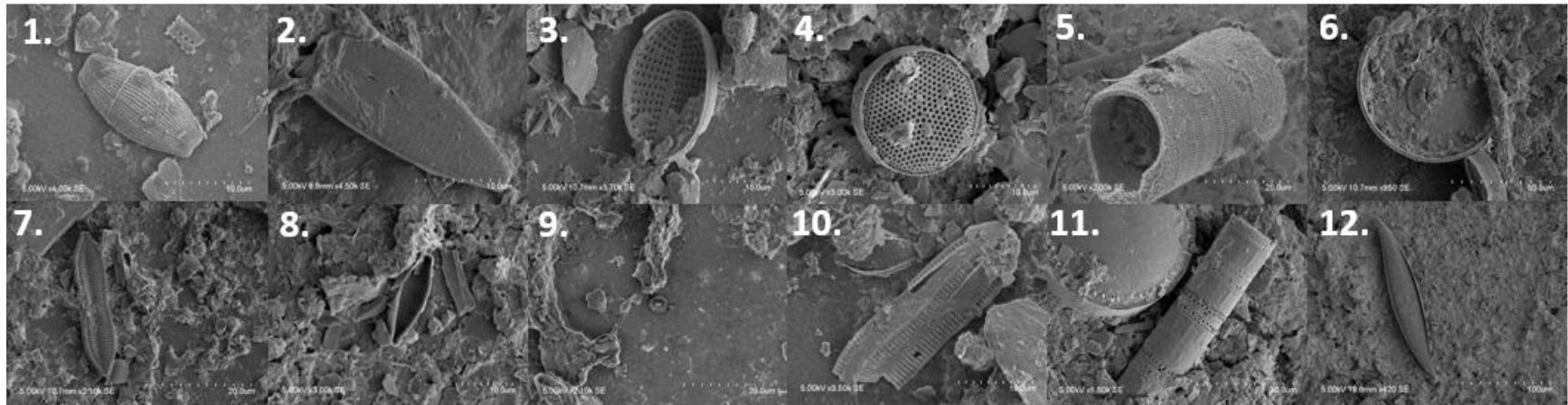


**Figure 3.7.** SEM micrographs showing the most commonly found diatom genera in the 12-month case study at Poolbeg Marina, Dublin Bay (1: *Amphora*; 2: *Navicula*; 3: *Cocconeis*; 4: *Minidiscus*; 5: *Melosira*; 6: *Thalassiosira*; 7: *Fragilaria*; 8: *Gonphonema*; 9: *Coccolithophore*; 10: *Cymatopleura*; 11: *Skeletonema*; 12: *Gyrosigma*).

### 3.5 Conclusion

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The aim of this work has been to characterize the development of a biofilm over a 12-month period in order to gather information on biofilm composition and diversity in Dublin Bay. To the authors knowledge, this study was the first long-term characterization of biofouling carried out at Poolbeg Marina in Dublin Bay. This study helped inform on the most common benthic diatom communities present in Dublin Bay, which will be a site for the deployment of future novel antifouling technologies reported in this thesis (Chapter 5 & 6).

The influence of environmental conditions (temperature, light irradiance, location) play a significant role in biofilm composition and diversity, with numerous reports found in the literature as mentioned in Section 4.1<sup>126,127,129,155</sup>. A significant increase in protein absorption was noted in October, in comparison to protein concentrations observed from May - September. However, in later months (December 2020 – March 2021), the significant reduction in seawater temperature was noted to decrease protein concentration in the biofilm matrix. This is consistent with studies previously mentioned by Briand *et. al.*, where environmental conditions affected biofilm diversity<sup>131</sup>. A similar response was observed in the quantification of carbohydrate, where protein absorption was highest in December. Protein and Carbohydrate absorption was lowest in samples extracted in May, which could be an indicator of reduced fouling community adhesion in the initial development of a biofilm.

DNA sequencing carried out for both prokaryotic (16S) and eukaryotic (18S) communities. *Proteobacteria* and *Cyanobacteria* were the dominant phyla present in bacterial communities, consistent with reports in the literature of early film colonizers <sup>145</sup>. Green algae (*Chlorophyta*), *Tunicata* and *Arthropoda* were among the dominant organisms seen with 18S sequencing. Generally, diversity amongst fouling communities identified through electron microscopy was low, with most diatom genus' occurring repeatedly. Interestingly, this was also observed on surfaces deployed in Galway Bay <sup>154</sup>, with *Amphora*, *Cocconeis* and *Navicula* dominant across the 12-month study. Diatoms were not identified at the phylum level as described previously using 18S sequencing and due to this, could not be compared to the genus' observed with electron microscopy. However, the appearance of Arthropods and Tunicates were consistent with those found and during visual inspection on-site at Dublin Bay.

# Chapter 4:

Assessment of the antifouling potential of novel transparent sol-gel coatings for application in the marine environment

## 4.1 Introduction

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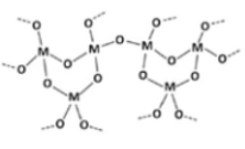
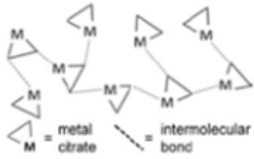
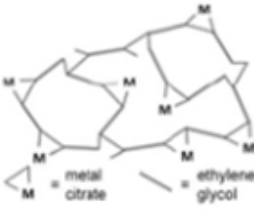
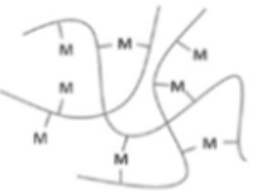
In 2008, with the ban on the use of antifouling (AF) coatings containing tributyl tin (TBT) by the International Maritime Organization (IMO) (as described in Chapter 1), the search for environmentally friendly, clean and effective AF coatings became increasingly important. Since then, sol-gel chemistry gained traction as a means of creating a low fouling surface (due to the physical and chemical properties of silica-based coatings and hybrid – inorganic – organic silicas. A sol-gel based approach to antifouling coatings has many advantages including low curing temperatures, improved chemical and physical stability, transparency (for application to optical devices) and application ease <sup>156</sup>. A sol is defined as ‘a stable suspension of colloidal particles (nanoparticles) in a liquid’. The particles in a sol can be of a crystalline or unstructured nature, with dense, porous or polymeric structures. A gel is described as a ‘porous, 3-D continuous solid network surrounding and supporting a continuous liquid phase’ <sup>157</sup>.

Sol-gel chemistry originated in the 19<sup>th</sup> century. It was observed that a gel was formed from the interaction of an alkoxide (prepared from SiCl<sub>4</sub>) with air. This gel formation was later found to have occurred due to atmospheric moisture causing hydrolysis of the silicon alkoxide followed by condensation. A gel can be formed in a variety of ways – even the same precursors can cause differences in gel structure with the slightest change in conditions. In 1974, Flory *et. al.*, first classified gels into different groups. These were (i) ordered; (ii) lamellar (i.e., clays, surfactant mesophases); (iii) covalent polymer networks; (iv) networks of physically aggregated polymers (i.e., hydrogels) and (v) disordered particulate gels. However, in 1996, a universal classification of the different gel types was given by Kakihana (Table 4.1) <sup>13</sup>.

The sol-gel process can be summarized as follows:

1. The sol-gel process begins with hydrolysis and condensation of alkoxides which allows for the synthesis of the sol <sup>13</sup>.
2. The gel is produced by a polycondensation reaction to form metal-oxo-metal or metal-hydroxy-metal bonds <sup>13</sup>.
3. Following this, an ageing process occurs whereby condensation continues through the gel network (causing the gel to shrink and solvent to be dispersed) <sup>13</sup>.
4. Drying of the gel to form either an xerogel (due to the collapse of the porous network) or an aerogel (via supercritical drying) <sup>13</sup>.
5. The last step can involve calcination whereby surface M-OH groups are removed at high temperature (i.e., 800 °C) (if required) <sup>13</sup>.

**Table 4.1.** Classification system given for the five different groups of gels found in sol-gel chemistry according to Kakihana (1996) (Reproduced from <sup>13</sup>).

| Type of gel                                                                | Bonding                                                                  | Source                                                                                                                                        | Gel schematic                                                                         |
|----------------------------------------------------------------------------|--------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| Colloidal                                                                  | Particles connected by Van der Waals or hydrogen bonding                 | Metal oxide or hydroxide sols                                                                                                                 |    |
| Metal-oxane polymer                                                        | Inorganic polymers interconnected via covalent or intermolecular bonding | Hydrolysis and condensation of metal alkoxides i.e., SiO <sub>2</sub> from tetramethyl orthosilicate                                          |    |
| Metal complex                                                              | Weakly interconnected metal complexes                                    | Concentrated metal complex solution i.e., aqueous metal citrate or ethanolic metal urea – often form resins or glassy solids rather than gels |    |
| Polymer complex I<br><i>In situ</i> polymerizable complex (Pechini method) | Organic polymers interconnected by covalent and coordinate bonding       | Polyesterification between polyhydroxy alcohol (i.e., ethylene glycol) and carboxylic acid with metal complex (i.e., metal-citrate)           |   |
| Polymer complex II<br>Coordinating and crosslinking polymers               | Organic polymers interconnected by coordinate and intermolecular bonding | Coordinating polymer (i.e., alginate) and metal salt solution (typically aqueous)                                                             |  |

Sol-gel derived functional coatings are commercially available for many practical applications (including self-cleaning, anticorrosion, antireflection etc.)<sup>158</sup>, and are emerging as suitable non-toxic alternatives to biocidal antifouling coatings<sup>9,159</sup>. The sol-gel process provides mild synthetic conditions and the capacity to include inorganic and organic components at the nanometric scale. This results in coatings that are able to provide both strength and durability typical of inorganic polymers but also a wide range of versatility typical of organic polymers<sup>158</sup>. The physicochemical properties and functionality of the final coating can be designed to meet various requirements by using metallic and organic precursors. Organic modified silicate (ORMOSIL) coatings<sup>160–162</sup> or metal nanoparticle doped ORMOSIL<sup>163</sup> are hybrid inorganic-organic silica coatings that have been shown to reduce biofouling. Sol-gel glassy coatings are transparent and can be effectively applied to submerged optical windows but also to other surfaces for self-cleaning applications<sup>164</sup>. Other attractive features of ORMOSIL coatings include the ease of preparation (i.e., one pot or step synthesis<sup>165</sup>), reactivity with a multitude of surfaces (i.e., glass, fiberglass, metal, wood etc.), low curing temperatures, prolonged chemical and physical stability and ease of application (i.e., spraying, dip coating, spin coating). There are three main strategies used in the design of antifouling coatings. Fouling-resistant coatings prevent the attachment of biofoulants to surfaces. Fouling-release treatments reduce biofoulant adhesion strength facilitating the removal of organisms from surfaces. Biocidal biofouling treatments kill organisms responsible for the formation of biofilms via the use of antimicrobials within the coating matrix<sup>165</sup>.

Fouling release surfaces rely on reduced adhesion of biofoulants and the least favourable surface energy for bioadhesion in microfouling is between 20 – 25 mN m<sup>-1</sup> <sup>166</sup>. In this region of the Baier curve (Figure 4.1), bioadhesion is minimal due to the formation of weak interactions between surface and EPS (i.e., proteins) <sup>9,166,167</sup>. Biofouling however involves a complex biochemical process, in which microfoulers (i.e., diatoms) adhere to surfaces via hydrophilic proteins<sup>168</sup> while macrofoulers (i.e., barnacles) adhere via hydrophobic proteins.

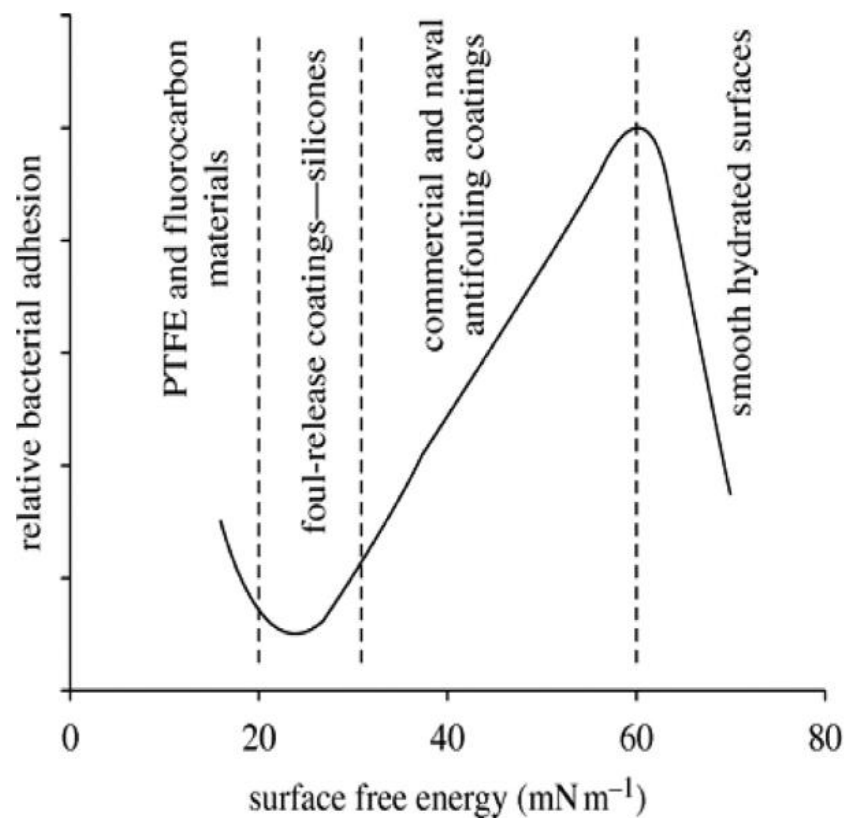


**Figure 4.1.** The Baier Curve (Reproduced from <sup>9</sup>).

For example, in a paper by Finlay *et al.*, fewer barnacles colonized hydrophobic xerogel coatings while the removal percentage of diatoms decreased with surface wettability <sup>162</sup>. Similarly, fouling release of juvenile barnacles has been achieved by using low wettability and low critical surface tension xerogel surfaces <sup>168</sup>. An increase in protein adsorption was noticed for superhydrophobic sol-gel coated surfaces with micrometer scale roughness when compared with smooth surfaces, however exposure to shear stress removed a larger proportion of protein from the superhydrophobic, rough surfaces than from the smooth surfaces <sup>169</sup>.

The marine environment poses a challenge to all deployed structures and biofouling affects all items that are deployed in the aquatic environment. In environmental sensing applications, optical sensors are impacted within hours of deployment by biofilm growth (Figure 4.2). Transparent materials used in sensing such as optical windows are prone to fouling that can impact data integrity. Therefore, it is necessary to employ countermeasures to reduce or remove biofilms on optical windows. The focus of this work is to study a number of novel transparent sol-gel materials, to determine their effectiveness as antifouling coatings for potential application to marine deployed sensors, camera lenses, solar panels, or other related technologies <sup>10,170</sup>.

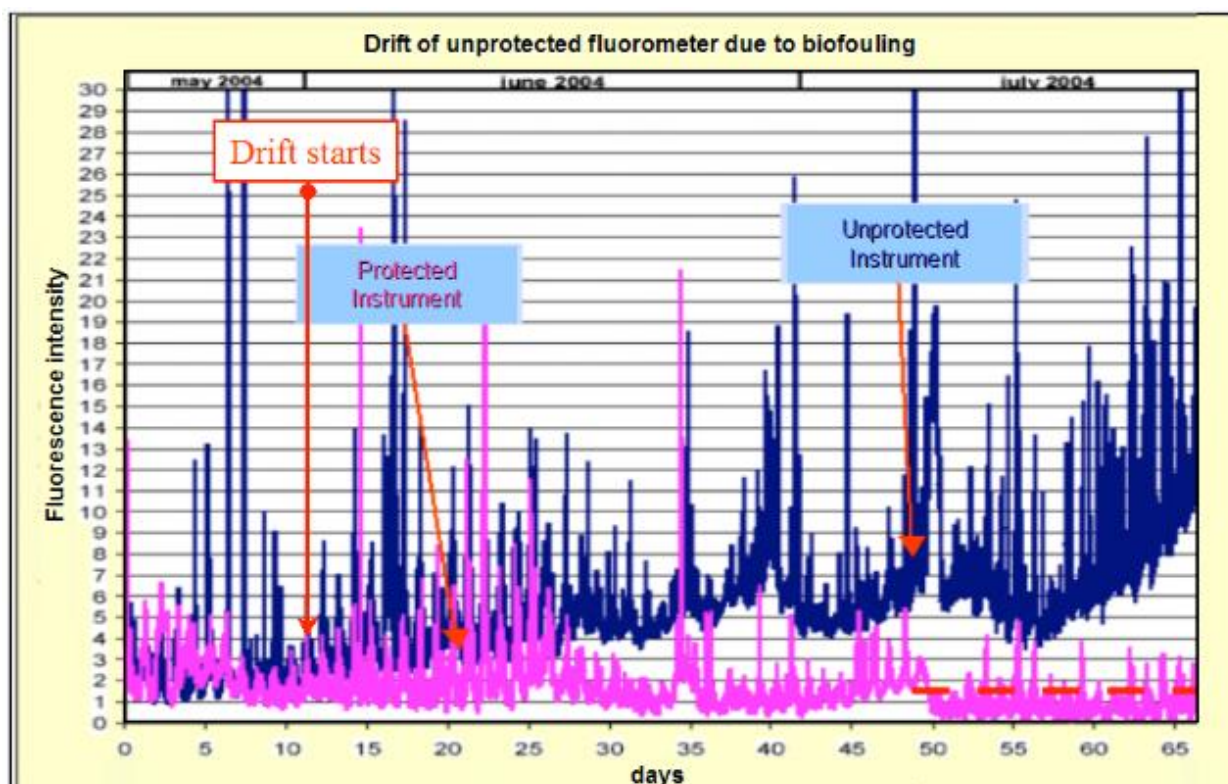


**Figure 4.2.** This graph illustrates the drift in sensor readings of an unprotected fluorometer due to marine biofouling (Reproduced from <sup>10</sup>).

In this context, a range of sol-gel coatings with increasing water surface wettability and roughness were developed. The surface energy of antifouling coatings was altered by modification of surface chemistry, while surface topology was roughened by the incorporation of amorphous fumed silica within the sol-gel. Sol-gel coatings with a selected range of surface wettabilities and topologies were deployed into a marine system in Galway Bay for periods of nine and thirteen months. A long-term initial study in the marine environment was performed to look at the colonization of diatom species to the sol-gel coatings, based on their surface chemistries and topologies and offer a comparison of performance against industrial antifouling technologies.

## 4.2 Aims and objectives

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The aim of this chapter is to investigate the potential of using chemistry by means of novel transparent or opaque ORMOSIL coatings for antifouling application to marine monitoring equipment.

The objectives include:

- To develop sol gel coatings for testing in the marine environment over a long time period.
- Characterization of the diversity of microfouling communities using scanning electron microscopy on prepared sol gel coatings against commercial AF paints deployed for 9-months.
- Determination of the abundance of fouling communities present on each of the deployed sol coatings and commercial paints by means of biochemical assays to determine protein and carbohydrate levels.
- Characterization of the abundance of macrofouling communities (i.e., barnacles) using digital imagery and image analysis software ImageJ on prepared sol coatings and commercial paints deployed at an Irish site for 13-months.

### 4.3 Materials and methods

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The methods used here are outlined in Chapter 2. Sol gel coatings were synthesized as discussed in Chapter 2, Section 2.9 using a number of sol gel precursors (Table 4.2). Sol gel coatings were characterized using scanning electron microscopy (SEM) (Hitachi S3400-N SEM). Samples were Au-coated (Agar Scientific) for 30 s followed by examination at 20 kV (Section 2.8.1). Surface wettability measurements were taken by means of a sessile drop technique using the FTA200 Dynamic Contact Angle Analyzer (FTA, USA) (Section 2.8.4). Surface roughness measurements were carried out using the Bruker Contour GT Profilometer (Section 2.8.5). Biofilm-covered slides were characterized for protein content using the Lowry Protein Assay (Section 2.7.1). Mean percentage cover (%), barnacle number and nearest neighbour (NN) distances were calculated for macrofouling coverage using Fiji (ImageJ) software as described in Section 2.13. Statistical analysis was completed as outlined in Section 2.14.

## 4.4 Results and discussion

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### 4.4.1 *Characterization of developed coatings*

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Five organically modified silica (ORMOSIL) coatings were developed and characterized prior to deployment into the marine environment (Chapter 2, Section 2.9.1). Water contact angles (WCAs) measured (Chapter 2, Section 2.8.3) on the coatings ranged from 50° to 130°, confirming the increasing wettability of the surfaces from hydrophobic to hydrophilic (Figure 4.3). Surface roughness measurements using white light interferometry show a wide range of surface topographies were obtained, with average surface roughness ranging from 43 nm (HCOO6) to 1600 nm (B Sol) (Figure 4.4). SEM imaging of the coatings (as described in Chapter 2, Section 2.8.1) provides more insight into the topography and shows smooth, homogeneous dense films were formed with sols HCOO6 and DMDEOS respectively, while porous films were formed when FmS was incorporated within the sol-gel network. Samples were gold coated for 30 s using a sputter coater before being imaged at an accelerating voltage of 20 kV. Coatings produced using HCOO6 and DMDEOS formulations generated robust, dense, and fully transparent films on glass substrates. DMDEOS was previously developed in the group for self-cleaning applications and its physical characteristics (i.e., material flexibility, mechanical robustness, transparency etc.) assessed <sup>171</sup>. HCOO6 shows very similar physical characteristics with DMDEOS, with slightly higher WCAs (110 – 115°). As both sols show similar average roughness values, the increase in contact angle can be attributed to a higher density of methyl (-CH<sub>3</sub>) groups on the surface of the coating.

These groups were purposely introduced within the sol-gel by addition of another precursor, CTMS. This precursor has only one hydrolysable group (-Cl) and it acts as a capping agent, which terminates chain growth and adds surface functionality by lowering the surface free energy<sup>172,173</sup>. Furthermore, CTMS is known to have a positive effect on the mechanical properties of films by reinforcing weak points within the network and preventing drying induced cracks<sup>133</sup>. T<sub>2</sub> sol gel used for the preparation of T<sub>2</sub>/FmS CH<sub>3</sub> (1:1) and T<sub>2</sub>/FmS (5:3) is similar in precursor composition with HCOO6 (CTMS replaced with TMES and adjusted molar ratios, see methods, Chapter 2, Section 2.9) but optimized to incorporate more Si-OH reactive groups. This increase in reactivity was introduced to increase the adhesive potential with both the substrate and the FmS particles. The final coatings produced with this approach had an average roughness of 846 nm for T<sub>2</sub>/FmS (5:3) and 519 nm for T<sub>2</sub>/FmS CH<sub>3</sub> (1:1), which is 1 order of magnitude higher than the average roughness of HCOO6, DMDEOS. The methylation of FmS was found to have a radical effect on the wettability of the coatings<sup>174–176</sup>, with a shift from hydrophilic (WCA = 50°) to hydrophobic (WCA = 130°).

**Table 4.2.** Chemical name, structure, CAS number and molecular weight ( $M_w$ ) for each of the sol-gel precursors.

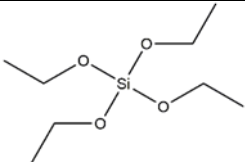
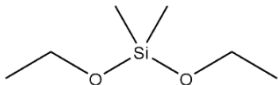
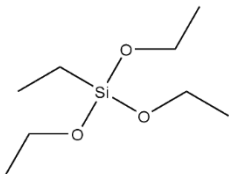
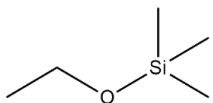
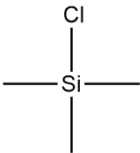
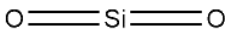
| Chemical name                     | Structure                                                                           | CAS         | $M_w$ (g/mol) |
|-----------------------------------|-------------------------------------------------------------------------------------|-------------|---------------|
| Tetraethyl orthosilicate (TEOS)   |    | 78-10-4     | 208.33        |
| Diethoxydimethylsilane (DMDEOS)   |    | 78-62-6     | 148.28        |
| Triethoxy(ethyl)silane (ETEOS)    |    | 78-07-9     | 192.33        |
| Ethoxytrimethylsilane (TMES)      |   | 1825-62-3   | 118.25        |
| Chlorotrimethylsilane (CTMS)      |  | 75-77-4     | 108.64        |
| Silica fumed (FmS, 0.007 $\mu$ m) |  | 112945-52-5 | 60.08         |
| Hydrochloric acid (HCl, 37%)      |  | 7647-01-0   | 36.46         |

**Table 4.3.** Robustness, transparency observations and water contact angle (WCA) measurements for each of the sol gel coatings used in this study (n = 3).

| Sol name                                     | Coating application | Ageing time | Transparency | Robustness | Mean WCA (°) | Mean SD |
|----------------------------------------------|---------------------|-------------|--------------|------------|--------------|---------|
| B Sol                                        | Spin                | 48 h        | ST           | Good       | 81           | 12.29   |
| DMDEOS                                       | Spray               | 13 d        | ST           | Good       | 100          | 1.47    |
| HC006                                        | Spin                | 90 h        | T            | Good       | 111          | 14.37   |
| T <sub>2</sub> /FmS (5:3)                    | Spray               | 48 h        | ST           | Medium     | 53           | 9.69    |
| T <sub>2</sub> /FmS CH <sub>3</sub><br>(1:1) | Spray               | 48 h        | O            | Good       | 130          | 3.41    |

**Legend:** ST = Semi-transparent; T = Transparent; O = Opaque

Two methods of application were used to apply the novel transparent sol-gel coatings onto glass microscope slides (75 x 25 mm): spin coating and spray coating. As detailed in Chapter 2 (Section 2.9.2), DMDEOS and HC006 sol-gels were spin coated using a Laurell WS-650-23 spin coater at 500 rpm. Sol-gels, B Sol, T<sub>2</sub>/FmS (5:3) and T<sub>2</sub>/FmS CH<sub>3</sub> (1:1) were spray coated for 10 s using a circular motion followed by heating at 110 °C. Robustness was assessed by means of scratch/abrasion test using a pencil hardness tester, Elcometer 501. The transparency of sol-gel coatings was assessed using an ocean optics USB2000+ spectrometer, an ocean optics LS1 tungsten halogen lamp (emission between 360 – 2000 nm) and a detector (QP1000-2-UV-Vis 1 mm internal diameter and 2 m length optical fibers) as described in previous work carried out in the group <sup>171</sup>.

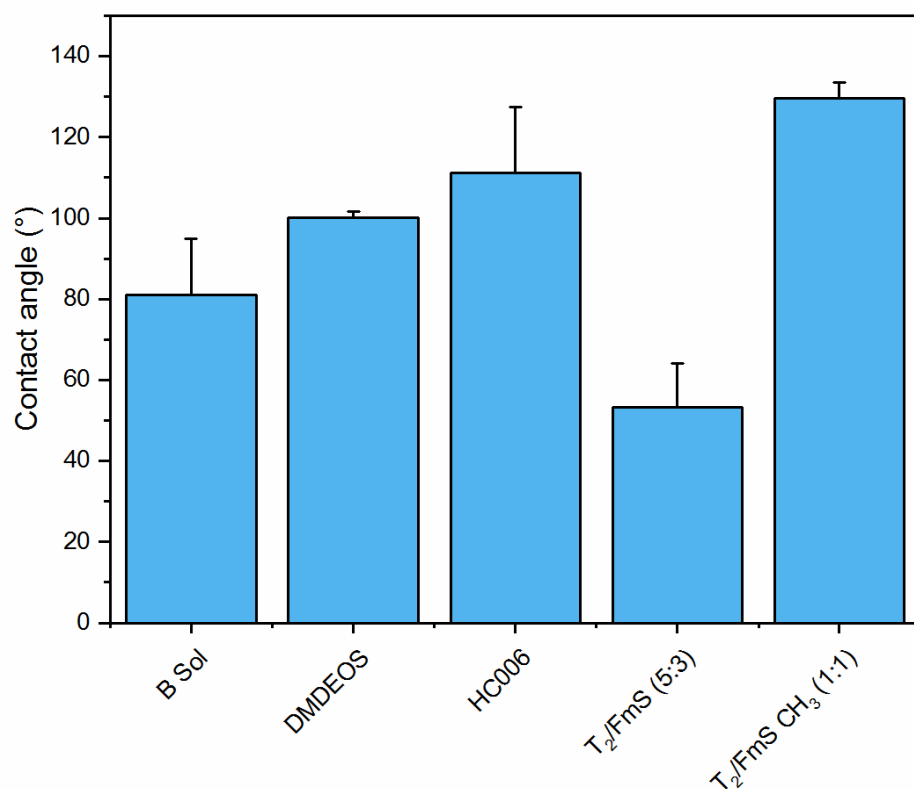


**Figure 4.3.** Mean Water Contact Angle (WCA) measurements taken for each of the sol gel coatings before deployment in Galway Bay, Ireland. Error bars represent 95 % confidence intervals (n = 3).

Surface roughness measurements were carried out using the Bruker ContourGT profilometer as described previously. The nm measurements of surface roughness presented reflect the average height of the features of a surface as the stylus is dragged across the surface, determining its roughness.

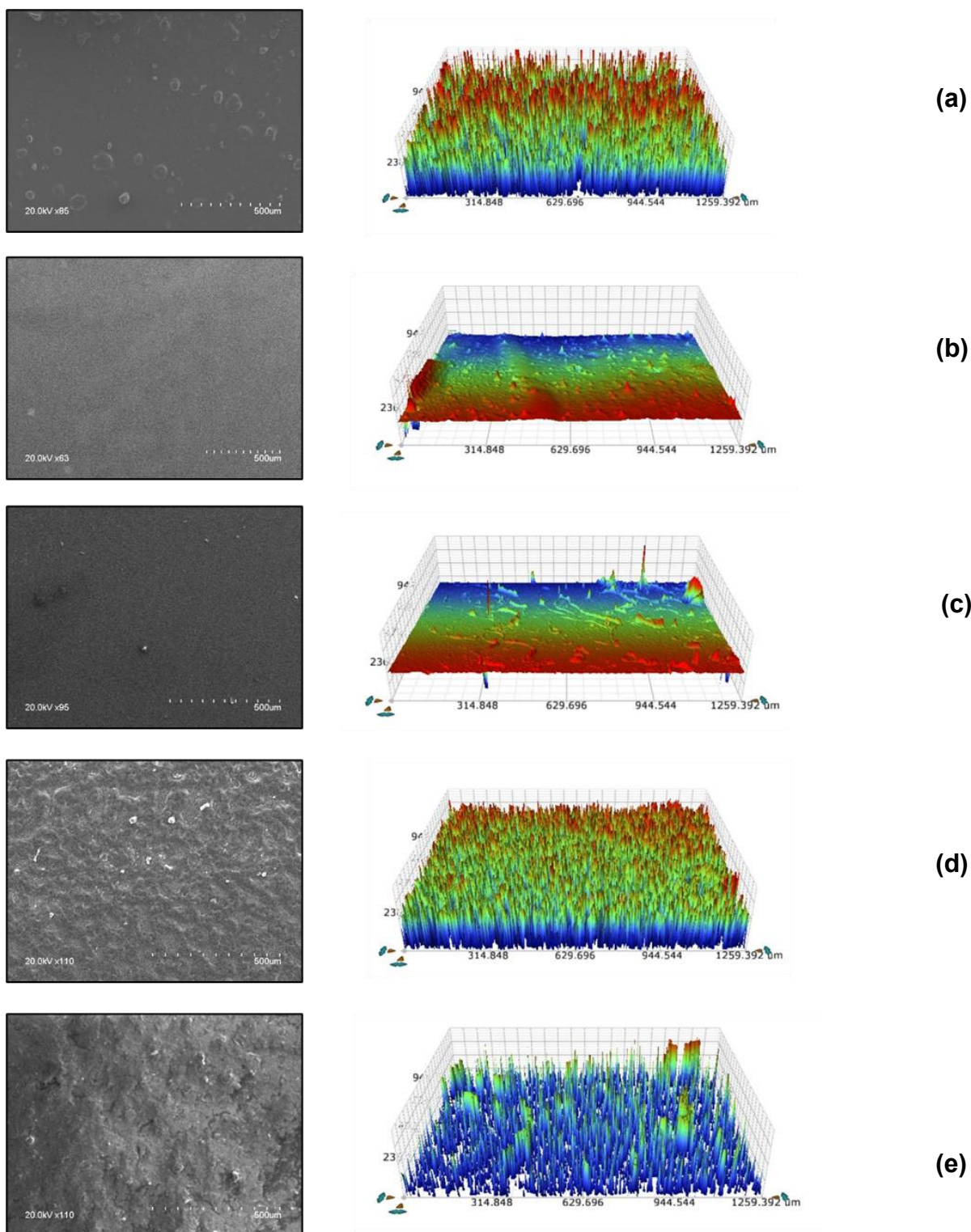


**Figure 4.4.** Surface characterization of the sol gel coatings with a profilometer (right) and SEM technique (left) (x 20.0 k, 500  $\mu\text{m}$ ). Average surface roughness ( $S_a$ ) of the coatings was recorded. (From top down (A – E); B sol ( $S_a = 1624.359 \text{ nm}$ ), DMDEOS ( $S_a = 0.068 \mu\text{m}$ ), HC006 ( $S_a = 43.517 \text{ nm}$ ),  $T_2/\text{FmS}$  (5:3) ( $S_a = 846.343 \text{ nm}$ ) and  $T_2/\text{FmS}$   $\text{CH}_3$  (1:1) ( $S_a = 519.934 \text{ nm}$ ) ( $n = 3$ ))

#### 4.4.2 Biofouling assessment

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The effects of different sol-gel coatings on biofilm build-up were assessed. Figure 4.5 shows the deployment set-up, where 10 replicate slides of each material were fixed. Figure 4.5 (a) shows the labelled slides, and Figure 4.5 (b) the panel before deployment. Figure 4.5 (c) and (d) shows panels retrieved at 9 months and 13 months respectively.

As can be seen from Table 3.3 below, a wide range of fouling organisms, marine benthic diatoms, were found on the slides deployed for nine months. Diversity is a good indicator of antifouling effectiveness but is also characteristic of the environment and time of year. The lack of diversity on deployed panels may indicate that this AF solution is effective at preventing certain organisms from adhering to a surface <sup>127</sup>. The European Chemical Agency (2018), consider antifouling paints under static immersion successful if they show macrofouling coverage of < 25 % <sup>177</sup>. Considering this, all short-term (in this case, 9-mo) deployed sol-gel coatings show antifouling potential. It was found that there was a low genus diversity overall with most genus' listed occurring on the slides repeatedly (i.e., *Amphora*, *Cocconeis*, *Navicula*). Some of the same diatom genus' (i.e., *Amphora*, *Cocconeis*) were observed in diversity studies carried out in Dublin Bay (Chapter 3) using electron microscopy. *Cocconeis* was found on all materials except DMDEOS.

In microscopy studies, the novel coating DMDEOS was found to be the most effective coating demonstrating minimal biofilm adhesion, which was comparable with the commercial paint, Trilux. HCOO6, B Sol and T<sub>2</sub>/FmS (5:3) coatings were found to contain a large diversity of diatoms and in large numbers (Table 4.4).

Generally, there are two types of antifouling (AF) coatings, self-polishing coatings (SPCs) (i.e., containing booster biocides) and fouling release coatings (FRCs), containing physiochemical and mechanical effects <sup>178</sup>. SPCs contain biocides, which steadily release over time as long as the antifouling paint film is present on the surface.

This was observed in a study by Briand *et al.* <sup>129</sup> in 2014, which demonstrated that biocide-free coatings presented higher densities of microfouling organisms than those containing biocides. Therefore, the use of copper (Cu) and biocide-containing paints <sup>177</sup> could explain the low biofilm observed on Trilux in comparison to some of the other sol-gel coating.

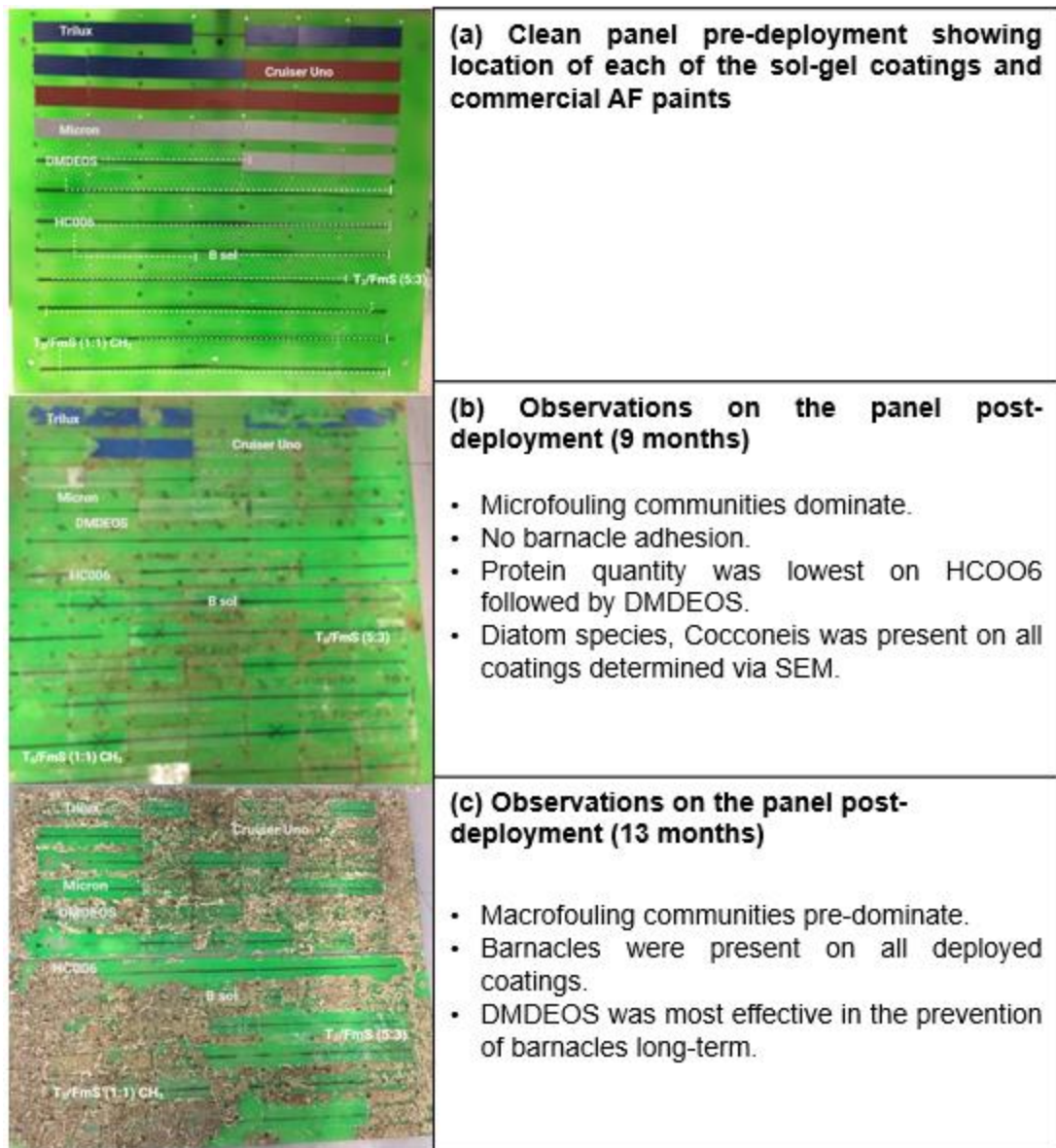


**Figure 4.5.** (a) Schematic of the layout of sol gel materials, (b) post-deployment biofouling following a 9-mo deployment from April 2017 – December 2018 during the height of the biofouling season (depth: 2.5 – 3 m), and (c) post-deployment biofouling after a 13-mo deployment from April 2017 – May 2018 including both dry and wet seasons (depth: 24 – 28 m).

**Table 4.4.** Possible observed diatom settlement on coatings deployed for 9 months in Galway Bay.

|    | Genus                  | B sol | DMDEOS | HC006 | T <sub>2</sub> /FmS (5:3) | T <sub>2</sub> /FmS (1:1)<br>CH <sub>3</sub> | Trilux |
|----|------------------------|-------|--------|-------|---------------------------|----------------------------------------------|--------|
| 1  | <i>Minidiscus</i>      |       |        | +     |                           | +                                            |        |
| 2  | <i>Coccolithophore</i> | +     |        | +     |                           | +                                            | +      |
| 3  | <i>Cocconeis</i>       | +     |        | +     | +                         | +                                            | +      |
| 4  | <i>Thalassiosira</i>   | +     | +      | +     |                           |                                              |        |
| 5  | <i>Sellaphora</i>      |       | +      |       |                           |                                              |        |
| 6  | <i>Proschkinia</i>     | +     |        | +     |                           | +                                            |        |
| 7  | <i>Navicula</i>        |       |        | +     | +                         |                                              |        |
| 8  | <i>Rhabdonema</i>      |       |        |       | +                         |                                              |        |
| 9  | <i>Asterionella</i>    |       |        |       | +                         |                                              |        |
| 10 | <i>Stephanopysis</i>   | +     |        |       | +                         |                                              |        |
| 11 | <i>Amphora</i>         | +     |        | +     | +                         |                                              |        |
| 12 | <i>Bleakeleya</i>      |       |        |       | +                         |                                              |        |

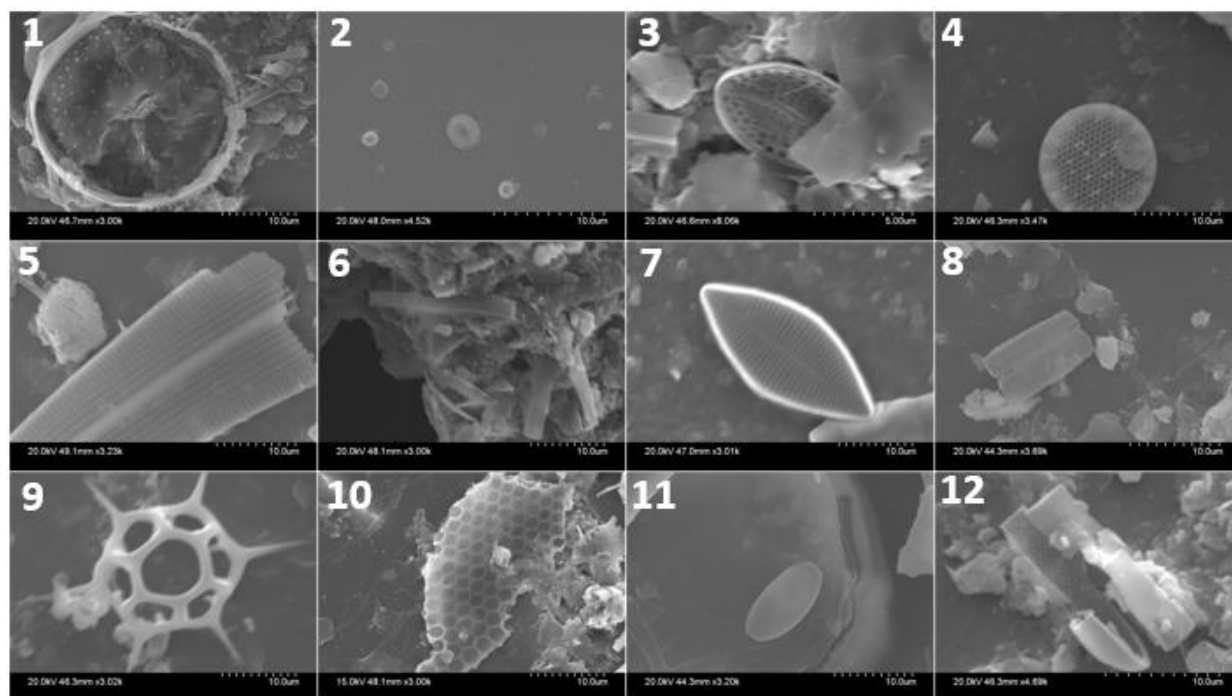


**Figure 4.6.** Observed scanning electron micrographs of possible diatom settlement on coatings deployed for 9-mo in Galway Bay (1: *Minidiscus*; 2: *Coccolithophore*; 3: *Cocconeis*; 4: *Thalassiosira*; 5: *Sellaphora*; 6: *Proschkinia*; 7: *Navicula*; 8: *Rhabdonema*; 9: *Asterionella*; 10: *Stephanopysis*; 11: *Amphora*; 12: *Bleakeleya*).

#### 4.4.2.1 Protein determination

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Determination of early fouling can be carried out in a number of ways. One of these is using a biochemical assay (i.e., Lowry Protein Assay) to determine protein associated with the exopolymeric substances associated with the initial biofilm (Figure 4.7) <sup>138,179</sup>. The results obtained from assessment of slides retrieved after 9 months deployment in Galway Bay indicate that the HCOO6 material had the lowest protein level, while B Sol had the greatest and was similar to T<sub>2</sub>/FmS (5:3). However, these results do not correlate well with the diversity data shown in Table 4.3, where a large variety of diatom species were observed settling on HCOO6. The latter, however, may be an indication of non-specific adhesion rather than biofilm attachment. Protein measurement is a better indicator of biofilm attachment <sup>154</sup>.

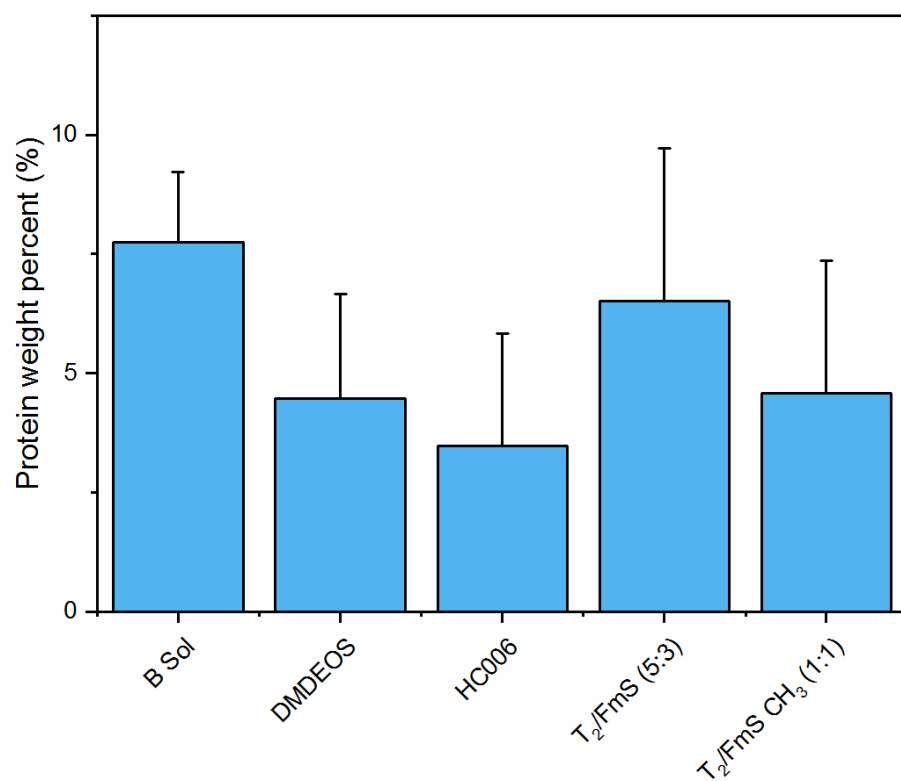


**Figure 4.7.** Protein determination using the Lowry assay carried out on the coated slides deployed in Galway Bay, Ireland for 9 months. The error bars represent 95 % confidence intervals.

#### *4.4.2.2 Assessment of macrofouling organism colonization*

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In the formation of biofilm there are many stages as illustrated in the schematic in Chapter 1 (Figure 1.1). Ideally coatings will lead to reduced fouling so that deployed devices do not require frequent maintenance. For the purpose of this study, the performance of novel sol-gel coatings is compared with commercial antifouling paints. Table 4.5 illustrates the loading of macrofouling organisms (i.e., barnacles) on materials deployed for 13 months. When evaluating the coatings deployed for 13-mo, it was found that macrofoulers predominate. Barnacle adhesion was present on all deployed coatings. The novel transparent coating DMDEOS was found to be most effective in preventing macrofoulers long-term followed by HCOO6 when compared with commercial paints Trilux, Micron and Cruiser Uno when looking at percentage barnacle coverage of the macro-fouled slides and using t-testing in Microsoft Excel 2016 (t-value of 0.76). There was no protein assessment completed of the coatings on the 13-mo study due to the build-up of macrofoulers. Figure 4.8 includes a subset of images of fouled materials and their analysis using ImageJ software. While it is clear that all materials support barnacle growth, the numbers are generally the same for all coatings. However, it is evident that some materials provide substrates that lead to a variety of settlement patterns. This provides useful information in understanding the chemistry and topography of effective coatings.

The results indicate that the lowest coating surface coverage of 24.97 %, occurs for the novel DMDEOS material, similar to commercial paint, Cruiser Uno, at the lowest paint surface coverage of 23.71 %. The highest surface coverage observed was with B sol at 56.78 %. However, more critically the data showing the nearest neighbour again supports the evidence that DMDEOS is effective in reducing macrofouling adhesion, with the fumed silica sol, T<sub>2</sub>/FmS (5:3) demonstrating an even distribution of barnacle settlement. This information regarding spacing and clustering of fouling organisms provides valuable evidence of the benefit of the new coatings, and the effectiveness of the differing chemistries of the materials leading to more effective antifouling. In the case of controlled adhesion in fumed silica sol (5:3), the average nearest neighbour is calculated to be 1.92, meaning that these organisms will not cluster and therefore the weight of fouling can be reduced. Where nearest neighbour values have higher averages, the degree of clustering is greater and therefore fouling is heavier.

**Table 4.5.** This table shows mean barnacle number, mean surface coverage (%) and mean nearest neighbour (NN) data for the macro-fouled coatings deployed for 13-mo (n = 2).

|   | Sol-gel/Coating                           | Barnacle Number | Mean Barnacle Area (mm <sup>2</sup> ) | Mean Biofilm Cover (%) | Mean Nearest Neighbour (mm) |
|---|-------------------------------------------|-----------------|---------------------------------------|------------------------|-----------------------------|
| 1 | B sol                                     | 218             | 9.77                                  | 56.78                  | 2.66                        |
| 2 | DMDEOS                                    | 157             | 5.96                                  | 24.97                  | 3.15                        |
| 3 | HC006                                     | 169             | 10.7                                  | 48.22                  | 3.12                        |
| 4 | T <sub>2</sub> /FmS (5:3)                 | 207             | 4.56                                  | 25.15                  | 1.92                        |
| 5 | T <sub>2</sub> /FmS (1:1) CH <sub>3</sub> | 200             | 7.22                                  | 38.51                  | 2.63                        |
| 6 | Cruiser Uno                               | 181             | 5.17                                  | 23.71                  | 2.35                        |
| 7 | Micron                                    | 161             | 7.72                                  | 33.14                  | 4.77                        |
| 8 | Trilux                                    | 179             | 10.38                                 | 49.57                  | 3.05                        |

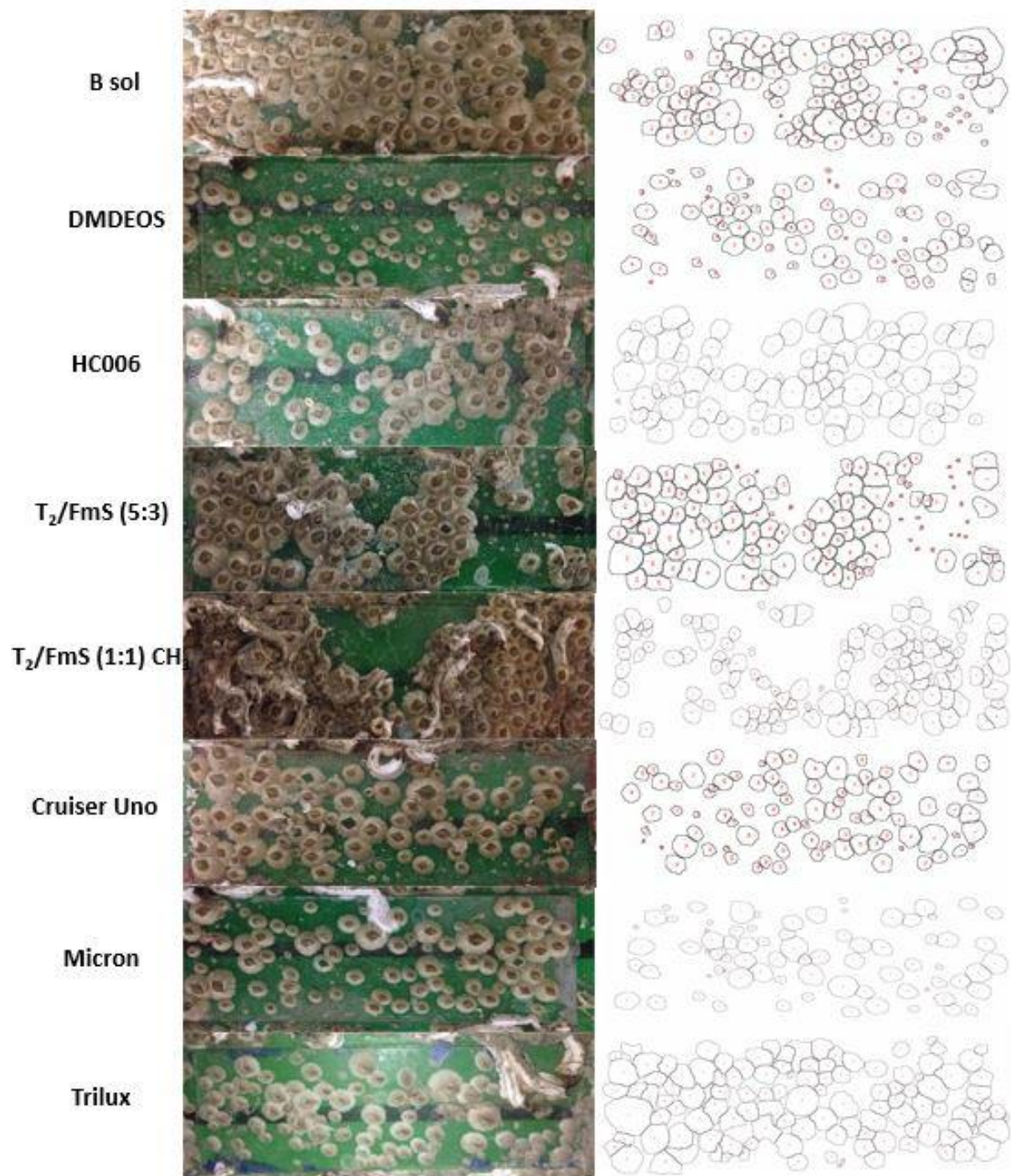


**Figure 4.8.** Examples of subsets of images of macro-fouled materials (left) (barnacles) and an outline of all counted barnacles determined in the analysis using Image J software (right) for determination of the nearest neighbour (NN) for each material ( $n = 3$ ).

Table 4.5 shows the detailed analysis data for determination of the nearest neighbour for each material. This data clearly shows the narrow range of values for  $T_2/FmS$  (5:3) compared with other materials. Even the commercial paints demonstrate clustering or aggregation of fouling organisms. This data was measured using ImageJ software. It is important to note that all coatings deployed in the macrofouling study showed barnacle and tubeworm colonization only. Barnacle colonization was the only organism accounted for in the calculation of mean surface coverage and mean NN data and no mixed communities (i.e., communities other than barnacles) were observed on the panels. It was expected that other macrofouling communities such as mussels and tunicates would be present alongside the barnacles.

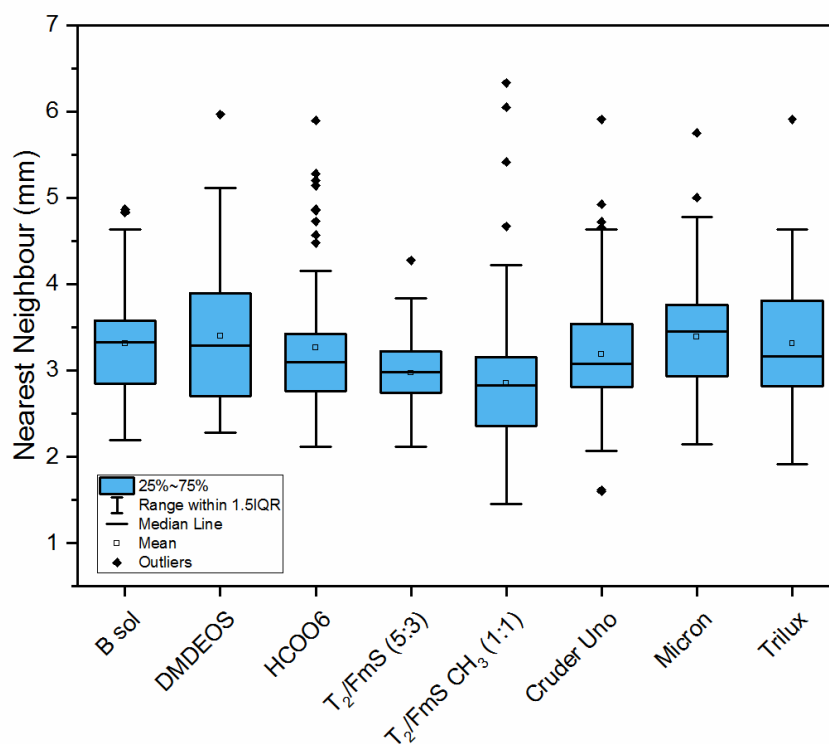


**Figure 4.9.** Nearest neighbour data for sol-gel coatings deployed in Galway Bay for thirteen months as part of the macrofouling study to test the overall effectiveness of our novel antifouling sol-gel coatings on a long-term basis ( $n = 2$ ). The average and median of each plate is shown as indicated in the figure. The bounds of the blue box show the proportion of data that is 25% above and 25 % below the median of the data (i.e., the distribution of the middle 50% of the data). If the top and bottom of the blue box are dissimilar sizes this indicates that the spread of values is skewed. The Interquartile range (IQR) is the size of blue box (middle spread of the data), and the whiskers are the upper and lower half of this box either added or subtracted from the IQR multiplied by 1.5 (showing the middle 75% of the data). Errors bars represent 95% confidence intervals.

Figure 4.10 shows the actual barnacle density data for each deployed coating, and Figure 4.11, the percentage coverage of the barnacles on each of the coatings. From this assessment, the overall settlement was found to be similar across all materials, but the format or pattern of adhesion is different on some materials. The most effective materials in this study arise from the T<sub>2</sub> sol-gel used for the preparation of T<sub>2</sub>/FmS (5:3). This is similar in precursor composition with HCOO6 (CTMS replaced with TMES and adjusted molar ratios, see methods section in Chapter 2 (Table 2.2)) but optimized to incorporate more Si-OH reactive groups. This increase reactivity was introduced to increase the adhesive potential of the coating, with both the substrate and the FmS particles. The final coatings produced with this approach had an average roughness of 846 nm for T<sub>2</sub>/FmS (5:3) which is 1 order of magnitude higher than the average roughness of HCOO6 and DMDEOS. The methylation of FmS was found to have a radical effect on the wettability of the coatings, with a shift from hydrophilic (WCA = 50°) to hydrophobic (WCA = 130°), with water rolling effect. From this study, it appears that a combination of roughness and wettability is most effective in these static field studies. In a study by Swain *et al.* the adhesion of diatoms to commercial coatings for ships under both static and dynamic conditions was investigated, whereby differences in community structure were observed<sup>180</sup>. Previous studies have examined variations in fouling communities under static conditions, but few have studied dynamic immersion<sup>3,19</sup>. The results concluded that hydrodynamic stresses significantly influenced the diversity of the microfouling communities observed on each of the commercial coatings<sup>180</sup>. It would be interesting to evaluate the effectiveness of the series of coatings tested in this study under dynamic flow conditions to see if a different response to fouling is observed.

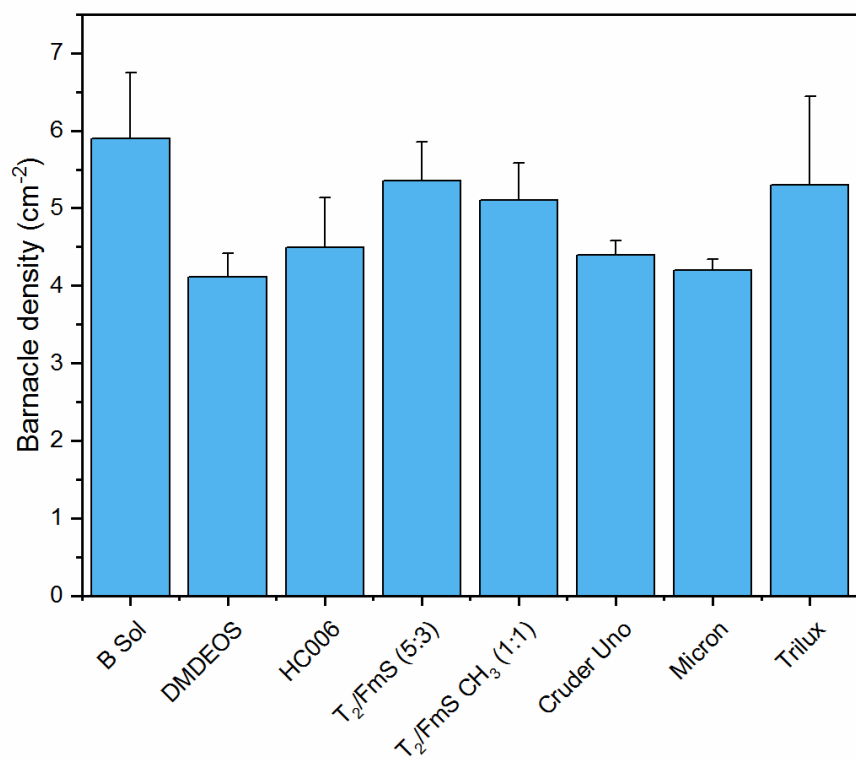


**Figure 4.10.** Barnacle attachment on sol-gel coatings deployed in Galway Bay for 13-mo as part of the macrofouling study to test the overall effectiveness of our novel antifouling sol-gel coatings on a long-term basis. Errors bars represent 95% confidence intervals (n = 2).

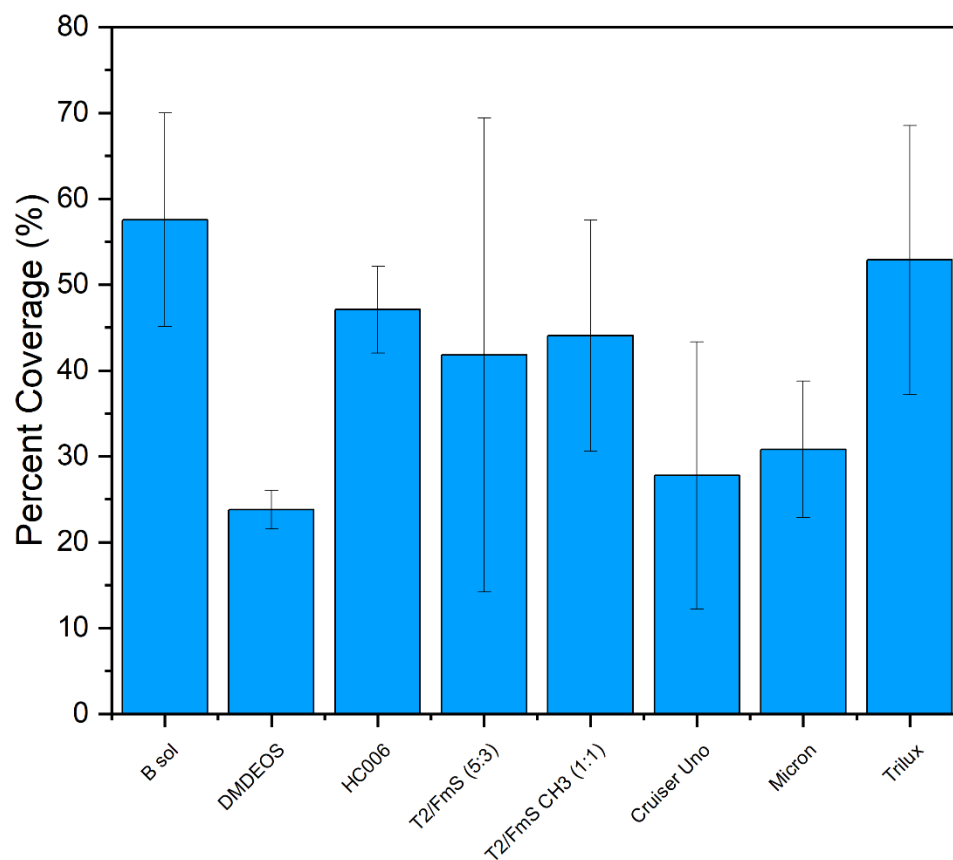


**Figure 4.11.** Percentage area surface coverage of barnacles attached to slides on sol-gel coatings deployed in Galway Bay for 13-mo as part of the macrofouling study to test the overall effectiveness of our novel antifouling sol-gel coatings on a long-term basis (n = 2).

## 4.5 Conclusion

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The purpose of this study was to investigate the antifouling potential of novel transparent sol-gel coatings deployed in the marine environment. Five novel materials were developed with varying chemistries leading to different hydrophobicities, wettability characteristics and roughness. The study extended greater than 1 year, with an initial evaluation of primary fouling (at 9 months), followed by a study of macrofouling (4 months later). It was found that materials that showed greatest diversity of organism colonization in the initial stages, did not lead to the heaviest biofilm (in terms of protein levels). However, there is no consistent pattern. This was the first time these developed sol-gel coatings had been tested in field studies and as a result, further study is required.

HCOO6 shows very similar physical characteristics with DMDEOS, with slightly higher WCAs (110 – 115°). As both sols show similar average roughness values, the increase in contact angle can be attributed to a higher density of methyl (-CH<sub>3</sub>) groups on the surface of the coating. Overall, the most effective coating in reducing the formation of an initial biofilm is HCOO6. The greatest diversity of organisms is seen, reducing the likelihood of permanent biofilm. However, this is not a very robust material and that may reduce its effectiveness over time. The material that has been most effective in reducing attachment of macrofouling organisms (barnacles) is DMDEOS. This is borne out by the overall coverage and nearest neighbour data. The roughness of the T<sub>2</sub>/FmS (5:3) also provides an effective material worthy of further study in dynamic conditions. Clearly sol-gels with defined chemistry, roughness and wettability provide real promise and antifouling transparent coatings for sensors or other devices that require such technology.

The biofouling observations on novel sol-gel coatings that have been concluded from this work are:

- a) Initial settlement of microfouling communities were inhibited by the HCOO6 sol gel coating, although in the long-term, is not as effective due to the range of microfouling organisms adhering on the glass substrate.
- b) The sol, DMDEOS, was shown to be the most effective at preventing the settlement of macro-fouling organisms to the surface, reducing film colonies, and potentially reducing further hard colonization.
- c) The T<sub>2</sub>/FmS (5:3) proved promising for antifouling and may provide a rationale for the further exploration of antifouling systems with defined roughness.

One of the main aims of this thesis is to evaluate the influence of antifouling mechanisms on biofouling. This chapter gave insight on the role of surface chemistry (i.e., roughness, wettability and hydrophobicity) on the colonization of novel transparent sol-gel materials in the marine environment. One particular coating mentioned, T<sub>2</sub>/FmS (5:3), looked hopeful for the design of environmentally benign antifouling systems in sensor technology. This chapter, in addition, focused on the diversity of attaching cell types on the different designed coatings – where biofouling organism species (i.e., diatoms) and numbers (i.e., barnacles) were variable, depending on the coating. As a result of this, it was proposed to carry out a long-term study (12-mo) of biofouling in the marine environment (Dublin Bay) to inform on biofilm diversity and composition in Irish waters.

# Chapter 5:

Bioinspired antifouling texture based on

*Scophthalmus rhombus* (Brill fish)

## 5.1 Introduction

---

*Scophthalmus rhombus* (Brill) is a small flatfish of the family *Scophthalmidae*, occurring in marine waters of the Mediterranean as well as in Norway and Iceland from 4 to 73 metres. It inhabits mainly inshore sandy and muddy coastal waters but can also inhabit muddy and gravel grounds. Its skin changes colour depending on the environment but is generally brownish with light and dark freckles and a creamy underside <sup>181,182</sup>. This study discusses the challenges faced in creating biomimetic surfaces using three commonly available microfabrication methods for the production of a synthetic fish scale texture inspired by brill (Figure 5.1).

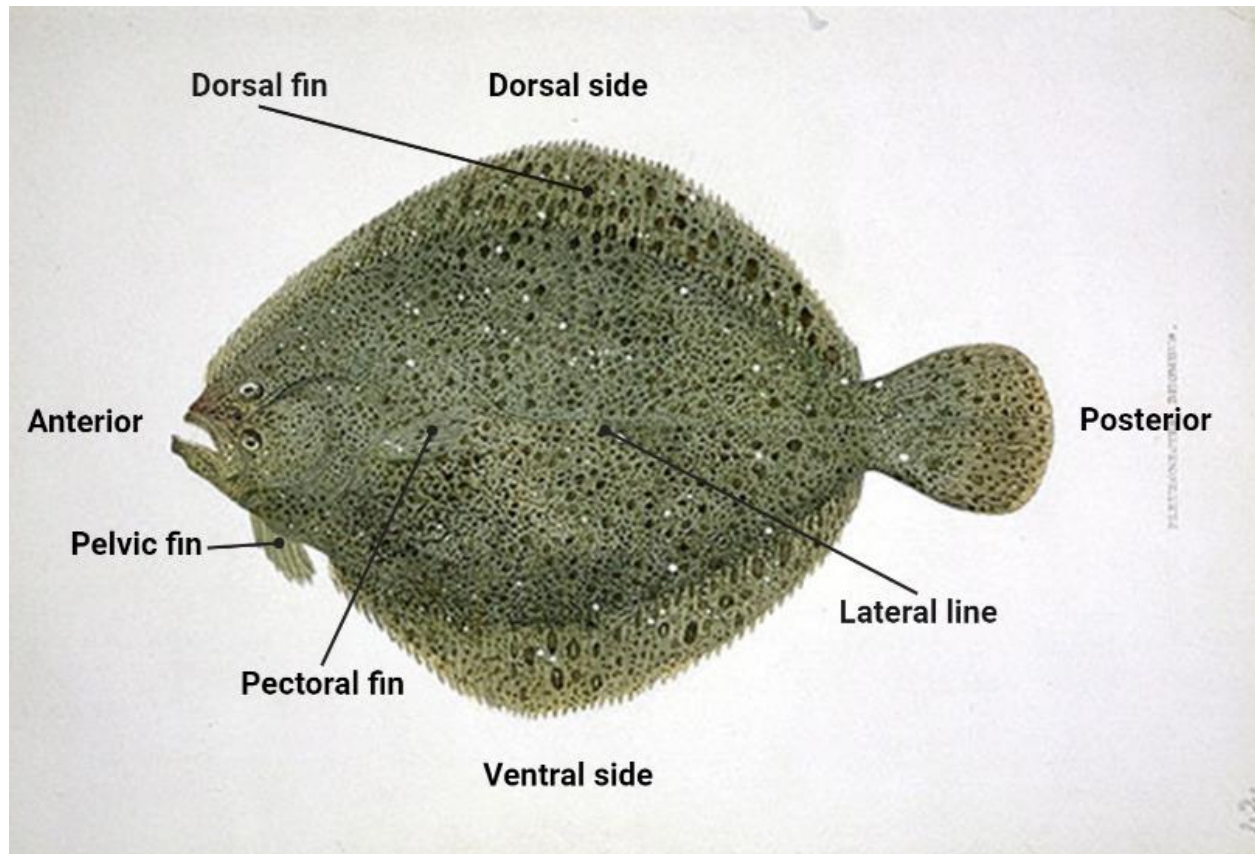


**Figure 5.1.** Anatomy of the marine brill fish, *Scophthalmus rhombus* (Taken from MacGillivray: <http://www.nhm.ac.uk>).

In this study, the topographical features of the Brill fish scale was characterized for the first time using an electron microscopy process. An adaptation of the Brill fish topography was decided upon and constructed in SolidWorks for the ability to control surface microstructure features such as length, width and peak-to-peak distances described within. Artificial replication of the *S. rhombus* scale was carried out using available microfabrication techniques, soft lithography (RM), 2-photon polymerization (2PP) and templating (Repliset-F5).

## 5.2 Aims and objectives

---

The aim of this chapter is to characterize and evaluate the topographical features of the common brill fish, *Scophthalmus rhombus*, for the development of a novel surface micro-texture for prevention of fouling. It was proposed that an antifouling effect could be induced by the production of a synthetic surface, inspired by the brill fish, *Scophthalmus rhombus*, whereby both chemistry and topography would play a role in enhancing antifouling performance. An attempt to characterize and evaluate the microtopography of the common brill fish, *Scophthalmus rhombus* for potential as an antifouling solution has been made.

The objectives of this work are to:

- 1) Assess the surface topography of the Brill fish, *Scophthalmus rhombus*, for the prevention of fouling in the marine environment.
- 2) Characterize the micro-topographical features of the Brill fish for the design of a novel bioinspired antifouling solution.
- 3) Produce artificial materials identical to the surface micro-texture of *Scophthalmus rhombus*, using 3-D printing and lithography methods, evaluating the fidelity of each of these replication methods.
- 4) Selection of microfabrication process for further study of cell-surface interactions.

### 5.3 Materials and methods

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The methods discussed here are outlined in Chapter 2. Scale structure and characterization was carried out using electron microscopy (Hitachi S-3400N SEM) as described in Section 2.8.1. Samples were Au sputter coated (Agar Scientific) for 30 s and observed under SEM at an accelerating voltage of 20 kV. Scale chemical composition using Energy dispersive X-ray analysis (EDX) was performed by Barry O' Connell as described in Section 2.8.2. The fabrication of designed microstructure by 3-D printing (2PP), soft lithography (PDMS casting) and templating (Repliset-F5 casting) was carried out as described in Section 2.10. Surface micro-textures were characterized using electron microscopy (Section 2.8.1), water contact angle (Section 2.8.4) and profilometry (Section 2.8.5).

## 5.4 Results and discussion

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The micro-texture selected for the design of a marine inspired antifouling surface was that of the Brill fish scale, *Scophthalmus rhombus* (Figure 5.2). Characterization using electron microscopy revealed only a small number of bacteria had adhered to surface micro-topographical features – with a larger proportion of the scale remaining free from biofouling. It was hypothesized that the micro-topographical features found on Brill could serve as inspiration for the design of a novel antifouling surface. The design was chosen based on (i) simplicity of design, (ii) novel application and (iii) ease of replication.

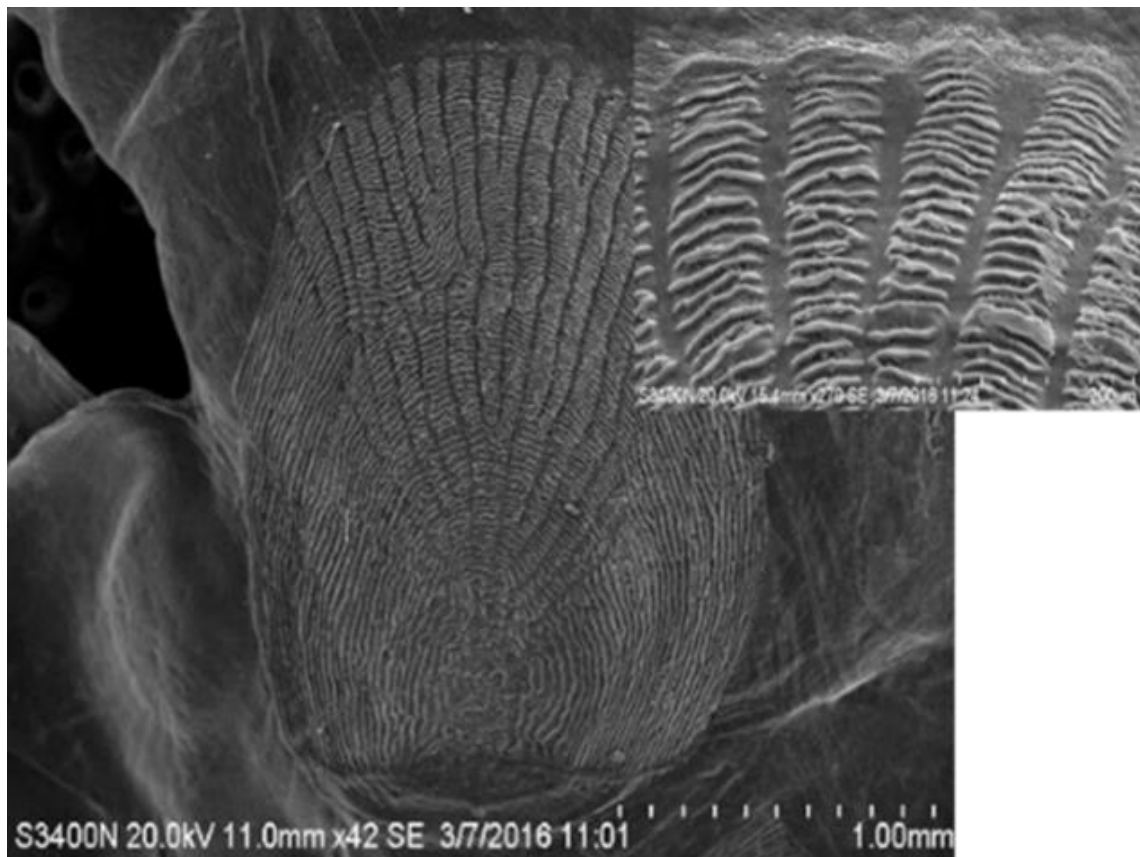


**Figure 5.2.** Single scale micrograph from *S. rhombus* (F1), showing characteristic growth rings interrupted by perpendicular rays (20 kV, 11 mm, x42) (inset: SEM micrograph showing interrupted growth rings by ray patterns (20 kV, 15.4 mm, x270))

#### 5.4.1 Scale structure

---

The structure of the Brill fish scale was examined using electron microscopy as described in Chapter 2, Section 2.8.1. A scale is perceived as an oval plate lying in the pocket of the dermis of the fish skin, with its posterior part sticking out above the skin surface in the direction of the fish tail. Scale is characterized by growth rings, which show the growth history of fish <sup>183</sup>, and are similar to rings observed in a finger print or cross section of a tree. Rings propagate spirally or concentrically from the lower central section of the scale, called a focus, and are observed as ridges called circuli<sup>183</sup>. These rings or circuli are being interrupted by perpendicular rays scattering radially also from focus of the scale (Figure 5.2). That interruption forms individual features observed as separated micro ridges and micro grooves (Figure 5.3).

Thus, presented below descriptive 3-D portrayal gives only the average shape of a general micro ridge, without distinction on longer or shorter micro ridges which dimensions depend on the level at which they are contained in parted, particular segments of posterior or anterior part of the scale.

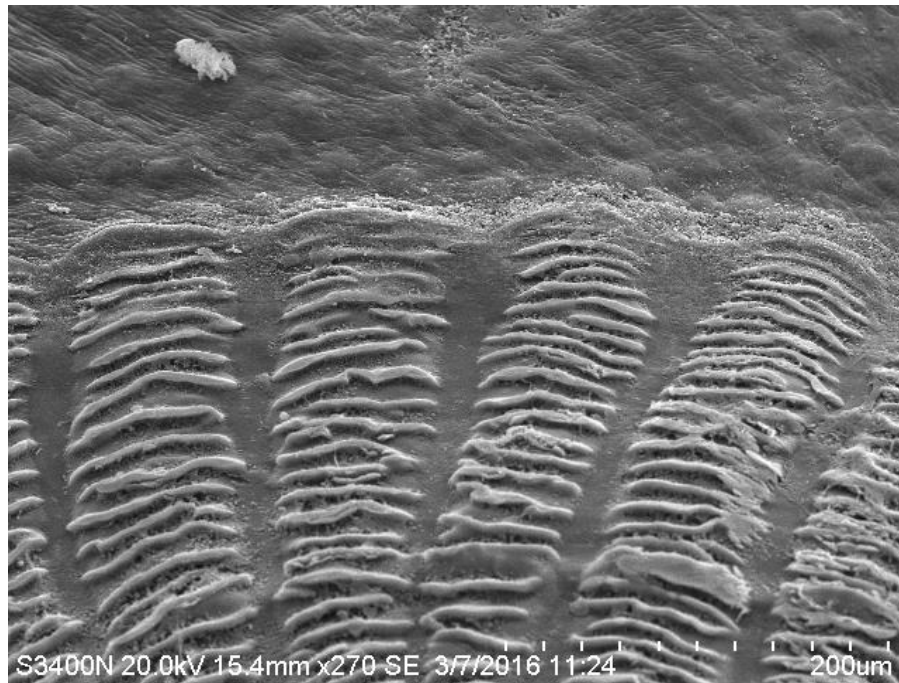


**Figure 5.3.** Scanning electron micrograph showing interrupted growth rings by ray-patterns and formed by its individual separated micro-ridges (F1) (20 kV, 15.4 mm, x270).

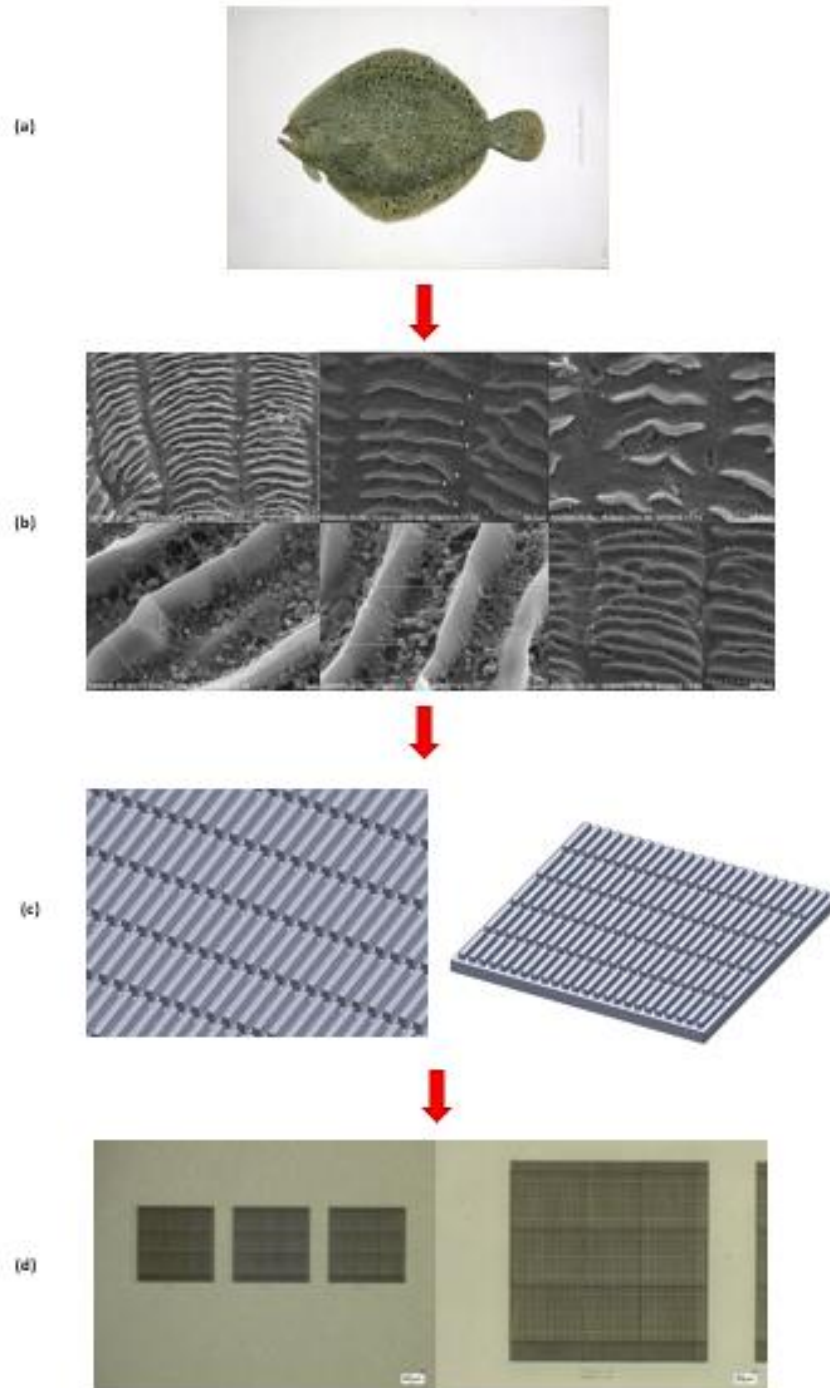


**Figure 5.4.** Schematic illustrating the process of designing bio-inspired materials for application in antifouling; (a) An image of the bioinspired surface, *S. rhombus* (Brill fish), (b) SEM micrographs showing the characterization of the Brill fish scale, (c) Rendered images showing the designed surface microtexture inspired by Brill and (d) Digital light microscopy images showing the produced surfaces fabricated using a 3-D printing technique.

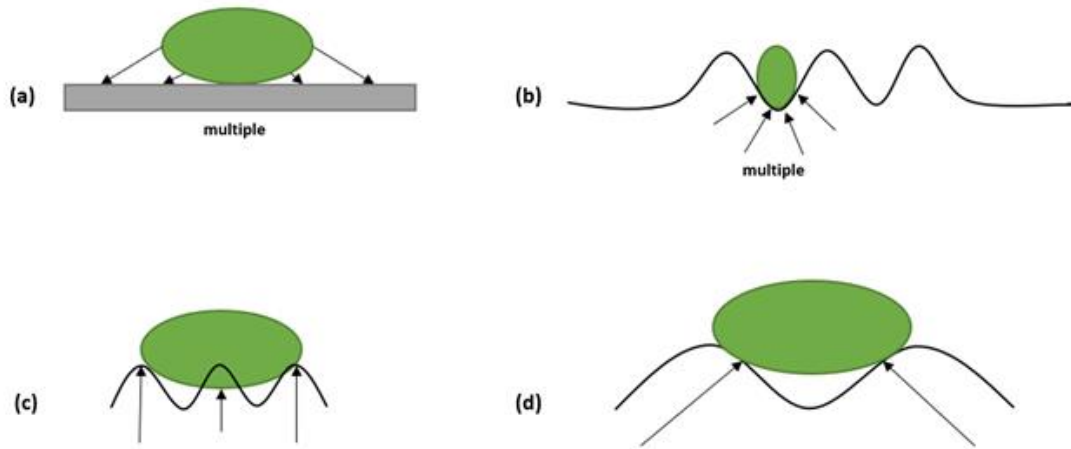
### 5.3.2 Scale characterization

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Scale characterization was carried out by Mr. Alan Barrett. On observation using scanning electron microscopy (Chapter 2, Section 2.8.1), all samples displayed shrinkage and visible disruption of analyzed epidermis. In characterization of the microtopography of brill scale, six different dimensions of micro ridges formed by the interruption of rings by ray-patterns, were considered. These dimensions were measured using the 'line tool' in Image J image processing software. Each dimension was assigned to a letter, which is shown below, and average dimensions are calculated from a number of SEM micrographs from four different samples and from different scales (Table 5.1: F1 – F4). Samples were examined at tilt of 0° and 40 – 42°. Average, standard deviation and standard error were performed using descriptive statistic (Excel 2013).

**Table 5.1.** Samples localization in *S. rhombus* body.

| <b>Sample Symbol</b> | <b>Location on <i>S. rhombus</i> body</b> |
|----------------------|-------------------------------------------|
| F1                   | Front, Anterior, Dorsal                   |
| F2                   | Front, Posterior, Dorsal                  |
| F3                   | Front, Anterior, Ventral                  |
| F4                   | Front, Posterior, Ventral                 |



## Legend

**A** = An example of a length measure

**D** = An example of a measure of height of a single micro-ridge

**E** = An example of a measure of peak-to-peak distance between two micro-ridges

**(a)** Diatoms on a smooth surface, i.e., multiple attachment points (Adapted from <sup>6</sup>)

**(b)** Diatoms settling on 2 µm ripples – multiple attachment points (Adapted from <sup>6</sup>)

**(c)** Diatoms settling on 2 µm ripples – 3 attachment points (Adapted from <sup>6</sup>)

**(d)** Diatoms settling on 4 µm ripples – 2 attachment points (Adapted from <sup>6</sup>)

**Figure 5.5.** Top: SEM micrographs showing an example of a length (A), height (D) and peak-to-peak distance (E) measure; Bottom: Illustration of the popular mechanism used to explain diatom attachment on textured surfaces, attachment point theory, whereby the cell will experience increased attachment with the addition of multiple 'attachment points'.

Below are presented SEM micrographs with examples of dimensions and assigned letters to them. Figures 5.6 and 5.7 are examples of how length of micro ridge was measured. Two types of length measurements were taken, the one with the assigned letter A, by a mean of straight line drawn from start point to the end point of the micro ridge (Figure 5.6), and the second by a mean of “freehand line”, with assigned letter B (Figure 5.7). The difference between averages of these two is not very significant (6.23  $\mu\text{m}$ ), but with the angle C (Figure 5.8 and Figure 5.9) and the height of the slope D (Figure 5.10), they could be taken as representative measurements of the shape of average micro ridge for the future biomimetic device. In further micrographs “peak to peak distance” assigned with a letter E is shown, which characterises the average distance between two micro ridges (Figure 5.11), and “interval between features” F (Figure 5.12), which illustrates the distance of detachment among two micro ridges interrupted by mentioned before ray patterns. In addition to characterized micro features on the brill scale, it is important to mention, that many bacteria were present on brill skin. However, this is not of concern in current study, therefore attempts of description were not taken.

In this study, an attempt to characterize Brill scale microtopography was taken for the first time. With using Image J software, an enormous number of measurements of six dimensions of a scale basic micro-component circuli were taken to deliver overall representation of its shape.

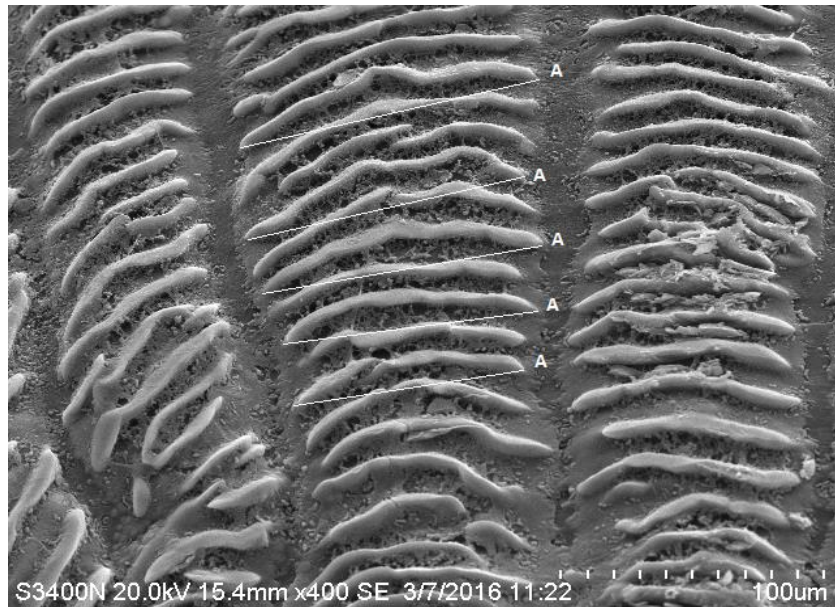


**Figure 5.6.** An example of a length measured by the mean of a straight line drawn from the start to the end of micro-ridge (F1) (20 kV, 15.4 mm, x400).



**Figure 5.7.** An example of a length measured by the mean of a 'freehand' line drawn from the start to the end of a micro-ridge (F4) (20 kV, 15.6 mm, x900).

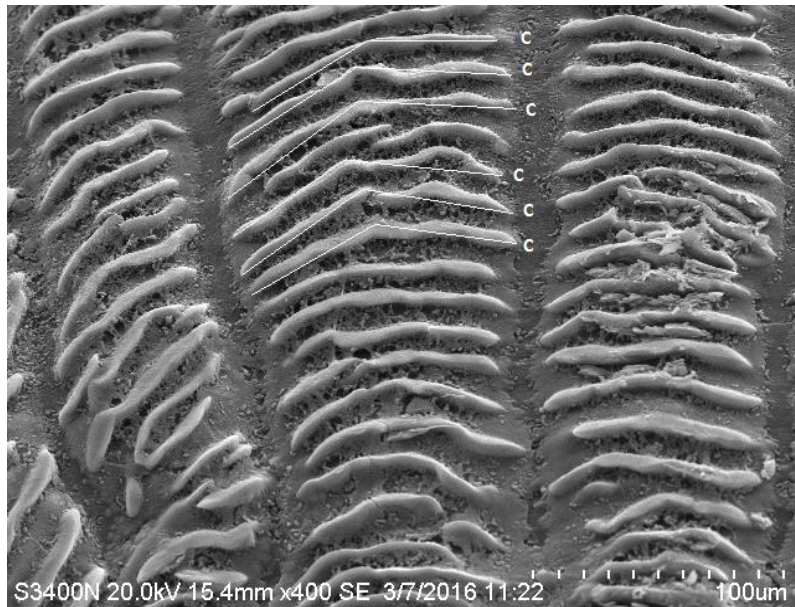


**Figure 5.8.** An example of a measure of an angle (F1) (20 kV, 15.4 mm, x400).



**Figure 5.9.** An example of measure of an angle (F3) (20 kV, 15.9 mm, x750).

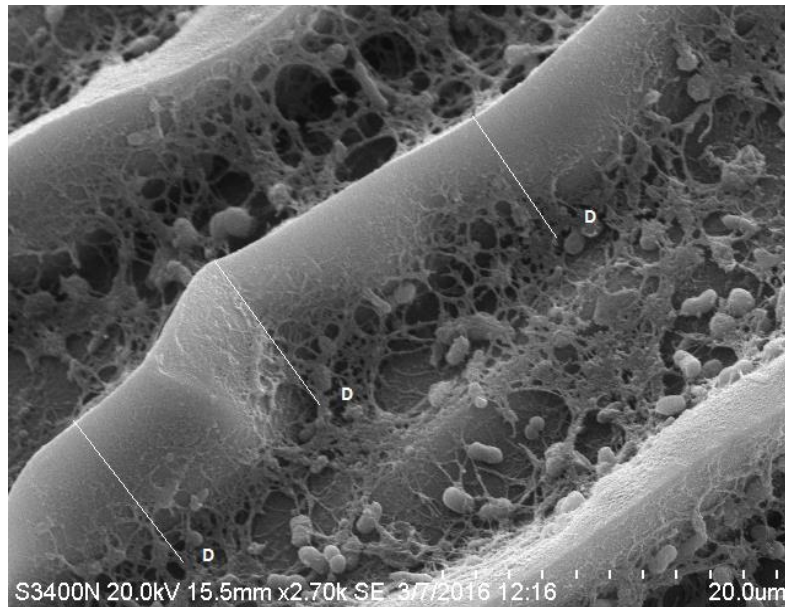


**Figure 5.10.** An example of a measure of height of a single micro-ridge (F1) (20 kV, 15.5 mm, x2.70k).

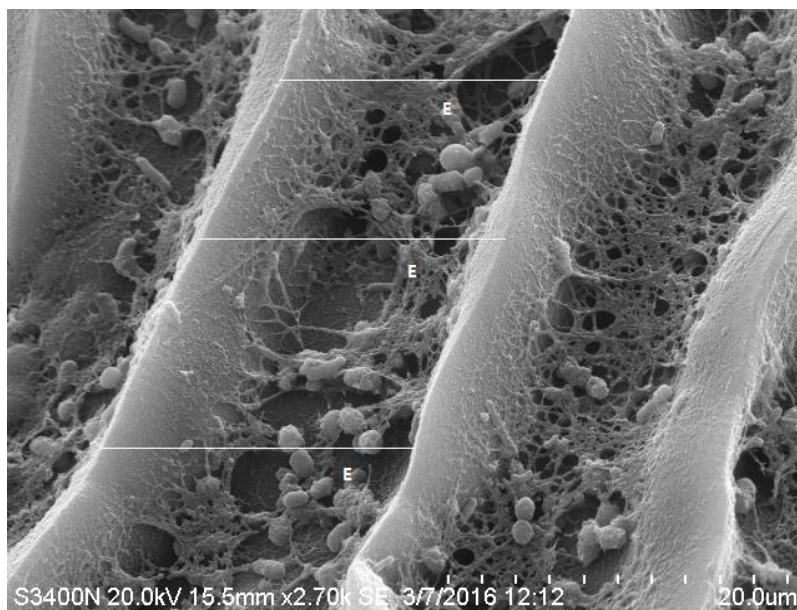


**Figure 5.11.** An example of a measure of peak-to-peak distance between two micro-ridges (F1) (20 kV, 15.5 mm, x2.70k). A large number of bacteria were present on brill skin. However, this is not of concern in this study, therefore attempts of description were not taken.

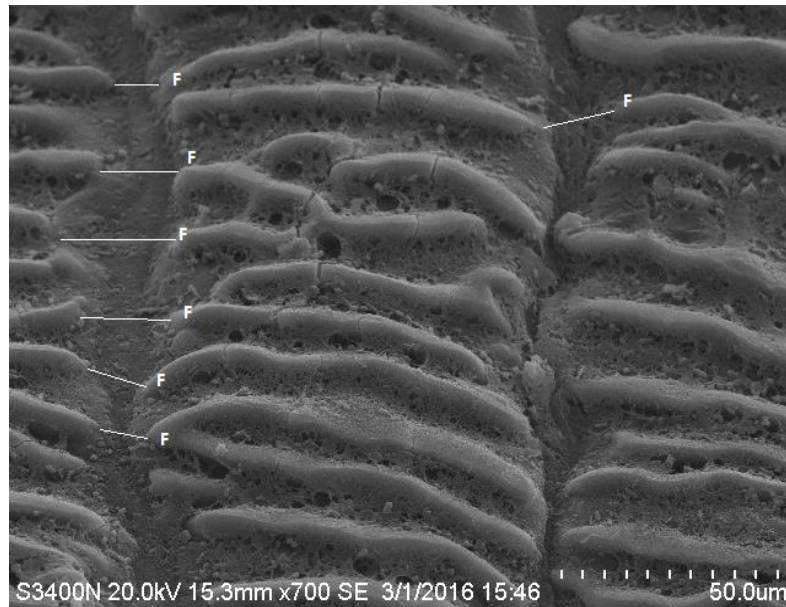


**Figure 5.12.** An example of a measure of the distance between features between two separated sections of micro-ridges (F3) (20 kV, 15.3 mm, x700).

The results of the characterization of the Brill fish scale (*Scophthalmus rhombus*) is summarized in Table 5.2. The measurements calculated here inform on the bio-design of novel antifouling (AF) materials based on Brill.

**Table 5.2.** Mean, standard error (SE) and standard deviation (SD) of dimensions of the *Scophthalmus rhombus* (Brill fish) scale (A: Length ( $\mu\text{m}$ ); B: Curvature length ( $\mu\text{m}$ ); C: Angle ( $^{\circ}$ ); D: Slope ( $\mu\text{m}$ ); E: Peak-to-peak distance ( $\mu\text{m}$ ) and F: Interval between features ( $\mu\text{m}$ )).

| Letter | Dimension                                      | Mean   | n   | SE  | SD   |
|--------|------------------------------------------------|--------|-----|-----|------|
| A      | Length ( $\mu\text{m}$ )                       | 74.84  | 235 | 1.1 | 16.5 |
| B      | Curvature length<br>( $\mu\text{m}$ )          | 81.07  | 222 | 1.2 | 17.5 |
| C      | Angle ( $^{\circ}$ )                           | 143.08 | 189 | 0.7 | 10.2 |
| D      | Slope ( $\mu\text{m}$ )                        | 11.7   | 163 | 0.4 | 5.3  |
| E      | Peak-to-peak<br>distance ( $\mu\text{m}$ )     | 16.6   | 226 | 0.4 | 5.8  |
| F      | Interval between<br>features ( $\mu\text{m}$ ) | 15.8   | 173 | 0.5 | 6.8  |

### 5.3.3 Scale chemical composition

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The chemical composition of marine organisms known to prevent fouling on their skin is also an important consideration. Energy Dispersive X-Ray analysis (EDX) was performed by Dr Barry O' Connell (see methods, Chapter 2, Section 2.8.2). EDX analysis is used in conjunction with electron microscopy to give insight into the elemental and chemical composition of a material. It allows for the identification of elements present in a compound through the generation of characteristic X-rays<sup>166</sup>. EDX measurements were taken at 30 kV. The peaks identified on the spectrum are evidence that an element is present (Figure 5.13). Quantitative analysis was also performed on the sample, however this is often inaccurate and is given as a guide only (Figure 5.14).

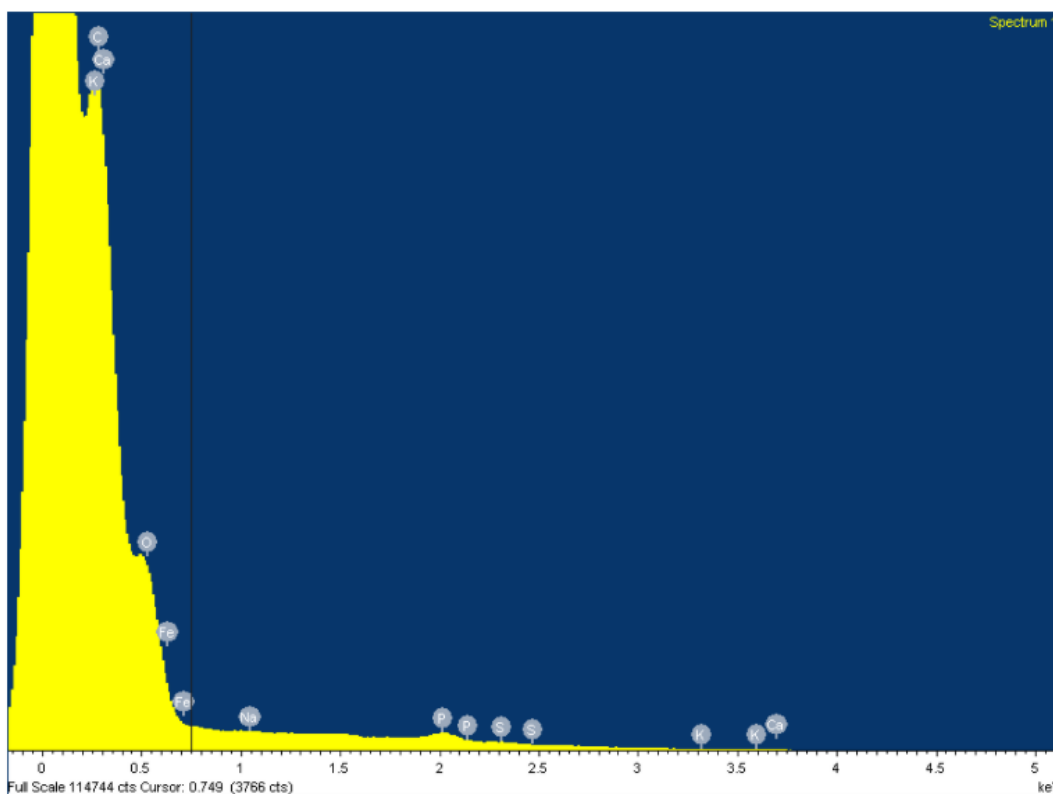


**Figure 5.13.** EDX spectrum obtained of the brill scale, *Scophthalmus rhombus*.

Fish skin is made up of two layers, an outermost layer called the epidermis and an inner layer, called the dermis. Unlike mammals, the dermis is formed externally by live cells and mucus cells that produce the mucus. Fish skin contain highly calcified fish scales, which may explain the Calcium peak observed in the EDX spectrum <sup>184</sup> Marine fish play a crucial role in the marine carbon cycle. A study by Wilson *et. al.*, revealed that fish excrete calcium carbonate within their intestines, previously cited to have come from marine plankton (i.e., shelled algae). Considering global fish biomass, it was suggested that fish may provide 3 – 15 % of total oceanic carbon creation with predicted increasing levels in response to the rise in carbon dioxide levels occurring with climate change <sup>185</sup>. EDX-SEM analysis performed on the fish scale indicated that C, O, Ca, and K were the main elements present in the *S. rhombus* scale. The presence of C, O and Ca is reflected in previously published reports of various marine organisms but particularly in Crustacean species <sup>186–188</sup>. It was also noted that  $\text{Ca}^{2+}$  can aid in the adherence of microfouling organisms to a surface in the marine environment. However, according to Sullivan *et al.*, in the case of the Crustacean, *Cancer pagurus*, the rise in the levels of  $\text{Ca}^{2+}$  was thought to reduce the settlement of diatoms to the cuticle <sup>105</sup>.

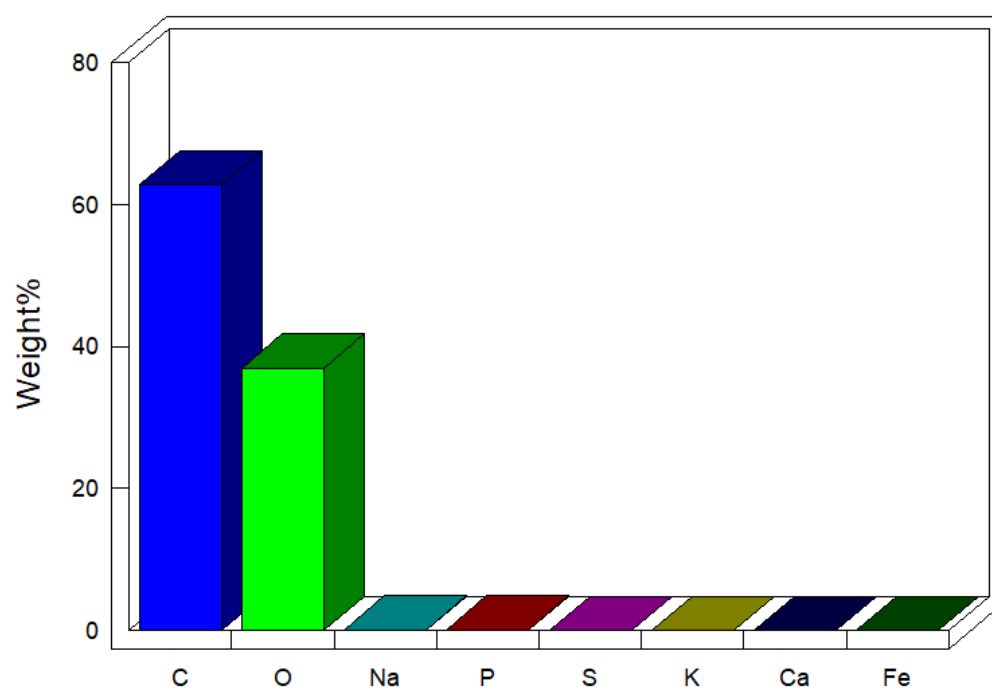


**Figure 5.14.** Quantitative analysis performed of percentage weight (%) of compounds present on the brill scale, *S. rhombus* (n = 3).

#### 5.3.4 Microfabrication

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Among many biological objects and their functions, aquatic animals deserve a special mention due to their antimicrobial capabilities resulting from chemical composition, surface topography or other behavioural defences, which can be used as an inspiration for antifouling technology. These include attempts of characterization of nano and micro surface topography such as the surface topography of dolphin <sup>189</sup>, shark <sup>190,191</sup>, catfish <sup>192</sup>, decapod crab <sup>193</sup>, shrimp <sup>194</sup>, mussels <sup>195</sup> or lobster <sup>196</sup> as previously described. After more than a decade of searching for a rational green method that reduces or prevents microbial attachment and colonization, it becomes increasingly known that topographic cues affect biofouling. Therefore, with the possibility of high-resolution manufacture techniques, the capacity to regulate nano and micro surface features provides a favourable research area for non-toxic antifouling solutions. In this study, the natural dermal scales of the *Scophthalmus rhombus* scale were artificially reproduced using three available methods; 3-D printing, PDMS casting and templating (i.e., Repliset-F5 casting) (see methods, Chapter 2, Section 2.10.1, 2.10.2, 2.10.3).

#### 5.3.4.1 2-photon polymerization (2PP)

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Based on previous characterization studies of *S. rhombus*, CAD designs were constructed using the modelling software, SolidWorks (2017 edition). The design was then fabricated using the Nanoscribe 2-photon 3-D printer as described previously (Section 2.10.1). Scanning electron microscopy and profilometry were employed to assess the fidelity of designed surfaces. The design constructed in SolidWorks (2017) consisted of a rectangular design with sharp edges. The initial design consisted of a micro-ridge with a feature length of 75  $\mu\text{m}$ , width, 10  $\mu\text{m}$ , surface feature height of 10  $\mu\text{m}$  with variable peak-to-peak distances of 10  $\mu\text{m}$  – 7  $\mu\text{m}$ . Outer dimensions were reported as 500  $\mu\text{m}$  x 500  $\mu\text{m}$  x 20  $\mu\text{m}$  (Figure 5.15).

$L_y = 10$  (a)  $\mu\text{m}$ , 9  $\mu\text{m}$  (b), 8  $\mu\text{m}$  (c) and 7  $\mu\text{m}$  (d)

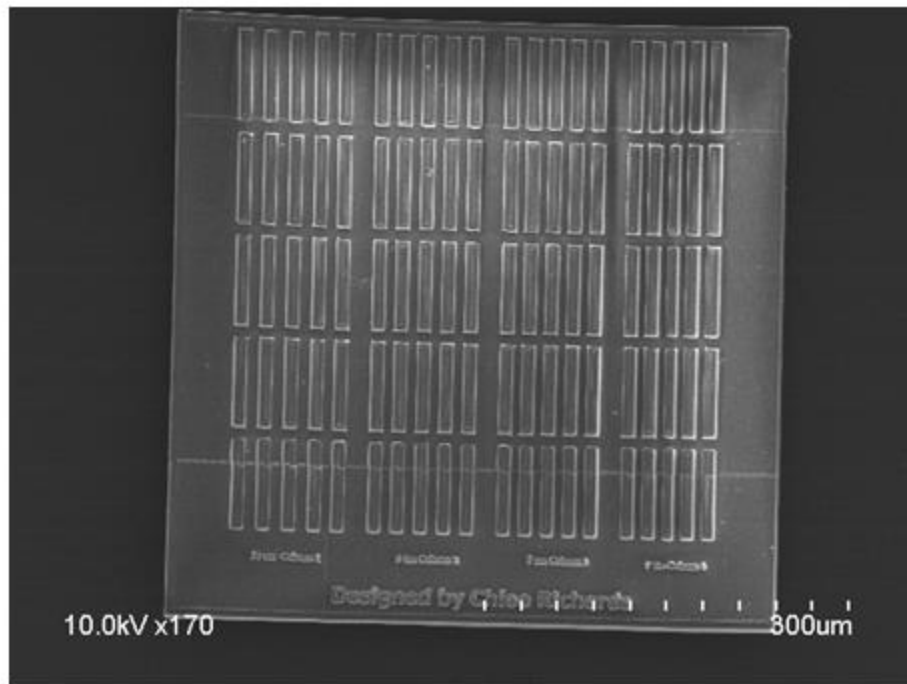
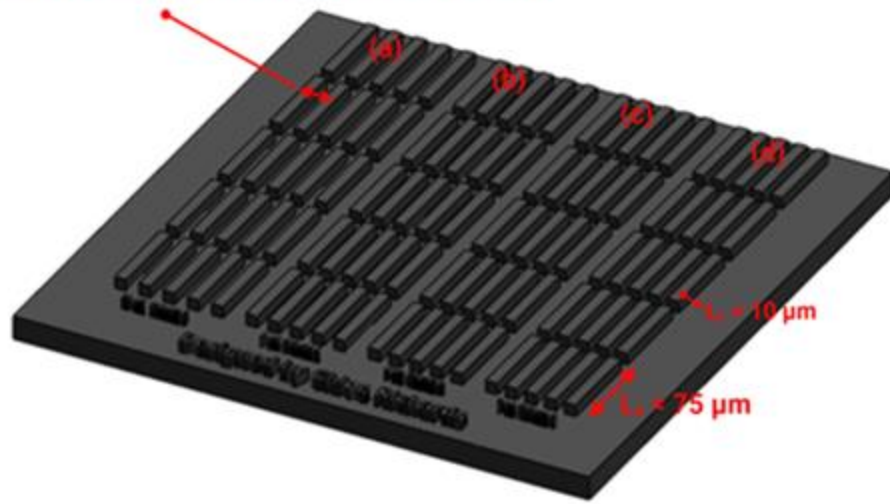


**Figure 5.15.** Top: Rendered CAD design of the initial design based on Brill (Version 1) designed in SolidWorks 2017 and fabricated using a 3-D printing process. Bottom: SEM of 3-D printed surface.

The reasoning for the use of variable peak-to-peak distances was based on the popular model used to explain cellular attachment on a surface: Attachment Point Theory <sup>6</sup>, whereby differences in contact points provided a different response to adhesion. It was proposed that cellular attachment would be lowered as the peak-to-peak distances between adjacent features was reduced.

#### *5.3.4.2 Mold casting*

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As previously described, a negative template of the 3-D CAD design was fabricated using the Nanoscribe 2-photon 3-D printer from a design constructed using SolidWorks (2017 edition). This negative template was vapour treated (i.e., hydrophobic treatment) in a vacuum chamber in a solution of 30 mL of heptane mixed with 4.7  $\mu$ L of Octadecyltrichlorosilane (OTS) for 2 hours, before drying in an oven at 100°C for 30 min. Sylgard 184k Polydimethylsiloxane (PDMS), 10:1 ratio was then poured onto the negative template to create a positive replica of the surface micro-texture. Figure 5.16 outlines the design of the negative mold created using 3-D printing, used in the production of textured PDMS surfaces.

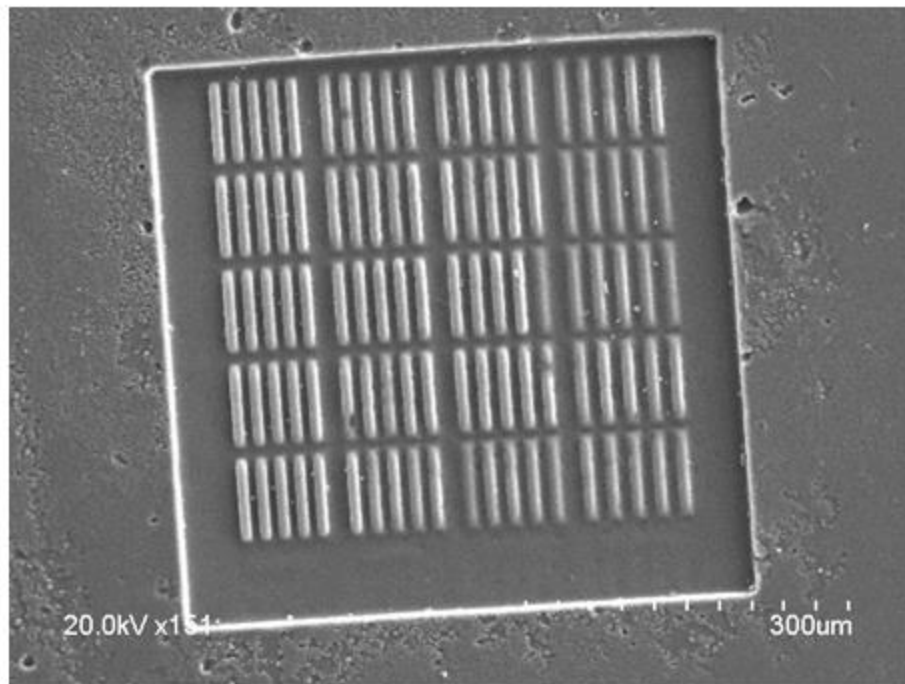
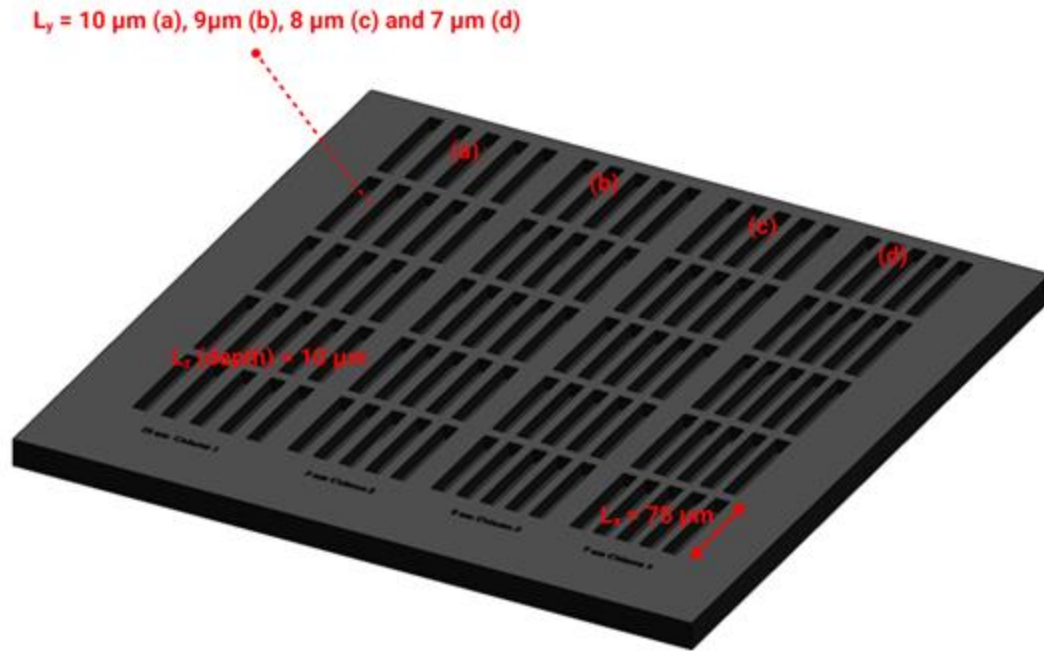


**Figure 5.16.** Top: Rendered CAD design of the negative mold design based on Brill designed in SolidWorks 2017 and fabricated using a 3-D printing process. Bottom: SEM of the positive textured PDMS surface.

#### 5.3.4.3 Repliset-F5 casting

As described in Chapter 2 (Section 2.10.3), the natural topography of the Brill fish scale (for which inspiration for the novel AF surface originates) was replicated using a templating process with the Repliset-F5 casting kit. The process of replication is illustrated in Figure 5.17 below.

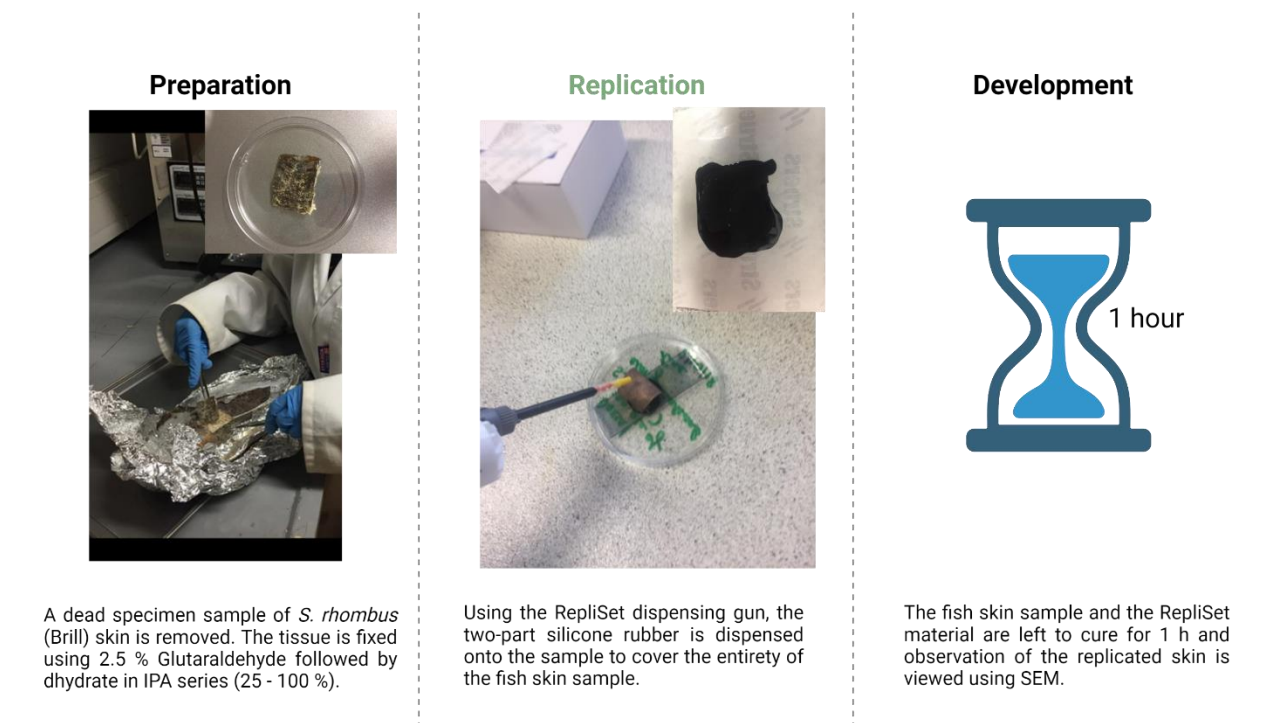


**Figure 5.17.** Schematic of the replication of the natural topography of the Brill fish using a Repliset-F5 casting technique.

### 5.3.5 Surface characterization

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The physical and chemical characterization was carried out on each of the produced surfaces fabricated using the three texturing methods. SEM was used to measure the dimensions of the created surfaces (i.e., feature length, peak-to-peak distance) whilst surface profilometry was used to measure feature height ( $n = 3$ ). Wettability of the materials was determined using static water contact angle measurement ( $n = 3$ ). The mean, standard deviation and % RSD were calculated for each of the textured surfaces. Generally, 2PP and PDMS casting (RM) methods proved efficient in replicating designed surface micro-textures, with some disadvantages. Two-photon polymerization allowed for accurate control over surface feature length (Table 5.3). Some advantages of the process include a relatively fast printing time ( $> 20$  min), high resolution (XY resolution = 400 nm; Z resolution = 1000 nm) and an ability to create a variety of patterned micro- and nano-structures. However, with high printing costs (i.e., €14 per hour) and limitations on printing size and polymer material, its restricted in its use as a large-scale antifouling solution (i.e., can be used in laboratory studies only).

**Table 5.3.** Precision-comparison of the replication of *S. rhombus* using a 2-photon polymerization process (2PP) (n = 3).

| Dimension                        | Mean  | SD   | % RSD |
|----------------------------------|-------|------|-------|
| Feature length ( $\mu\text{m}$ ) | 77.26 | 1.66 | 2.15  |
| Feature height ( $\mu\text{m}$ ) | 9.68  | 0.41 | 4.26  |
| 10 $\mu\text{m}$ spacing         | 10.58 | 0.99 | 9.37  |
| 9 $\mu\text{m}$ spacing          | 9.09  | 0.63 | 6.94  |
| 8 $\mu\text{m}$ spacing          | 8.31  | 0.52 | 6.31  |
| 7 $\mu\text{m}$ spacing          | 6.84  | 0.71 | 10.31 |

Mold (PDMS) casting had a number of advantages, and some disadvantages. Advantages include its ease of use and high resolution. However, disadvantages such as the requirement of a clean room and the potential incorporation of a cleaning step to reduce imperfections in subsequent replications of the master mold, should be carefully considered. In this case, the PDMS master mold also requires hydrophobic vapour treatment before casting to prevent the sticking of the two PDMS layers. The geometry of the replication in PDMS was also altered. PDMS casting produced surfaces with rounded edges (similar to an effect observed in laser micromachining), instead of the designed sharpened edge for mechanical stiffness. Table 5.4 below shows the precision comparison for the replication of *S. rhombus* using a mold casting process.

**Table 5.4.** Precision-comparison of the replication of *S. rhombus* using a casting process (PDMS) (n= 3).

| Dimension           | Mean  | SD   | % RSD |
|---------------------|-------|------|-------|
| Feature length (μm) | 76.49 | 1.92 | 2.52  |
| Feature height (μm) | 9.83  | 0.07 | 0.71  |
| 10 μm spacing       | 10.98 | 1.52 | 13.82 |
| 9 μm spacing        | 8.58  | 0.99 | 11.53 |
| 8 μm spacing        | 9.86  | 0.97 | 9.80  |
| 7 μm spacing        | 10.59 | 1.22 | 11.51 |

Static water contact angle (WCA) was measured via a sessile drop technique for both the 3-D printed (2PP) PDMS and Repliset-F5 casted surfaces (n = 1) (Figure 5.18). The polymer used in the 2PP process is hydrophilic, having a water contact angle measurement under 85.41 °. Although, surface chemistry has been noted to play a role in biofilm prevention, the focus of this study was on the physical modification of a surface for antifouling. Textured surfaces made using 2PP could only be fabricated with the material specified by the manufacturer (Nanoscribe). The photoresist used was IP-S which contains 4-BIS-(diethylamino)-benzophenone (CAS: 90-93-7, EG: 202-025-4) (< 1 %) and 4-Hydroxybutyric acid lactone (CAS: 96-48-0) (< 2%). PDMS is a reported hydrophobic surface <sup>2</sup>, with a water contact angle of 90° and over. Here, PDMS was discovered to have a static water contact angle of approximately 94.16°. The two-part silicone rubber used in the Repliset-F5 casting process had a water contact angle of 88.13°.

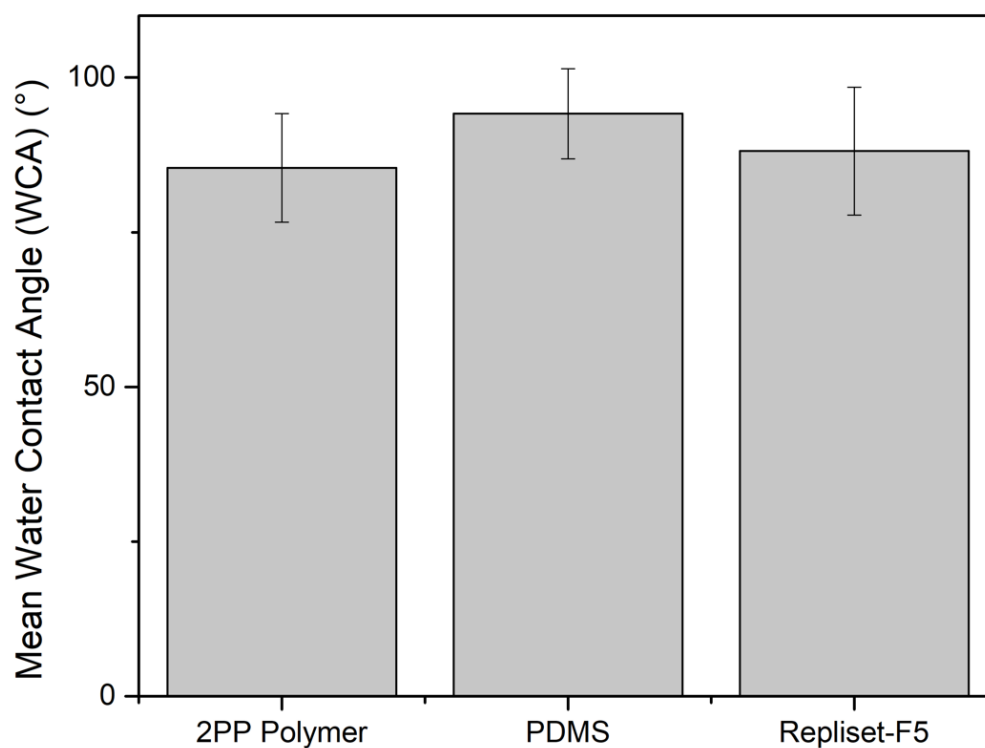


**Figure 5.18.** Mean WCA measurements for each of the materials used in the production of designed textures replicated by 3-D printing (2PP), mold casting (PDMS) and Repliset-F5 casting (n = 3).

Below are presented electron micrographs showing the process of replication from a natural fish scale using Repliset-F5 technology (Figure 5.19). As evident, large proportions of the Repliset-F5 material did not produce a textured surface after PDMS casting, causing imperfections to the PDMS area (Figure 5.19 (a)). In addition, Repliset-F5 casting is not an efficient means of replicating large quantities of textured surfaces, due to fragments of the PDMS material getting trapped in the master mold after each replication. An extensive characterization of each produced surface by Repliset-F5 casting would be required before cell adhesion studies could take place. It would also be difficult to detect cells on the surface of the replicated fish skin using microscopy techniques, due to the complex geometry and orientation of each of the replicated micro-ridges. As a result, Repliset-F5 casting was not chosen going forward.

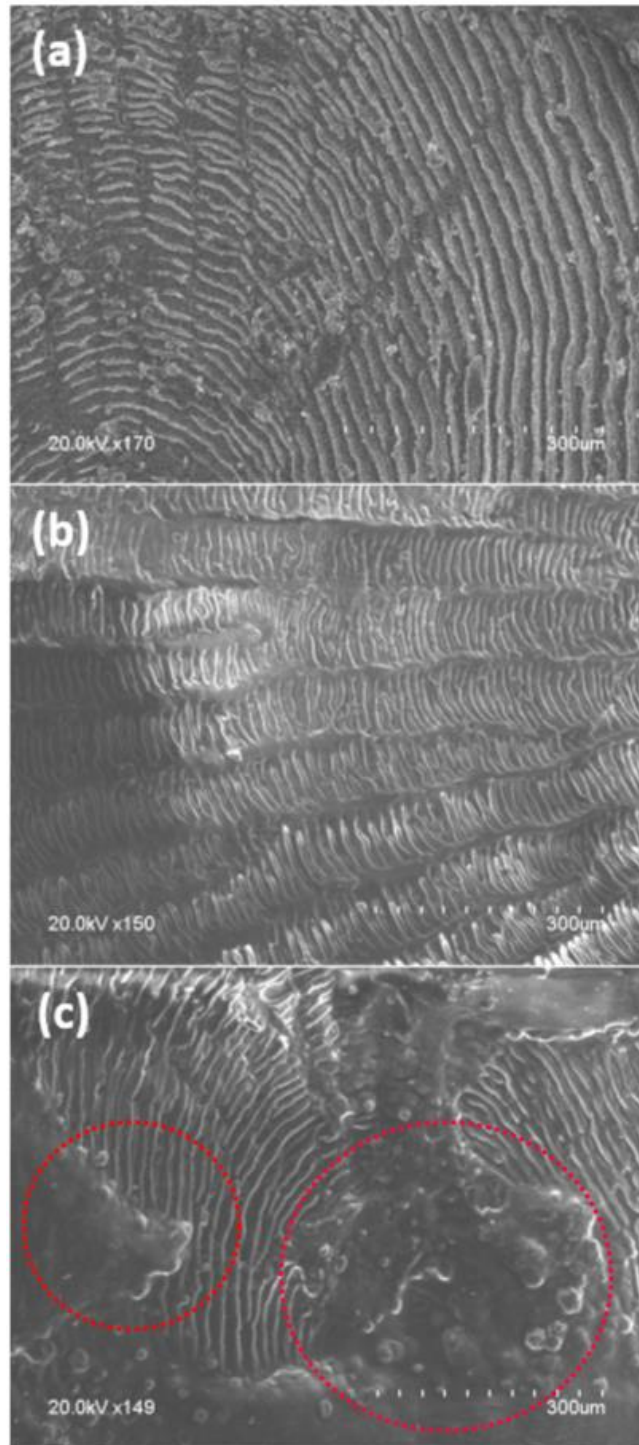


**Figure 5.19.** (a) Electron micrography showing the Brill fish scale; (b) A negative mold of the postive fish scale surface replicated using Repliset-F5 and (c) Imperfections from PDMS casting of the master mold – red innotations marking areas of the replication that have not been produced as required (i.e., no texturization).

## 5.4 Conclusion

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In this study, the topographical features of the Brill fish scale were characterized in detail using electron microscopy and Fiji (imageJ) software for the first time. It was revealed that a micro-ridge found on a scale had dimensions of length, 74.84  $\mu\text{m}$ ; slope of 11.7  $\mu\text{m}$  and peak to peak distances of 16.6  $\mu\text{m}$ . The scale was also characterized for its chemical properties, which was similar to those found in Crustacean species, whereby the surface is made up mostly of  $\text{Ca}^{2+}$ . A simple initial design was constructed based off the characterization of the scale consisting of length 75  $\mu\text{m}$ , width 10  $\mu\text{m}$  and peak-to-peak distances of 10 – 7  $\mu\text{m}$ .

A number of methods of producing engineered surface topographies were examined with respect to robustness, cost, and control of surface features in this study. Ultimately, 2-photon polymerization (Nanoscribe 2-photon 3-D printing) was chosen as the method of choice for the study, despite the associated costs. This method offered the ability for the user to easily control surface feature proportions, with the least number of imperfections arising from the manufacturing process in comparison to other available methods.

From this work – six textures have been designed with varying feature height ( $L_z = 10, 20 \mu\text{m}$ ) and peak-to-peak distances ( $L_y = 10, 5, 2.5 \mu\text{m}$ ) (Table 6.1). These are selected based on microfouler attachment theory, ability to replicate and future scalability (i.e., texturization of a larger surface area). Scalability is extremely important to go from a proof of concept of antifouling effectiveness in the laboratory to long-term studies carried out in the field including application on marine monitoring systems (i.e., tidal energy blades, sensing equipment, marine buoys).

# Chapter 6:

Evaluation of diatom attachment on marine inspired surface micro-textures for application in tidal energy systems

#### 6.4.2 Pattern fidelity

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Engineered surfaces ( $n = 3$ ) were checked for imperfections using SEM (i.e., to check for accuracy of surface feature length, width, and peak-to-peak distances) and surface profilometry ( $n = 3$ ) was to check the accuracy of surface feature heights ( $\mu\text{m}$ ). A series of electron micrographs were taken ( $n = 10$ ) using the Hitachi S3400-N. The scale bar on each image was used to scale the image into micron ( $\mu\text{m}$ ) units. The line tool on ImageJ (Fiji) software was used to measure feature length ( $\mu\text{m}$ ) and peak-to-peak distance ( $\mu\text{m}$ ). Total surface area ( $\text{mm}^2$ ) was measured using the internal measuring tool on the Hitachi S3400-N system. The accurate reproduction of the designed micro-textures is recorded below. As evident, all designs were replicated to their desired specifications. Designed micro-textures that did not meet the dimensions required for testing were discarded. Table 6.2 gives a summary of the produced surfaces dimensions as calculated using electron microscopy and ImageJ (Fiji) software ( $n = 3$ ).

**Table 6.2.** Summary table of reported mean dimensions of textured materials (n = 3).

| Dimension                                | T1                | T2                | T3                | T4                | T5                | T6                |
|------------------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Feature length (μm)                      | 85 ± 1.17         | 85 ± 1.08         | 85 ± 0.88         | 85 ± 0.76         | 85 ± 1.13         | 85 ± 1.5          |
| 'Peak-to-peak'<br>distance (μm)          | 10 ± 0.49         | 10 ± 0.63         | 10 ± 0.39         | 5 ± 0.34          | 2.5 ± 0.22        | 10 ± 0.51         |
| Feature height/depth<br>(μm)             | 10 ± 0.21         | 10 ± 0.11         | 20 ± 0.81         | 10 ± 0.74         | 10 ± 0.55         | 10 ± 0.1          |
| Total surface area<br>(mm <sup>2</sup> ) | 1 mm <sup>2</sup> | 1 mm <sup>2</sup> | 1 mm <sup>2</sup> | 1 mm <sup>2</sup> | 1 mm <sup>2</sup> | 1 mm <sup>2</sup> |

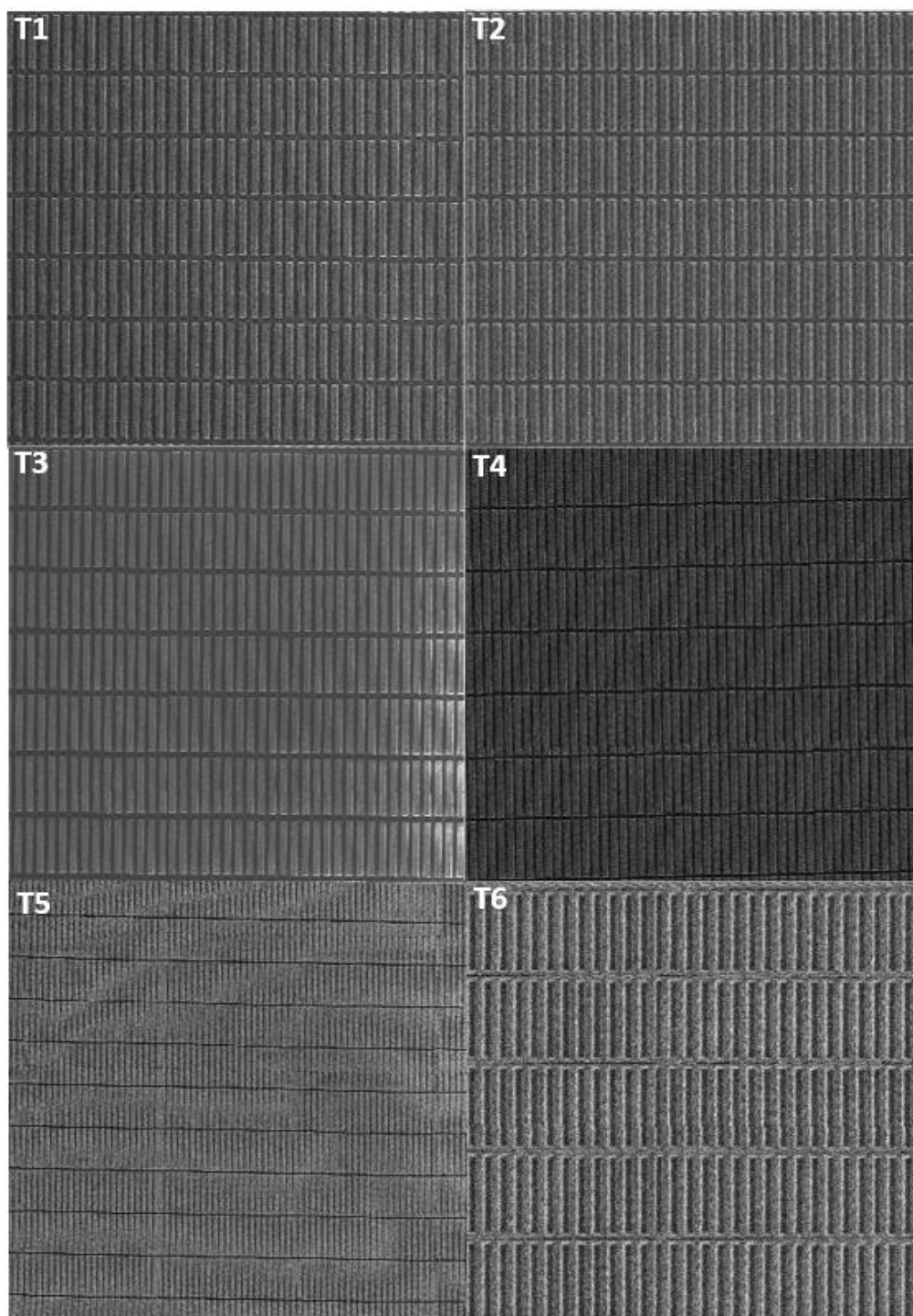


**Figure 6.3.** Top-down scanning electron micrographs of the surfaces produced (T1 – T6) as described in Table 6.1 using a 2-photon polymerization process ( $n = 3$ ).

In this study, the cell-surface interaction of two laboratory grown diatoms, *A. coffeaeformis* and *N. ovalis* on micro-textured materials was investigated for application as an antifouling solution.

Cell adhesion strategy is an important consideration and understanding the attachment behaviour of microfouling organisms (such as diatoms) can aid in the design of effective AF materials. Presented here are laboratory studies of two diatom species, *A. coffeaeformis* and *N. ovalis* using rapid 3 h biofouling adhesion assays, which helps in the understanding of cell-surface interaction.

#### 6.4.3 Diatom species, *A. coffeaeformis*, settlement on textured surfaces

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The results of static adhesion studies by means of 1-way ANOVA followed by Tukey's *post hoc* test with diatom species, *A. coffeaeformis* are displayed in Figure 6.4. All samples displayed visible reduction in the attachment of *Amphora coffeaeformis* cells to the surface. As shown in Figure 6.4, biofilm percentage cover was found to be greatest on T2 (Mean =  $71.28 \pm 3.38$  %). Cell settlement was found to be lower than that of a smooth surface (Mean =  $55.92 \pm 5.40$  %) and glass surface (Mean =  $58.56 \pm 5.03$  %) on T1 (Mean =  $54.34 \pm 4.67$  %), T3 ( $P < 0.05$ ) (Mean =  $39.20 \pm 1.52$  %), T4 (Mean =  $51.37 \pm 5.11$  %), T5 ( $P < 0.05$ ) (Mean =  $40.69 \pm 3.69$  %) and T6 ( $P < 0.05$ ) (Mean =  $41.20 \pm 5.04$  %). This is consistent with previously cited reports in a study by Scardino *et. al.*,<sup>6</sup> where the behaviour of four benthic fouling organisms (*Fallacia carpentariae*, *Nitzschia cf. paleacea*, *Amphora sp.* and *Navicula jeffreyi*) were assessed on a range of laser etched polyimide surfaces to test 'attachment point theory'. The study concluded that diatoms attached in significantly higher numbers to surfaces with higher attachment points. This is evident in this case on T2 and control surfaces (i.e., glass microscope slide and smooth 3-D printed polymer material). In particular, on T2, the rounded edges of the micro-textured surface provide multiple attachment points for cells in comparison to the other micro-textured surfaces (T1, T3, T4, and T5) with sharpened edges.

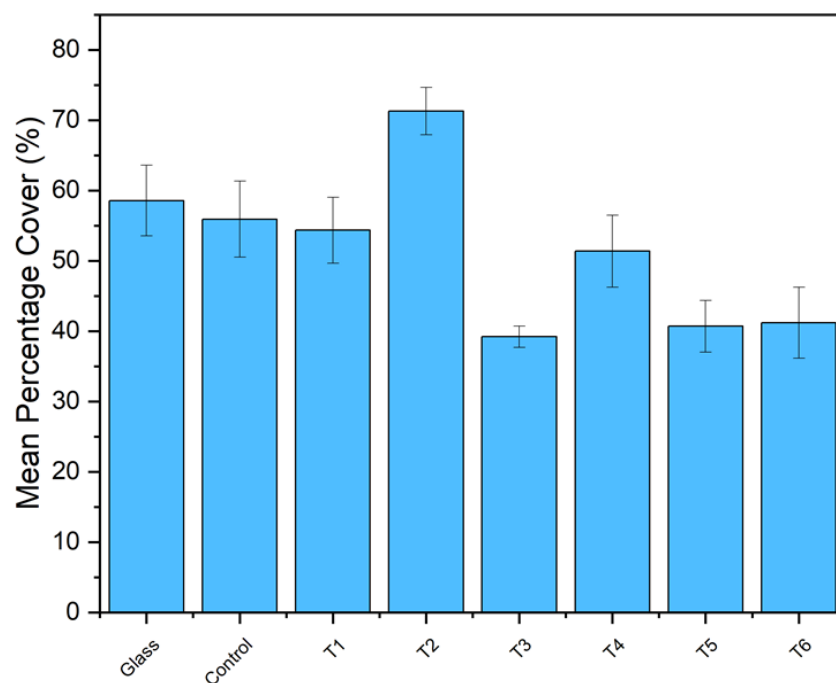


**Figure 6.4.** Mean biofilm percentage cover of adhered *Amphora coffeaeformis* cells after 3 h laboratory assays on produced surfaces. Cells were kept at a chlorophyll  $\alpha$  concentration of  $0.3 - 0.4 \mu\text{g mL}^{-1}$  (Section 2.6) and cell numbers were estimated by counting using an improved Neubauer bright-line hemocytometer to achieve a cell density of approximately  $3 \times 10^6$  cells per mL (Section 2.11) ( $n = 3$ ) ( $P < 0.05$ ).

Figure 6.5 gives an example of an electron micrograph taken of *A. coffeaeformis* cells settling on the surface of produced surface, T1. Laboratory grown diatom species, with cell dimensions of approximately 6 x 3 µm are seen to settle neatly in the spaces between micro-ridges of dimensions around 10 µm. Callow *et al.*, previously noted that the 'highest number of attachment points is possible where the diatom fits within the most attractive microtexture' <sup>88</sup>. Typically, *A. coffeaeformis*, grown in the marine environment (dissimilar to the diatoms grown under laboratory conditions) have dimensions of around 14 x 7 µm <sup>203</sup>. However, under stressed laboratory conditions, the diatoms here are much smaller than those observed in the field, explaining their attachment between surface features. It is important to note that little settlement was evident on the tops of the surface features, illustrating the ability of a micro-texture to break up colonization of microfouling organisms on a surface <sup>202</sup>.

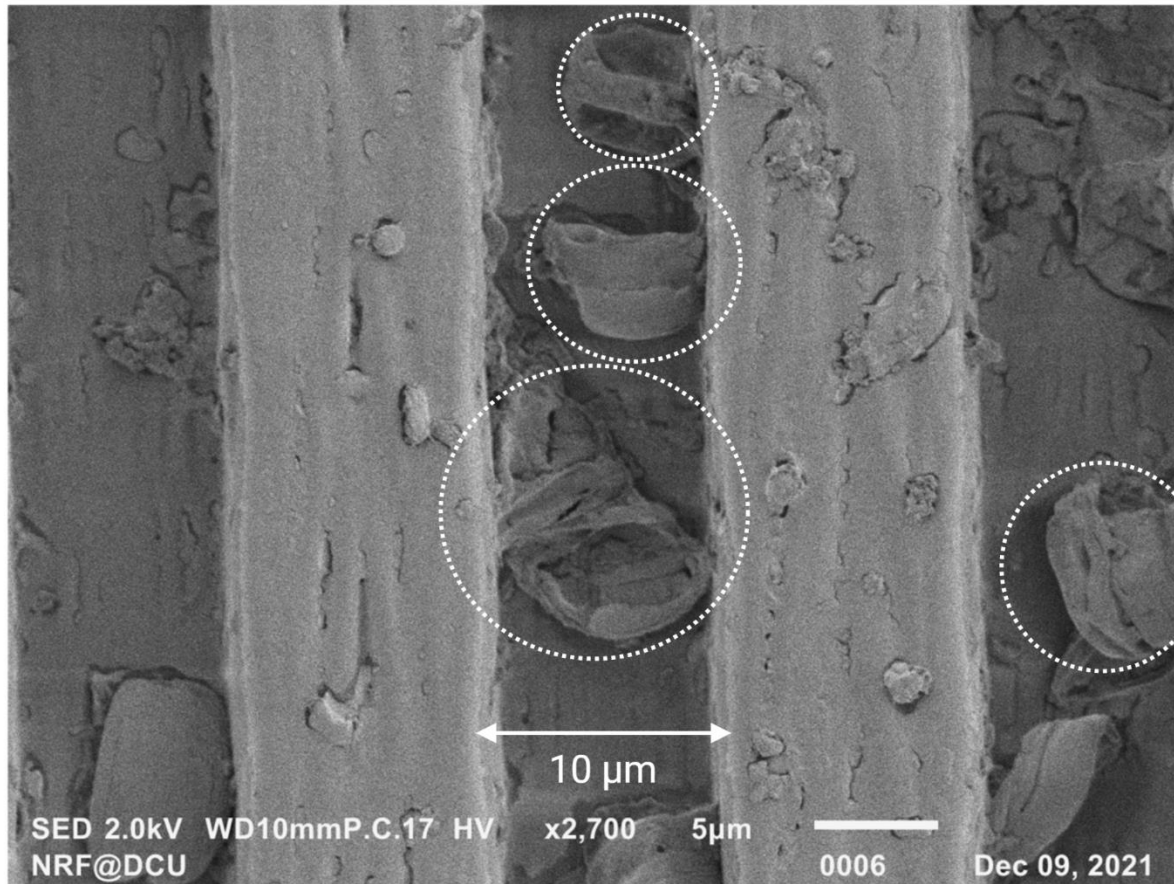


**Figure 6.5.** An example of an SEM micrograph showing *A. coffeaeformis* settlement between features on designed surface micro-texture, T1 (with average cell sizes of  $6 \times 3 \mu\text{m}$ ,  $n = 10$ ) (Mean =  $54.34 \pm 4.67\%$ ) ( $n = 3$ ).

Figure 6.6 gives an example of an electron micrograph of *A. coffeaeformis* cells settling on engineered surface microtexture, T4 ( $L_y = 5 \mu\text{m}$ ). Here, there is very little settlement of cells between surface features but instead there is an increase in the attachment of *A. coffeaeformis* cells on top of surface features. This study reinforces attachment point theory observed in previously cited work by Scardino <sup>6,202</sup>, where settlement is reduced when the number of attachment points are decreased. In this way, cellular behaviour is controlled where the peak-to-peak distances between surface features are reduced from  $10 \mu\text{m}$  to  $5 \mu\text{m}$ .

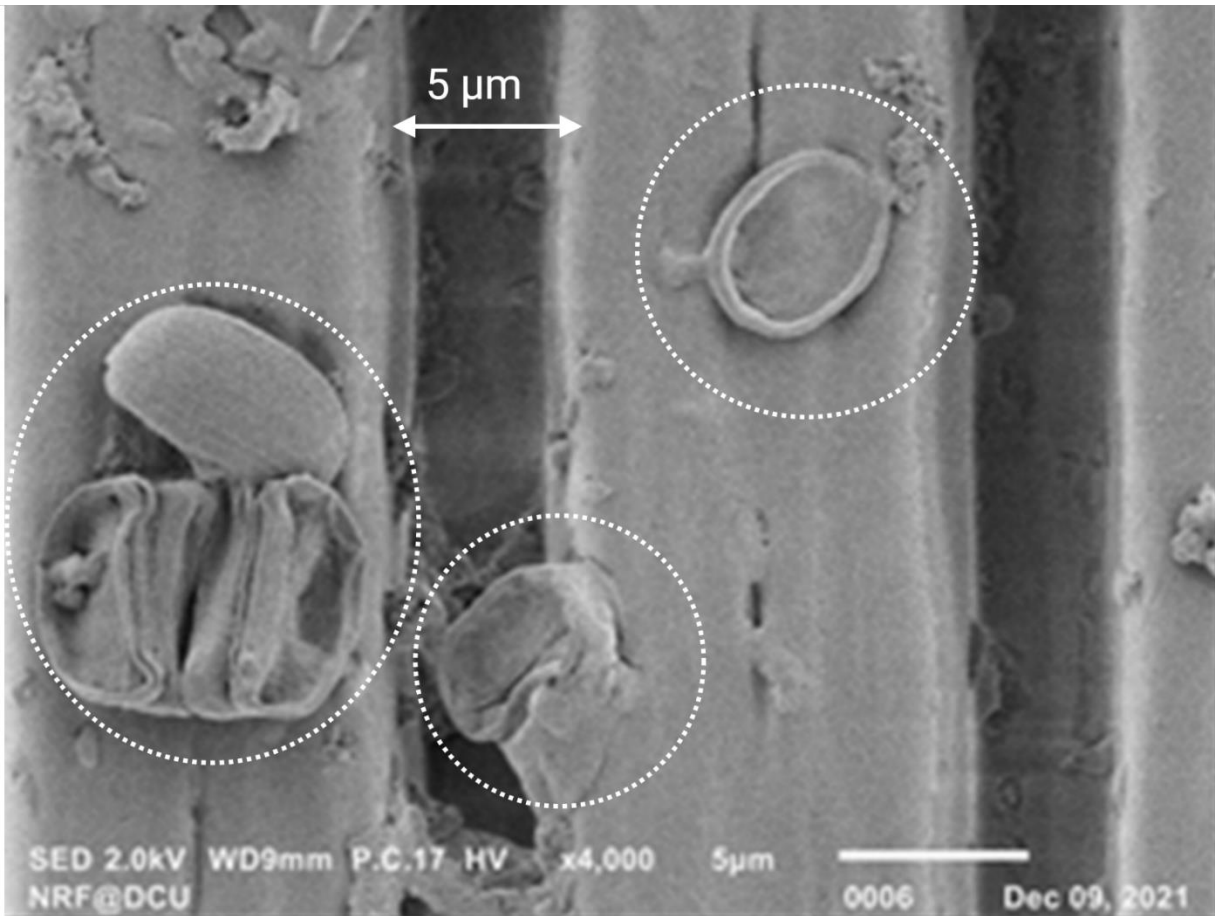


**Figure 6.6** An example of an SEM micrograph of *A. coffeaeformis* settlement on top of features on designed surface micro-texture, T4 (with average cell sizes of  $6 \times 3 \mu\text{m}$ ,  $n = 10$ ) (Mean =  $51.37 \pm 5.11 \%$ ) ( $n = 3$ ).

The same response is observed on microtextured surface, T5, with a further reduction of favourable attachment points with the decrease of peak-to-peak distances between adjacent surface features from  $10 \mu\text{m}$  (T1) to  $5 \mu\text{m}$  (T4) to  $2.5 \mu\text{m}$  (T5). However, in this case, a clustering of cells of *A. coffeaeformis* occurs, similar to that observed on smooth control surfaces, which was expected with the reduction in peak-to-peak distances between surface features, allowing cells multiple attachment points for colonization of the surface.

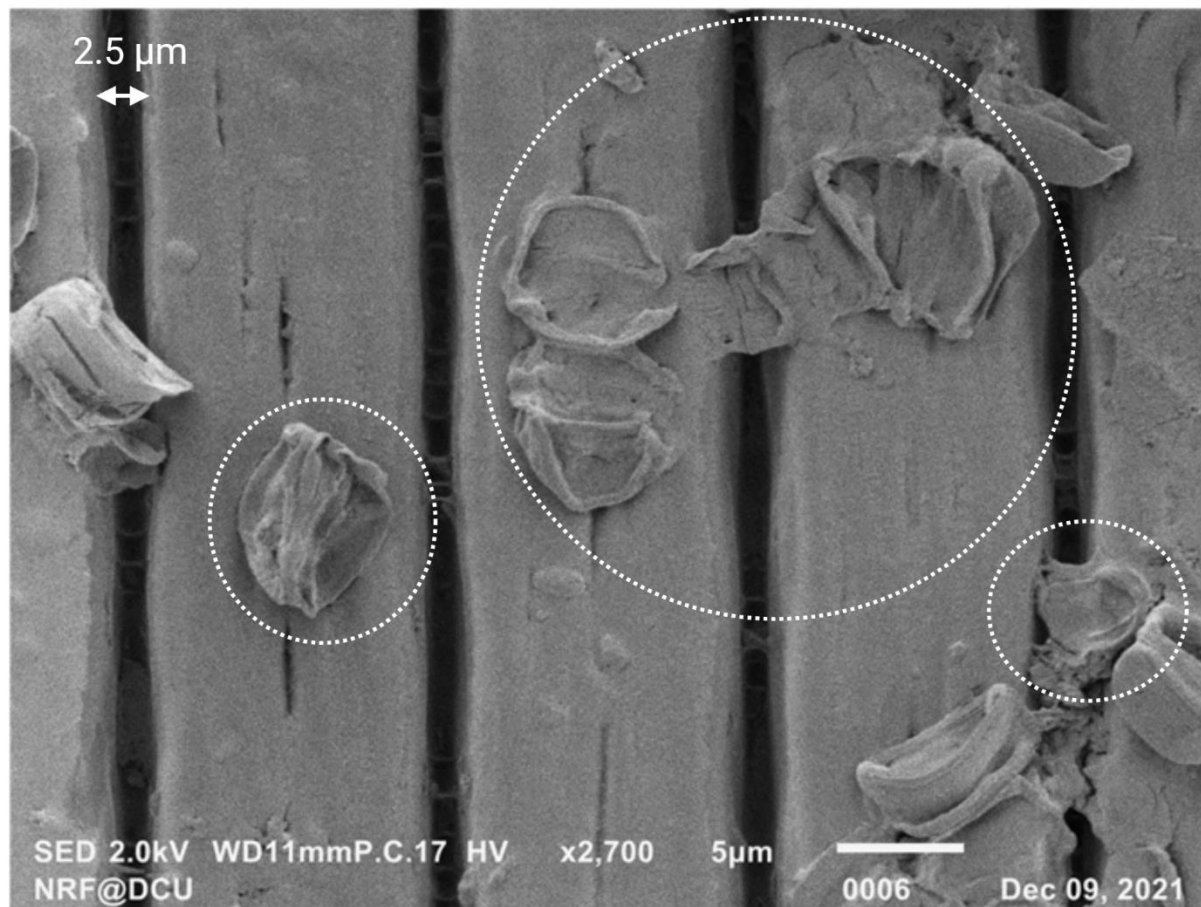


**Figure 6.7.** An example of an SEM micrograph of *A. coffeaeformis* settlement on designed surface microtexture, T5 (Mean =  $40.69 \pm 3.69$  %) (with average cell sizes of  $6 \times 3 \mu\text{m}$ ,  $n = 10$ ) ( $n = 3$ ).

The average size of a cell colony was calculated for each of the designed micro-textures presented in Figure 6.8 below. A colony is defined as 'two or more cells that group together'. The formation of larger colonies on immersed underwater surfaces can be difficult to remove, requiring rigorous cleaning to break the physiochemical bonds that hold the biofilm matrix together. Colonies may form from the same cell or appear over time in response to spore production and are usually embedded in an EPS matrix <sup>7</sup>. As shown in Figure 6.8, a reduction in colony formation of *A. coffeaeformis* cells was observed on surfaces, T1 (Mean =  $32.54 \pm 5.41 \mu\text{m}$ ), T2 (Mean =  $40.19 \pm 6.45 \mu\text{m}$ ), T3 (Mean =  $47.53 \pm 9.96 \mu\text{m}$ ), T4 ( $25.49 \pm 5.37 \mu\text{m}$ ), T5 (Mean =  $25.45 \pm 4.79 \mu\text{m}$ ) and T6 (Mean =  $13.49 \pm 2.03 \mu\text{m}$ ) in comparison to smooth surfaces.

A reduction in *A. coffeaeformis* settlement was greatest on T6. For T6, groves are filled along the full length of the array creating a series of rectangular cavities. This type of super-hydrophilic surface has been linked to reduced cavitation erosion as the gas trapped in the cavity prevents direct impingement of the liquid jets produced by the collapse of cavitation bubbles onto the surface <sup>201</sup>. However, it is worth noting that these studies involve rapid 3 h biofouling tests and the performance of T6 over time should also be investigated as colonization of the cavities is expected to occur over time.

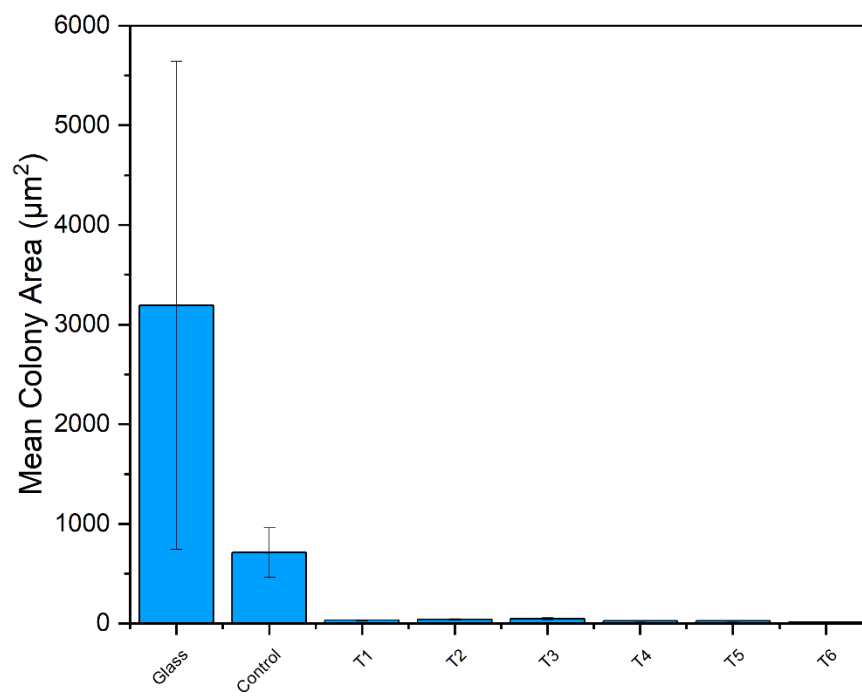


**Figure 6.8.** Mean colony area ( $\mu\text{m}^2$ ) of adhered *A. coffeaeformis* cells on each on the engineered surfaces. Standard error was used to calculate error bars ( $n = 3$ ) ( $P < 0.05$ ).

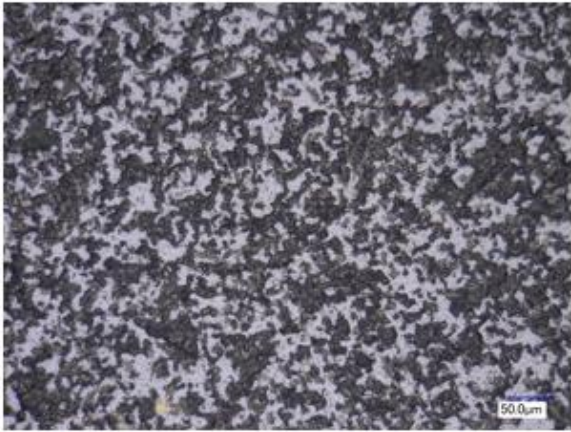
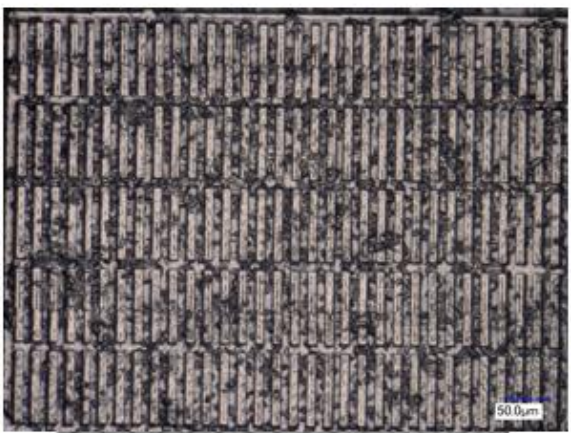
| Image                                                                               | Observations                                                                                                                                                                                                                                                                                                                            |
|-------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|    | <p><b>(a) Glass:</b></p> <ul style="list-style-type: none"> <li>• Huge aggregation of cells (i.e., forming colonies) on glass microscope slide surface.</li> </ul>                                                                                                                                                                      |
|   | <p><b>(b) Control:</b></p> <ul style="list-style-type: none"> <li>• Again, large clustering of cells occurs (aggregation).</li> <li>• The wettability (i.e., hydrophilic nature) of the polymer 3-D printing material may have some effect on cell adhesion.</li> </ul>                                                                 |
|  | <p><b>(c) Microtexture, T1:</b></p> <ul style="list-style-type: none"> <li>• The disruption of the aggregation of cells occurs here (i.e., cells settle singularly compared with patterns observed on the glass and control samples).</li> <li>• Cells adhere between features and at the end of features instead of on top.</li> </ul> |

**Figure 6.9.** An example of the images taken using the digital light microscope of cell settlement behaviour of *N. ovalis* on (a) a glass microscope slide, (b) control (i.e., polymer 3-D printing material) and (c) designed micro-texture, T1.

#### 6.4.4 *N. ovalis* settlement on textured surfaces

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Laboratory assessment of *N. ovalis* settlement on textured surfaces was performed using the 3 h rapid adhesion assay described previously. The results of the static adhesion assay is presented below in Figure 6.10. Significance testing was determined via 1-way ANOVA followed by Tukey's *post hoc* test. Textured surfaces, T1 (Mean =  $47.90 \pm 4.51$  %), T4 (Mean =  $43.92 \pm 3.23$  %) and T6 (Mean =  $39.89 \pm 3.04$  %) indicated a reduction in the adhesion of *N. ovalis* cells to the surface however this was not significant in comparison to glass (Mean =  $44.25 \pm 10.53$  %) and a smooth surface (Mean =  $30.36 \pm 4.47$  %). This indicates that the diatom species, *N. ovalis* preferentially settled on textured surfaces. The highest cell abundances were found on surfaces, T2 (Mean =  $68.20 \pm 0.87$  %), T3 (Mean =  $52.56 \pm 2.92$  %) and T5 ( $53.08 \pm 3.55$  %).

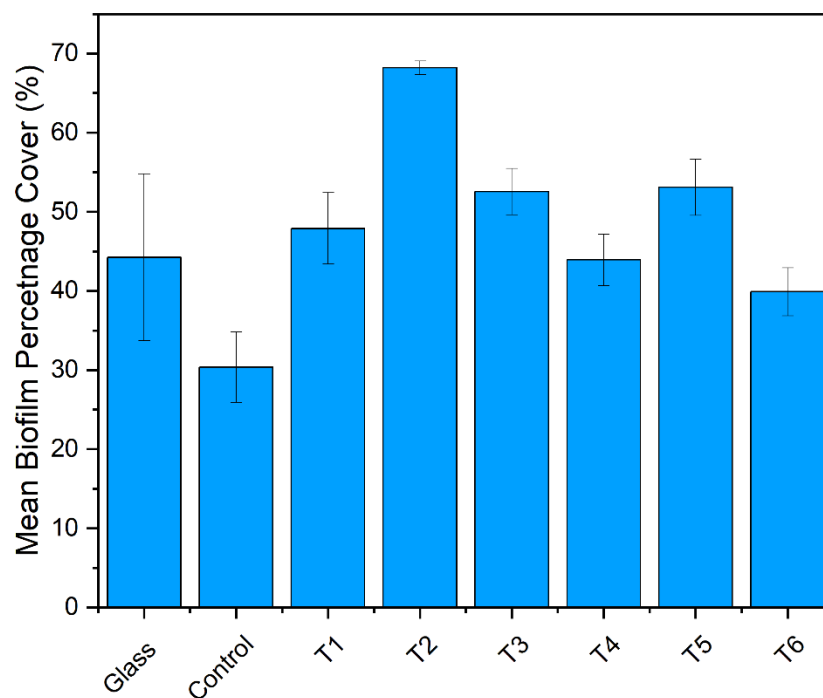


**Figure 6.10.** Mean percentage cover (%) of adhered organism, *N. ovalis*, cells on produced textured surfaces. Cells were kept at a chlorophyll  $\alpha$  concentration of  $0.3 - 0.4 \mu\text{g mL}^{-1}$  (Section 2.6) and cell numbers were estimated by counting using an improved Neubauer bright-line hemocytometer to achieve a cell density of approximately  $3 \times 10^6$  cells per mL (Section 2.11) ( $n = 3$ ) ( $P < 0.05$ ).

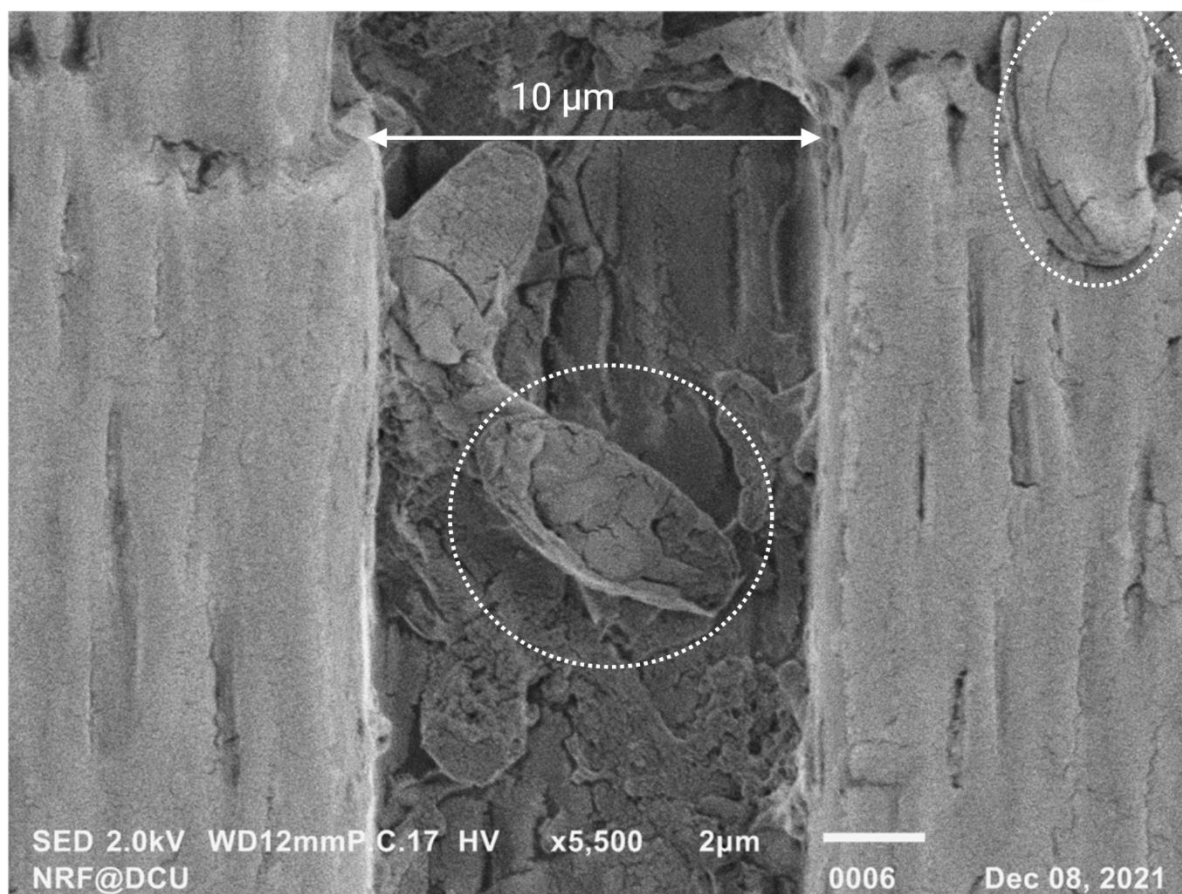


**Figure 6.11.** Scanning electron micrographs showing observed cell settlement of *N. ovalis* (with average cell sizes of  $7 \times 3 \mu\text{m}$ ,  $n = 10$ ) on textured surface, T1 ( $n = 3$ ).

A similar response to topography is observed for *N. ovalis*. Cell dimensions (i.e.,  $7 \times 3 \mu\text{m}$ ) smaller than that of the peak-to-peak distances ( $10 \mu\text{m}$ ) allow cells to preferentially settle between surface features <sup>202</sup> (Figure 6.14). As the peak-to-peak distance decreases from  $10 \mu\text{m}$  to  $5 \mu\text{m}$ , cells settle mainly on top of surface micro-structures similar to what was observed in *A. coffeaeformis* tests (Figure 6.12).

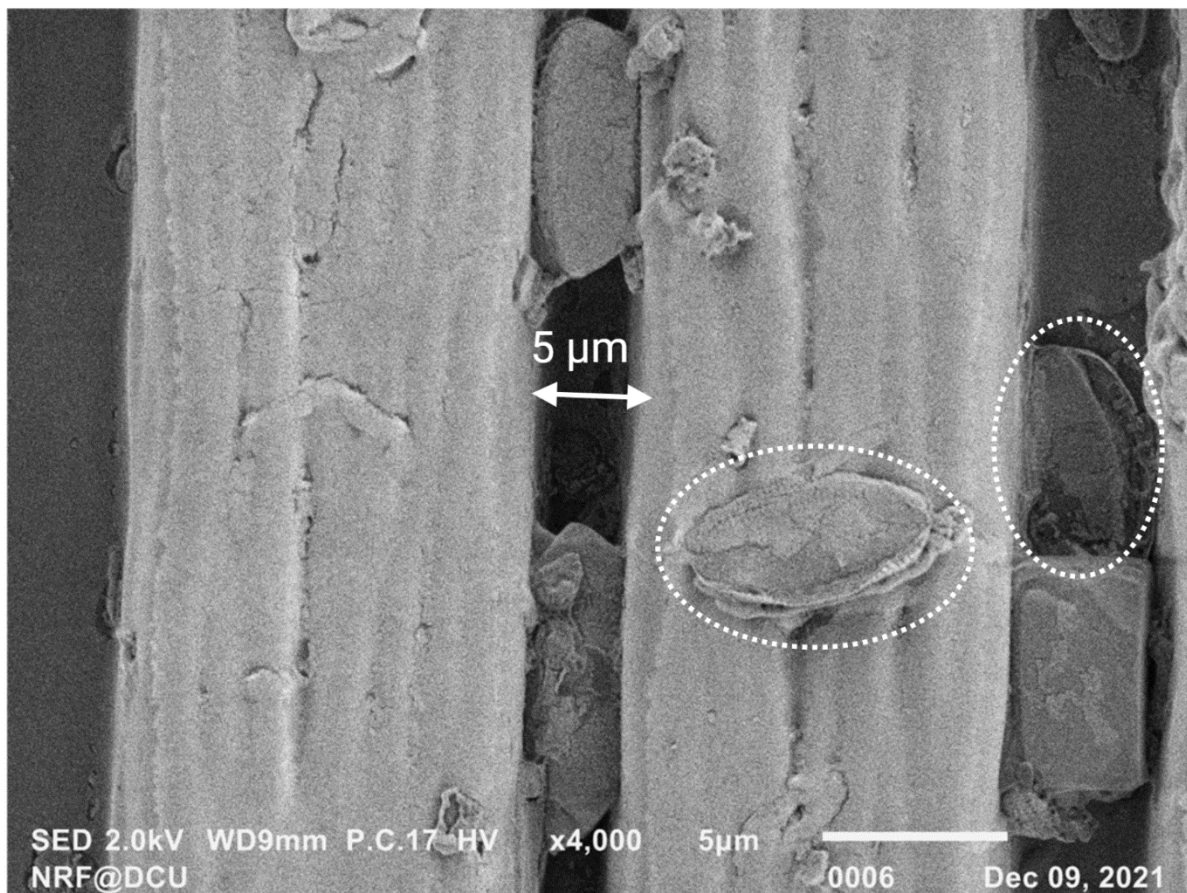


**Figure 6.12.** Scanning electron micrographs showing observed cell settlement of *N. ovalis* on textured surface, T4 (with average cell sizes of  $7 \times 3 \mu\text{m}$ ,  $n = 10$ ) ( $n = 3$ ).

Previous reports in the literature have investigated the influence of surface topography on cell size and orientation. Cell orientation is interesting here, with both *A. coffeaeformis* and *N. ovalis* cells orientating themselves to find a favourable place for attachment <sup>3</sup>. A study by Ren *et. al.*, discovered a similar response with *Escherichia coli* on 5 µm in height poly (dimethylsiloxane) (PDMS) patterns. *E. coli* cells attached on top of the line patterns were found to align themselves perpendicularly to the line patterns. Cells that settled between the surface topographic patterns preferred to settle parallel to the orientation of the surface feature. In addition, colony formation of *E. coli* was reduced <sup>204</sup>. Hsu *et al.*, carried out a study on the attachment of microorganisms, *E. coli*, *L. innocua* and *P. fluorescens* on silica and alumina surfaces with micro- and nano- topographical patterns <sup>199</sup>. Results from the study concluded that cell orientation was dictated by the way in which a cell can maximize their contact with a surface (i.e., for stronger adhesion). The same response is observed here. Cells settling on T1 (Figure 6.11) maximize their contact with the surface by attaching in between surface features allowing for increased stability and protection from external stresses. On T4 (Figure 6.12) and T5 (Figure 6.13), cells are noted as attaching horizontally across the top of surface microridges or tightly packed between the 5 µm distance amongst adjacent features.

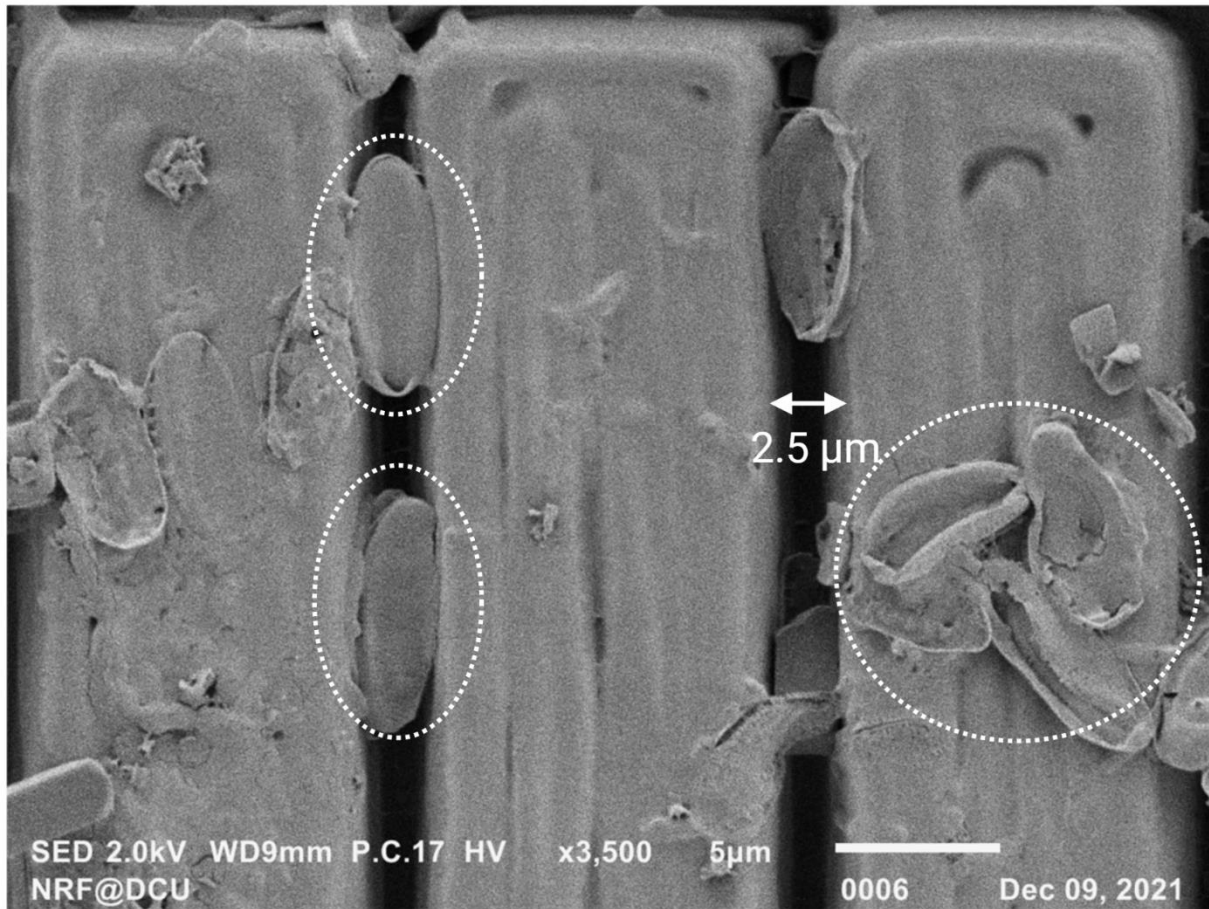


**Figure 6.13.** Scanning electron micrographs showing observed cell settlement of *N. ovalis* on textured surface, T5 (with average cell sizes of  $7 \times 3 \mu\text{m}$ ,  $n = 10$ ) ( $n = 3$ ).

The average size of a colony on each of the textured surfaces and smooth surfaces were calculated using Fiji (ImageJ) software ( $n = 3$ ) as described in Chapter 2 (see methods, section 2.13). Significance was determined via 1-way ANOVA testing followed by Tukey's *post hoc* test using Sigma Plot 11.0 software. The results of these tests are presented below in Figure 6.17. Significant differences ( $P < 0.05$ ) were noted on all produced surfaces T1 (Mean =  $22.3 \pm 3.57 \mu\text{m}$ ), T2 (Mean =  $76.09 \pm 11.41 \mu\text{m}$ ), T3 (Mean =  $49.11 \pm 11.7 \mu\text{m}$ ), T4 (Mean =  $15.59 \pm 2.61 \mu\text{m}$ ), T5 (Mean =  $36.1 \pm 7.88 \mu\text{m}$ ) and T6 (Mean =  $9.06 \pm 3.9 \mu\text{m}$ ), in comparison with both control (Mean =  $152 \pm 23.34 \mu\text{m}$ ) and glass (Mean =  $1061.43 \pm 477.64 \mu\text{m}$ ) samples. The results presented here indicated that the average size of a colony was significantly reduced through the incorporation of surface topographical features on a surface.

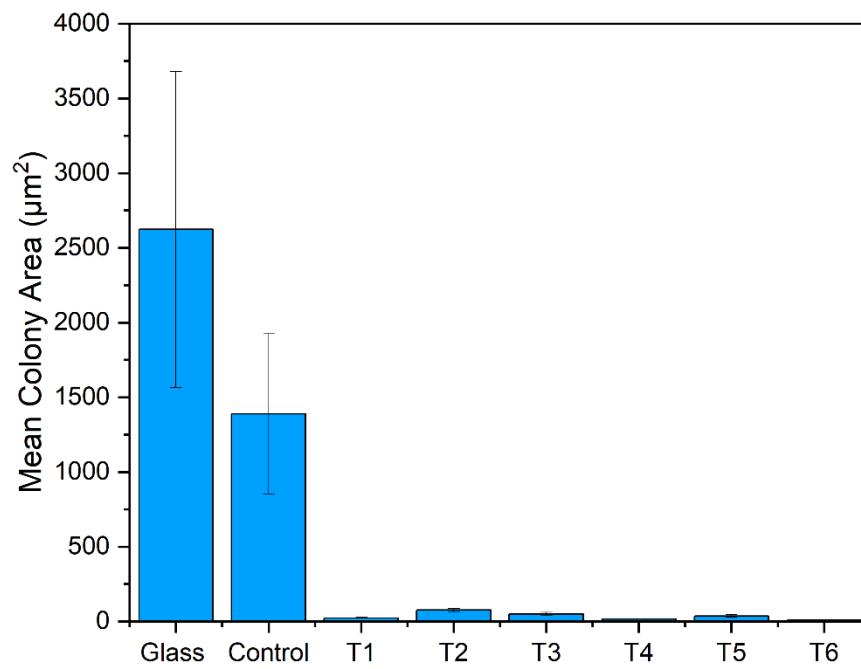


**Figure 6.14.** Mean colony area ( $\mu\text{m}^2$ ) of *N. ovalis* cells on produced textured surfaces. Standard error was used to produce error bars ( $n = 3$ ) ( $P < 0.05$ ).

#### 6.4.5 *Dynamic adhesion studies*

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The objective of this work was to disrupt the adhesion of biofouling organisms using the designed surface micro-textures. The static studies indicate that test organisms behave differently on micro-textures versus the control (i.e., the form of the organism's structure is different). The spinning of cells using centrifugal force was used to determine changes in the detachment of biofouling organisms in response to topographical pattern on a commercial spin stand and modified spin coater as described previously. 1-way ANOVA testing was performed to test for significant differences. No significant differences ( $P > 0.05$ ) were observed for studies involving the spin stand in terms of a reduction in biofouling. The cause of this could be linked to the type of stresses generated by centrifugal force. In centrifugal force, stresses are applied uniformly in the radial direction. A different result could be anticipated in a marine environment, whereby forces change direction with the passing of eddies. The spinning frequency used in this case (70 Hz) which was the maximum capacity of the spin stand is also generating stresses lower than the expected hydrodynamic stresses.

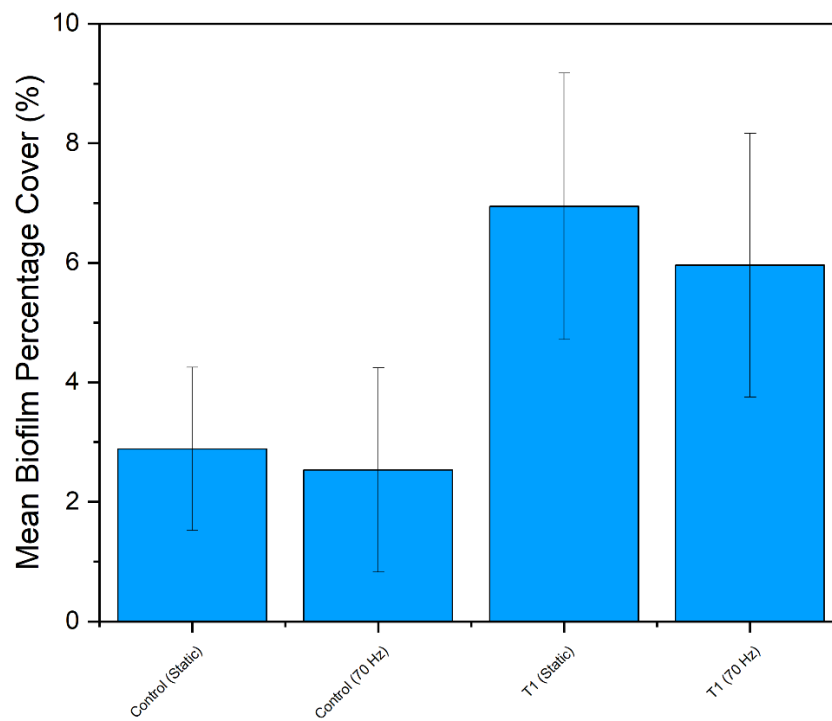


**Figure 6.15.** Mean biofilm percentage cover (%) of *N. ovalis* cells on produced surface, T1, in comparison to a control sample under static and dynamic (70 Hz) conditions (n = 3). There were no significant differences found here determined by 1-way ANOVA.

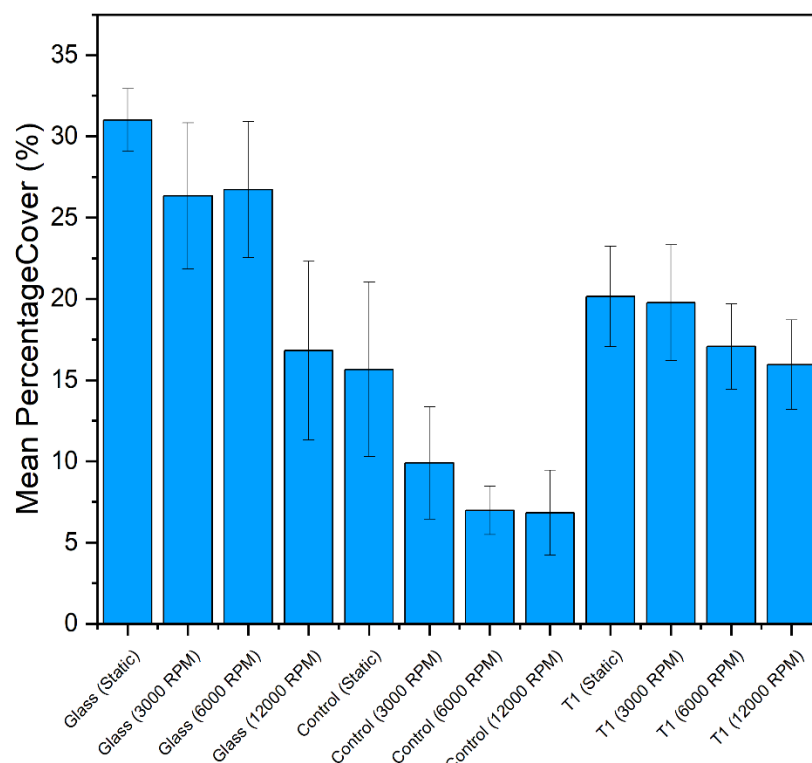


**Figure 6.16.** Mean biofilm percentage cover (%) of adhered *N. ovalis* cells on engineered surface microtexture, T1, in comparison to control and glass samples (n = 3).

A reduction in biofilm cover (%) was observed on glass, the smooth control (3-D printed polymer material) and T1 from static to dynamic conditions of 12000 RPM. Biofilm levels were higher on glass and the textured surface, T1, in comparison to the smooth 3D printed surface, indicating that *N. ovalis* cells preferentially settled within textured surfaces. As described previously, centrifugal stresses are applied radially, which could mean that *N. ovalis* cells are being trapped in the microtexture, making it difficult to dislodge them from the surface in dynamic studies. A huge reduction (14.19 % decrease) in biofilm cover was observed for the glass microscope slide and an 8.82 % decrease in cell attachment was observed for the control sample. This may indicate that smooth surfaces (i.e., with

multiple attachment points for effortless cell adhesion) allow for the removal of settled cells. A 4.20 % decrease in biofilm cover was observed for cells on a microtextured surface under static to dynamic (12000 RPM) conditions, which signifies cell entrapment within the microtextured material.

#### 6.4.6 Comparison of observed diatom settlement to theoretical models

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Initial results from adhesion studies involving *A. coffeaeformis* and *N. ovalis* under static laboratory conditions indicate that textured surfaces T3, T4, T5, and T6 show promise as an alternative non-toxic antifouling (AF) solution. Textured surface, T3, highlighted the influence of feature height (20  $\mu\text{m}$ ) on adhesion of *A. coffeaeformis* cells to the surface. In this case, the increase in surface feature height was found to influence cells to preferentially settle between peak-to-peak distances and reduced cell adhesion on the top of surface features. T4 (feature width = 5  $\mu\text{m}$ ) and T5 (feature height = 2.5  $\mu\text{m}$ ) reduced the adhesion of biofouling organisms to the intervals between features with most settling on top of surface microfeatures in comparison to surface T1, where cell settlement is evident on and between surface features. A similar response was noted in a study by Scardino *et. al.*,<sup>202</sup>, whereby lower fouling was observed when the width of the designed microtexture was slightly smaller than the width of the attaching microorganism. T6, mentioned previously to be linked with reduced biofouling adhesion through the entrapment of gas in cavities showed real promise – however over time, these cavities have the potential to ‘clog’ and provide a favourable surface for attachment. There was no significance observed in dynamic adhesion studies carried out via spin stand and spin coater processes however further work is required in the optimization of these dynamic studies.

The theory behind the attachment observed in this chapter is that of Attachment Point Theory as previously discussed in Section 6.1. It was found that increasing the number of attachment points a surface has greatly influences cell adhesion on surfaces described within this chapter. The reduction of the interval between features from 10  $\mu\text{m}$  (T1) to 5  $\mu\text{m}$  (T4) and 2.5  $\mu\text{m}$  (T5) greatly limited biofilm adherence from settling between features on T1 to settling on top of features on T4 and T5. Colony formation was also noted to be decreased with the introduction of a surface topography with *A. coffeaeformis* cells.

In conclusion, T1 was chosen as the designed micro-texture for incorporation onto tidal energy blades due to its ability to disrupt cell colony formation in rapid biofouling studies conducted using the two diatom species, *A. coffeaeformis* and *N. ovalis* as well as meeting requirements for scale-up for application on tidal energy blades.

## 6.5 Conclusion

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The introduction of a topographical pattern causes a disruption in the settlement of diatom cells, *A. coffeaeformis* and *N. ovalis* cells to the surface. The aim of this work was to produce a successful antifouling technology based on surface topography modification for application to tidal energy blades. From this study, the ability to control cell response with the introduction of topography was evident. It was found that cells smaller ( $< 10\ \mu\text{m}$ ) than the peak-to-peak distances between surface features will sit comfortably between adjacent features, with little to no settlement observed on top of surface features (T1). As the peak-to-peak distances were decreased, as observed in T5 ( $2.5\ \mu\text{m}$ ), cells begin settling on top of surface features instead of between uniform gaps (to allow for stronger binding to the surface as previously explained). Micro-textures with sharpened edges (T1, T3, T4, T5 and T6) were noted to have reduced attachment in comparison to micro-texture, T2, with rounded edges – potentially due to reduced ‘contact points’ a sharpened edge provides in comparison to rounded edges (which essentially increases the surface area of a micro-texture). A significant reduction in colony formation was observed on all micro-textured surfaces with both diatom species in comparison to control glass and 3-D printed polymer material.

Overall, T1, was successful in disrupting biofilm formation (i.e., colony formation) of two diatom species, *A. coffeaeformis* and *N. ovalis* and was chosen as the antifouling technology to be incorporated onto tidal energy blades as part of the Horizon 2020 NEMMO project, which will be laser micromachined on composite blade material and tested on a rotating blade in field studies this year. This study illustrated effectively for the first time the potential of controlling cellular settlement on a surface through the modification of topographical pattern inspired by the Brill fish, *S. rhombus*.

# Chapter 7:

Significance of the study and future research  
directions

The prevention of biofouling in the marine environment and the development of novel non-toxic antifouling systems have become increasingly important in recent years. The development of antifouling technology requires careful evaluation of cell-surface interaction to be able to inhibit cellular attachment at the microfouling and macrofouling levels. The aim of this thesis was to investigate a number of antifouling solutions for the control of biofouling in the marine environment. The work presented in this thesis demonstrates the potential for the use of developed environmentally benign sol-gel coatings on transparent materials used in sensor development, reducing the requirement for regular maintenance and cleaning. In addition, the thesis demonstrates the potential of using surfaces inspired by nature, in this case, *S. rhombus*, for application in tidal energy systems.

A 12-month case study was conducted in Dublin Bay, to inform on biofilm composition and diversity in Irish waters. Biofilm composition was determined to be heavily affected by environmental parameters (i.e., light, temperature, location), with the highest concentration of protein and carbohydrate found during the Winter fouling season. *Proteobacteria* and *Cyanobacteria* were among the dominant taxa of prokaryotic communities, consistent with reports found in the literature. *Arthropoda*, *Tunicata* and *Chlorophyta* were dominant phyla in eukaryotic communities, which were also observed using visual inspection of the panels. Diatom phyla were not identified in molecular sequencing which is could be due to the unavailability of diatom library databases for use in 18S sequencing, which highlights that the use of microscopy to identify diatom genera can be an efficient means of identifying common biofouling organisms. Scanning electron microscopy

identified *Amphora*, *Navicula*, *Minidiscus* and *Cocconeis* as the most common fouling organisms found on the panels, with overall low monthly diversity. This study helped inform on the most common diatom species present in Irish waters. Diatom species, *Amphora coffeaeformis* and *Nitzschia ovalis* were chosen for use in diatom adhesion assays to evaluate cell behaviour on microtextured materials.

Environmentally benign sol-gel coatings were investigated for application as an antifouling (AF) system in sensor technology. Water quality monitoring using autonomous sensors suffers many challenges in the aquatic environment of which biofouling is one that impacts cost of maintenance, data quality and integrity. In high fouling season, sensors often require maintenance and cleaning every two weeks adding cost to the monitoring programme. The findings of this study revealed the most successful coating for microfouling was one that consisted of a precursor, tetraethyl orthosilicate (HC006) that has a water contact angle  $> 100^\circ$ , without significant roughness (43.52 nm), determined over a 9-mo deployment. However, the improved roughness and wettability of a second coating, Diethoxydimethylsilane (DMDEOS), showed real promise in relation to the reduction of macrofouling over a 13-mo deployment.

This thesis also presented the characterization of the physical and chemical properties of the Brill fish scale for the first time. The dimensions of the Brill fish scale were determined to be 74  $\mu\text{m}$  (length) x 15.8  $\mu\text{m}$  (width) x 11.7  $\mu\text{m}$  (height). From this characterization, an adapted replication of the Brill fish scale, *S. rhombus*, was made using three different microfabrication methods: 2-photon polymerization (2PP), mold casting and Repliset-F5 casting. The fidelity of each of the replications were recorded using SEM, WCA and profilometry. It was determined that the replications made using the 2-photon polymerization process offered the greatest control over surface features – an advantage for understanding cellular attachment on surface topography. 2PP was chosen as the method of choice for the fabrication of micro-textured materials for future antifouling studies.

An investigation into cellular attachment on development marine-inspired microtextured materials was also conducted within this thesis using both static and dynamic trials. It was determined that the incorporation of a surface topography, caused a disruption in the growth of cell colonies of *A. coffeaeformis* and *N. ovalis* cells on the surface in comparison to control smooth surfaces. Microtexture, T1 offered the ability to control cellular response at the microfouling level (i.e., diatoms). The idea for this was that if you disrupt the growth of microfouling communities, you may also disrupt the growth of macrofouling communities, which add significant costs to the cleaning of marine monitoring equipment, for example. The dimensions of microtexture, T1, were observed to cause a disruption in biofilm percentage cover (%) and colony formation. T1 could also be easily replicated and scaled-up using commercial micromachining methods. Following on from this work, designed surface

micro-texture, T1, has been selected and will be scaled-up (i.e., by a laser micromachining process) onto a tidal energy blade as part of the EU-funded Horizon 2020 project 'NEMMO'. T1 will then be tested for its antifouling effectiveness in field trials on the energy blade system this year.

This thesis successfully demonstrated the novel work for developing an antifouling mechanism, based on a biomimetic approach. The thesis outlines the complete process of developing AF technology, from the initial design concept (i.e., the Brill fish, *S. rhombus*), to the implementation of a modified 3-D printed micro-textured material (T1) for application in tidal energy, following successful laboratory antifouling studies using two grown diatom species, *A. coffeaeformis* and *N. ovalis*. It is hoped that the knowledge gained through the development of a biomimetic AF technology in this thesis can be used to progress the development of future environmentally friendly AF technologies, reducing the requirement for regular cleaning and maintenance of marine industrial equipment.

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