Biodegradation of poly(ethylene terephthalate) by marine bacteria, and strategies for its enhancement

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Abstract

Plastic accumulation, particularly in the world’s oceans is of increasing environmental concern. One of the major components of plastic waste is poly(ethylene terephthalate) (PET), a polymer frequently used in many applications, including textiles and food packaging. The current methods of disposal of PET waste, landfill, incineration and recycling, each have inherent drawbacks and limitations, and as such there is a need for efficient and cost-effective alternative.

Biodegradation is an attractive option for environmentally friendly and efficient disposal of plastic waste. To date, no protocol has yet been developed to feasibly dispose of PET by biodegradation con a commercial scale. The current works aims to investigate the potential of PET biodegradation as a plastic disposal procedure by providing fundamental knowledge of biodegradation processes, and to develop strategies for improving biodegradation efficiency.

PET samples were incubated in marine bacterial community enrichment cultures, and the dynamics of the polymer – bacterial interactions traced. Modifications to polymer surfaces were monitored using a variety of surface characterisation techniques, including atomic force microscopy (AFM), x-ray photoelectron spectroscopy (XPS) and infrared microspectroscopy using Synchrotron radiation. Taxonomic members of the bacterial enrichment cultures that developed in the presence of PET were recovered and identified via 16S rRNA gene sequencing.

Marine bacteria were shown to possess the ability to degrade PET surfaces. Larger topographical surface features were degraded preferentially, which resulted in overall smoothening of the polymer surface. Subtle changes in the chemistry of the PET surface were also detected, which were indications of polymer chain cleavage and oligomer excision.

Bacterial enrichment communities were dominated by members of α- and γ-proteobacteria, with contributions from β-proteobacteria. Among the isolates with the highest potential for PET biodegradation, many returned nearest taxonomic relatives of species of Thalassospira, Kordiimonas and Alteromonas. Commensal relationships
appeared to be required for effective PET biodegradation, with several sulphur-cycling strains showing potential participation in PET degrading communities.

Several strategies for enhancement of the rate of PET biodegradation were also put forward. Increasing the size of bacterial enrichment communities by supplementation with essential elemental nutrients in nitrogen and phosphorus proved effective in increasing the rate of biodegradation, as did alteration of the composition of the communities by increasing the selective advantage of PET-degrading strains through incubation in dark conditions. An alternative approach through manipulation of the polymer was another effective option; pre-treatment of PET samples with surfactants increased their surface roughness, which enabled bacterial cells greater access to the polymer chains.

Limited cell attachment to PET surface was identified as one of the major barriers to biodegradation. To address this, several unrelated surface topographies were tested for the ability to promote cell adhesion, for eventual application on PET surfaces. Of those tested, a superhydrophobic topography based on the structure of the lotus leaf showed the most promise for enhancement of biodegradation through promotion of cell attachment.

The results of this study represent a significant contribution to the fundamental understanding of PET biodegradation mechanisms. The knowledge obtained here can be used for the further development of biodegradation as a technique for disposal of PET waste.
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Declaration

I, Hayden Webb, declare that this thesis is my original work and contains no material that has been accepted for the award of Doctor of Philosophy, or any other degree or diploma, except where due reference is made.

I declare that to the best of my knowledge this thesis contains no material previously published or written by any other person except where due reference is made. Wherever contributions of others were involved every effort has been made to acknowledge the contributions of the respective workers or authors.

Signature ______________________________________________________________
List of publications

**Online Book:**


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**Peer-reviewed articles:**


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Table 5.8. Susceptibility of Celeribacter neptunius H14\textsuperscript{T} to various antibiotics.

Table 5.9. Comparison of biochemical characteristics of Celeribacter neptunius to those of closely related strains.

Table 5.10. Comparison of biochemical characteristics of Alteromonas australica to that of other species of Alteromonas.

Table 5.11. Susceptibility of Alteromonas australica H17 to various antibiotics.

Table 6.1. Quantitative roughness analysis of titanium thin films deposited on silicon wafer substrata.
Chapter 1: Introduction
1.1. Overview

As a material, poly(ethylene terephthalate) (PET) possesses a number of properties that make it extremely popular for a wide range of applications. PET is strong and durable, chemically and thermally stable, has low gas permeability, high clarity and low electrical conductance, and is easily handled and processed (Kint and Muñoz-Guerra 1999, Awaja and Pavel 2005). These properties, among others, make PET an appealing material for use in a number of applications, such as synthetic fibres and textiles, packaging applications, sheets and films, electronics, automotive parts, houseware, lighting products, power tools, sports goods and photographic and x-ray materials (Awaja and Pavel 2005, Sinha et al. 2010). The primary functions of PET are in food packaging and synthetic fibres; PET accounts for more than half of the synthetic fibre production around the world, and global consumption of the polymer exceeds $17 billion (Sinha et al. 2010).

However some of the very properties that make PET such a popular material also lead to the polymer posing a significant environmental threat, particularly to the world’s oceans (Moore 2008, Ryan et al. 2009). The stability and durability of PET mean that it is exceedingly slow to degrade in the environment, and this coupled with the high popularity and manufacture of PET leads to its accumulation (Derraik 2002, Moore 2008). Also, as plastics often have a tendency to float, much of the PET in the environment is washed away via storm water etc. and ultimately makes its way to the ocean. Once there it can cause considerable harm to marine wildlife and damage to marine environments (Azzarello and Van Vleet 1987, Baird and Hooker 2000, Barreiros and Barcelos 2001, Rios et al. 2007, Gregory 2009, Denuncio et al. 2011, Ivar do Sul et al. 2011).

To date, only three plastic disposal methods are routinely used on a large scale: landfill, incineration and recycling. Each technique has disadvantages and drawbacks. Landfill and incineration both lead to the release of dangerous secondary pollutants into the environment, and landfill also has an additional drawback in the requirement of large portions of land space (Zhang et al. 2004, Simoneit et al. 2005, Urase et al. 2008, Valavanidis et al. 2008). Recycling addresses the environmental concerns of landfill and incineration however the process is relatively inefficient and less cost effective, and
subsequently there is less incentive for investment in recycling facilities (Paci and La Mantia 1999, Awaja and Pavel 2005).

Biodegradation is an attractive alternative plastic disposal method which has the potential to be both environmentally friendly and cost effective. However as of yet, no viable biodegradation protocol has been developed for PET. Several attempts have been made in the literature, however the only study in which substantial PET biodegradation was achieved used a protocol that would be unsuitable for large-scale commercial application due to prohibitive expense (Müller et al. 2005).

While considerable work has gone into understanding the abiotic environmental degradation mechanics of PET (Andrady 2011, Raquez et al. 2011), there is little literature available that specifically addresses biological processes in degradation of the polymer. Most studies simply assume that biodegradation of PET occurs by depolymerisation through breakage of the ester bonds, and although this is certainly a possibility there is a tendency not to consider alternatives. Many cases exist in nature where different organisms evolve different techniques to achieve the same goal.

1.2. Aims of the study

The primary objective of this study is to contribute toward the development of biodegradation as an effective and viable alternative PET disposal procedure, using marine bacteria as the degradative agents. As mentioned above, a large proportion of PET waste is found in the ocean, and as such the marine environment is a good prospective source for organisms which are already starting to develop PET degrading metabolic pathways. Three intermediate objectives were identified as stepping stones toward novel PET biodegradation techniques.

The first intermediate objective is to determine whether or not marine bacteria possess the capability for biodegradation of PET, and to assess their potential to do so. This involves characterisation and comparison of PET surfaces both before and after exposure to bacterial interactions, to identify what modification, if any, the bacteria can induce. Achievement of this objective should contribute significantly to the fundamental understanding of the mechanisms by which PET biodegradation occurs.
The second intermediate objective is to characterise the composition of any bacterial communities which prove to be capable of biodegradation of PET. Taxonomic identification of the bacterial species which are required for PET biodegradation will provide clues as to what culture conditions may help to promote degradation. Additionally, an important question to be answered under this objective concerns the number of individual taxonomic strains required for effect biodegradation of PET, i.e. can PET be degraded by a single bacterial species, or does it require the co-operation of an entire bacterial community.

The third intermediate objective is the development of one or more strategies to enhance the rate at which marine bacteria are able to degrade PET. Biodegradation processes need to be as efficient as possible for potential application on a commercial scale. This objective can only be addressed after fundamental knowledge of the mechanisms of biodegradation and the bacterial strains responsible, however depending on their results some possible approaches may be supplementation of growth media with trace nutrients or growth factors, or manipulation of PET surfaces to make them more conducive for biodegradation.

In the coming chapters, the current state of knowledge concerning PET biodegradation will be reviewed, after which an overview of the methods and techniques used in this study is presented. Following this, experimental results detailing the way in which PET biodegradation occurs, which bacterial strains are responsible and possible degradation enhancement strategies are presented, followed by general discussion of the topic and conclusions drawn. This work increases the fundamental understanding of PET biodegradation processes, and provides a significant contribution toward the development of new PET disposal protocols.
Chapter 2: Literature review
2.1. Overview

This chapter aims to present a detailed overview of the current state of knowledge in areas that related to biodegradation of poly(ethylene terephthalate) (PET). This includes an outline of the problems associated with plastic pollution in the marine environment, description of the properties and application of PET, analysis of the current state of research on the biodegradation of PET and discussion of some of the analysis techniques that can be used for tracking biodegradation.

2.2. Plastics

2.2.1. Environmental impacts of plastic pollution

Plastic pollution in the marine environment is causes several hazardous and ecologically damaging effects. Plastic debris poses a direct threat to wildlife, with many and varied species documented as being negatively impacted by plastic items. The main dangers associated with plastic objects for most species surround entanglement in and ingestion of said items (Derraik 2002). Juvenile animals in particular, often become entangled in plastic debris, which can result in serious injury as the animal grows (Pemberton et al. 1992, Sazima et al. 2002), not to mention restriction of movement, preventing animals from properly feeding, and, in the case of mammals, breathing (Gregory 2009). A wide variety of species have been reported to be impacted by plastic debris; marine birds (Azzarello and Van Vleet 1987, Blight and Burger 1997), sea turtles (Barreiros and Barcelos 2001), cetaceans (Baird and Hooker 2000), fur seals (Pemberton et al. 1992), sharks (Sazima et al. 2002), and filter feeders (Moore et al. 2001) are just a few of the affected species documented. Marine birds are particularly susceptible to ingestion of plastic objects that they mistake for food (Azzarello and Van Vleet 1987, Blight and Burger 1997). Plastic ingested by these animals persists in the digestive system, and can lead to decreased feeding stimuli, gastrointestinal blockage, decreased secretion of gastric enzymes, and decreased levels of steroid hormones, leading to reproduction problems (Azzarello and Van Vleet 1987).

Plastic particles in the ocean have been shown to contain quite high levels of organic pollutants. Toxic chemicals such as polychlorinated biphenyls (PCBs), nonylphenol (NP), organic pesticides such as DDT, polycyclic aromatic hydrocarbons
(PAHs), polybrominated diphenyl ethers (PBDEs), and bisphenol A (BPA) have been consistently found throughout oceanic plastic debris (Mato et al. 2001, Rios et al. 2007, Hirai et al. 2011). The presence of these compounds further increases the risks associated with ingestion of plastic debris by wildlife, and additionally many of these compounds can undergo significant biomagnification and may potentially pose a risk to humans (Hirai et al. 2011). PCBs are associated with developmental impairment (neurological impairment, growth abnormalities, and change in the onset of puberty), cancer and endocrine disruption (Schecter et al. 2010). PBDEs cause neurobehavioural changes and endocrine disruption (Trudel et al. 2011). Organic pesticides, especially DDT, have been linked to arthritis, breast cancer, diabetes, neurobehavioural changes and DNA hypomethylation (Schecter et al. 2010). PAHs are carcinogenic, cytotoxic and mutagenic (Chung et al. 2011), and NP and BPA have been reported to be endocrine disruptors (Zhou et al. 2011).

Plastic debris also provides increased opportunity for dispersal of many potentially invasive species to new environments (Wheeler 1916, Censky et al. 1998, Masó et al. 2003, Barnes and Milner 2005, Gregory 2009). Colonisation and subsequent dispersal of marine species is, unsurprisingly, common, with many reports of barnacles (Barnes and Milner 2005, Gregory 2009), bryozoans (Barnes and Milner 2005, Gregory 2009), polychaetes (Barnes and Milner 2005, Gregory 2009), dinoflagellates (Masó et al. 2003), algae (Gregory 2009), and molluscs (Gregory 2009) found adhered to plastic debris. Terrestrial animals are also capable of riding marine debris to new areas, ants have been reported to ride debris from the Brazilian mainland to San Sebastian Island, several kilometres away (Wheeler 1916). There are even examples of animals as big as iguanas riding flotsam to new islands in the Caribbean (Censky et al. 1998).

2.2.2. Levels of plastic in the marine environment

There have been many efforts at quantifying the level of plastic pollution in the marine environment, the vast majority of which focus on the deposition of debris on beaches (Frost and Cullen 1997, Walker et al. 1997, Martinez-Ribes et al. 2007, Morishige et al. 2007, Santos et al. 2008). This is probably due to the fact that plastics generally float, and therefore tend to accumulate on beaches (Santos et al. 2008). The bulk of debris washed onto beaches studied, in terms of numbers, is composed of plastic.
items; often three quarters of items washed onshore are made of plastic (Morishige et al. 2007, Santos et al. 2008). Such high levels of plastic debris in the environment can be attributed to the high availability of plastic products and its ability to persist in the environment (Frost and Cullen 1997, Ivar do Sul and Costa 2007).

In a study investigating the amount of plastic in the open ocean as opposed to on beaches, the levels of plastic particles was compared to zooplankton in surface waters in the North Pacific Ocean (Moore et al. 2001). Zooplankton outnumbered plastic particles approximately five to one, however, the total plastic collected outweighed zooplankton by approximately 6 times. Other studies have reported similar abundances of plastic pollution. During an expedition in the Atlantic Ocean, the total floating marine debris (including naturally occurring debris such as kelp) was characterised from as far south as the Southern Ocean, all the way north to the high Arctic (Barnes and Milner 2005). In most samplings plastic was the most abundant type of debris, reaching as high as 92% of items collected in one sampling, and plastic was present in every 10° latitudinal belt. A separate study monitoring plastic debris in the Atlantic over a period of 22 years found that 62% of all net tows contained plastic items large enough to be easily detected, and in some regions reaching an average of more than 20000 pieces km\(^{-2}\) (Law et al. 2010). It has been reported that in the North Pacific subtropical gyre plastic levels contain up to 335000 plastic items km\(^{-2}\), or 5.1 kg km\(^{-2}\) (Ryan et al. 2009).

2.2.3. Poly(ethylene terephthalate)

Poly(ethylene terephthalate) (PET) is a semicrystalline, thermoplastic polyester (Kint and Muñoz-Guerra 1999, Levchik and Weil 2004, Awaja and Pavel 2005, Bergeret et al. 2009). It is manufactured by a number of separate companies, under different trade names (Table 2.1). PET is strong and durable, chemically and thermally stable, has low gas permeability, and is easily processed and handled (Kint and Muñoz-Guerra 1999, Awaja and Pavel 2005). This combination of desirable properties makes PET a suitable material for a wide range of properties, and a significant component of worldwide plastic consumption. More than 50% of synthetic fibres produced around the world consist of PET, and global consumption of PET has been reported to exceed $17 billion per year (Sinha et al. 2010). PET is primarily used as fibres, sheets and films, more specifically its uses include food and beverage packaging (esp. softdrink and
water bottles), electronics, automotive parts, houseware, lighting products, power tools, sports goods, photographic applications, x-ray sheets and textiles (Kint and Muñoz-Guerra 1999, Awaja and Pavel 2005, Sinha et al. 2010). Depending on the intended application and desired properties, PET can be manufactured to specification by controlling the polymerisation conditions. Some of the specific properties of PET are summarised in Table 2.2.

Table 2.1. Manufacturers and commercial names of PET

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Commercial name</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSM Engineering Plastics</td>
<td>Arnitel®</td>
</tr>
<tr>
<td>Du Pont De Nemours &amp; Co., Inc.</td>
<td>Mylar®</td>
</tr>
<tr>
<td>Du Pont De Nemours &amp; Co., Inc.</td>
<td>Rynite®</td>
</tr>
<tr>
<td>Eastman Chemical Company</td>
<td>Eastapac®</td>
</tr>
<tr>
<td>ENKA-Glazstoff</td>
<td>Diolen®</td>
</tr>
<tr>
<td>Farbwerke Hoescht AG</td>
<td>Hostadur®</td>
</tr>
<tr>
<td>Imperial Chemical Industries Ltd.</td>
<td>Melinex®</td>
</tr>
</tbody>
</table>

Table 2.2. Intrinsic properties of PET polymers

<table>
<thead>
<tr>
<th>Property</th>
<th>Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average molecular weight</td>
<td>30000-80000 g mol⁻¹</td>
</tr>
<tr>
<td>Density</td>
<td>1.41 g cm⁻³</td>
</tr>
<tr>
<td>Melting temperature</td>
<td>255-265 °C</td>
</tr>
<tr>
<td>Glass transition temperature</td>
<td>69-115 °C</td>
</tr>
<tr>
<td>Young’s modulus</td>
<td>1700 MPa</td>
</tr>
<tr>
<td>Water absorption (24 hrs)</td>
<td>0.5 %</td>
</tr>
</tbody>
</table>

*Values taken from Awaja and Pavel, (2005). Values with ranges indicate properties which vary depending on crystallinity and degree of polymerisation.

2.2.4. Chemistry, synthesis and manufacture

Commercial synthesis of PET begins with one of two chemical reactions: ethylene glycol (EG) is reacted with either (1) terephthalic acid or (2) dimethyl terephthalate (DMT) (Kint and Muñoz-Guerra 1999, Awaja and Pavel 2005). Reaction 1 is performed
at 240-260 °C and 300-500 kPa, and reaction 2 is performed at 140-220 °C and 100 kPa, both reactions yield bis(hydroxyethyl) terephthalate (BHET) (Kint and Muñoz-Guerra 1999, Awaja and Pavel 2005). After the initial reaction, two or three polymerisation steps are then performed, depending on the required molecular weight. The first polymerisation step is transesterification between BHET molecules, displacing EG, at 250-280 °C and 2-3 kPa (Amari and Ozaki 2001, Awaja and Pavel 2005). The resulting oligomers are then polycondensed at 270-280 °C and 50-100 kPa (Kint and Muñoz-Guerra 1999, Awaja and Pavel 2005). At this stage the polymer is suitable for applications that do not require high molecular weight chains, however if higher molecular weight is required, the polymer is subjected to a third, solid state polymerisation, at 200-240 °C and 100 kPa (Kint and Muñoz-Guerra 1999, Awaja and Pavel 2005, Sinha et al. 2010). After synthesis of the raw polymer, it can then be processed into the required form, via extrusion, injection or blow moulding (Awaja and Pavel 2005).

![Figure 2.1. Chemical reactions of PET manufacturing](image-url)
2.2.5. Degradation

As a rule, widely used plastics do not naturally degrade to a large degree when released into the environment (Yamada-Onodera et al. 2001, Bonhomme et al. 2003, Zheng et al. 2005, Marqués-Calvo et al. 2006). This is perhaps unsurprising, as one of the primary reasons for the popularity and widespread application of many polymers is their exceptionally high stability and durability (Yamada-Onodera et al. 2001, Zheng et al. 2005). There are four mechanisms by which plastics degrade in the environment: photodegradation, thermooxidative degradation, hydrolytic degradation and biodegradation by microorganisms (Andrady 2011). Generally speaking, natural degradation of plastic begins with photodegradation, which leads in to thermooxidative degradation. Ultraviolet light from the sun provides the activation energy required to initiate the incorporation of oxygen atoms into the polymer (Andrady 2011, Raquez et al. 2011). This causes the plastic to become brittle and to break into smaller and smaller pieces, until the polymer chains reach sufficiently low molecular weight to be metabolised by microorganisms (Zheng et al. 2005, Andrady 2011). These microbes either convert the carbon in the polymer chains to carbon dioxide or incorporate it into biomolecules (Yamada-Onodera et al. 2001, Andrady 2011). However this entire process is very slow, and it can take 50 or more years for plastic to fully degrade (Müller et al. 2001). This is not aided by the fact that the photodegradative effect is significantly decreased in seawater due to the lower temperature and oxygen availability, and that the rate of hydrolysis of most polymers is insignificant in the ocean (Andrady 2011).

Many studies have investigated the degradability of a wide range of polymers (Yamada-Onodera et al. 2001, Bonhomme et al. 2003, Zhang et al. 2004, Müller et al. 2005, Herzog et al. 2006, Artham and Doble 2009, Kondratowicz and Ukielski 2009). Zheng et al. (2005) observed that in most cases, polymers with pure carbon backbones are particularly resistant to most methods of degradation, but polymers that include heteroatoms in the backbone (e.g. polyesters, polyamines) show higher susceptibility to degradation. While this is often true, there is however a secondary qualifier in the latter case; aromatic polymers tend to be resistant to degradation, despite the presence of bonds that are normally readily hydrolysed (Müller et al. 2001, Zheng et al. 2005). PET is a classic example of such a polymer; the ester bonds that form part of the polymer chain could normally be quite easily broken by a number of mechanisms, however due
to its aromatic groups the polymer is essentially non-degradable under normal conditions.

While the PET polymer is particularly stable and durable, it has been shown that microbial communities are capable of utilising diethylene glycol terephthalate (DTP), a subunit of PET, as a sole carbon and energy source (Zhang et al. 2004). This suggests that the reason for the extreme stability arises from being in a polymeric state. There have been a few studies that have established a link between plastic degradability and the degree of crystallisation of the polymer (Kint and Muñoz-Guerra 1999, Marten et al. 2003, Müller et al. 2005, Herzog et al. 2006, Asakuma et al. 2009, Kondratowicz and Ukielski 2009). Increased crystallisation limits chain movement and decreases the availability of polymer chains for degradative agents such as microbial lipases or other ester lysing molecules (Fig. 2.1). Lipase catalysed degradation of poly(hydroxybutyrate-co-valerate) (PHBV) has been shown to occur preferentially in the amorphous regions of the polymer, exposing polymer crystals (Mueller 2006).

Figure 2.2. Schematic representation of the effect of polymer crystallinity on enzymatic degradadation.
2.2.6. Biodegradable polymers

Over the last ten years, there has been a shift away from investigation of the degradability of traditional plastics, with more and more emphasis placed on the development of novel biodegradable polymers. Many biodegradable polymers currently exist, both natural and synthetic, however the two major barriers to their incorporation in current plastic-based applications are increased production costs and inferior material properties, e.g. decreased durability (Amass et al. 1998, Zheng et al. 2005). Production costs can be minimised through continued development of manufacturing protocols and increasing efficiency, but substantial research is still required to produce biodegradable polymers with comparable physical properties to conventional plastics. Regardless, some progress has been made in the field of biodegradable plastics, and a number of strategies have emerged for their development.

One technique for production of biodegradable plastics is to attempt to produce materials based on conventional plastics with increased degradability without compromising physical properties. For example, polymers with additional functionality on the polymer chains have been produced by both post-polymerisation treatments (Yamada-Onodera et al. 2001, Artham and Doble 2009) and copolymerisation with equivalent functionalised monomers (Marqués-Calvo et al. 2006). The rationale behind this was to create increased opportunities for microbial enzymes to attack the polymer chains. However, biodegradation of these polymers is still relatively limited, and for degradation to occur there is a requirement for significant energy input, especially in the post-polymerisation treatments. Better degradation rates have been achieved when producing block copolymers of conventional plastics with readily hydrolysable polymeric molecules. Common examples are starch, lactic acid, ethylene glycol, and caprolactone (Kint and Muñoz-Guerra 1999, Russo et al. 2009, Brandelero et al. 2011, Kim et al. 2011b, Raquez et al. 2011). Unfortunately these types of polymers are significantly less durable than many conventional plastics, and in many cases it is unclear whether these polymers are truly biodegraded, or whether they simply disintegrate into small pieces.

Development of plastics based on biological molecules has been a popular area of research. Polymers have been produced based partially or entirely on starch (Russo et al. 2009, Brandelero et al. 2011, Canché-Escamilla et al. 2011, Raquez et al. 2011),
lactic acid (Nampoothiri et al. 2010, Ye et al. 2011), caprolactone (Neppalli et al. 2011), proteins (Verbeek and van den Berg 2010), cellulose acetate (Puls et al. 2011) and other polysaccharides (Ghasemlou et al. 2011), and in many cases the mechanical durability of these polymers has been improved through addition of plasticisers (Verbeek and van den Berg 2010, Ghasemlou et al. 2011) or nanoparticles (Neppalli et al. 2011), or by controlling production conditions (Brandelero et al. 2011). However the most significant group of biopolymers is poly(hydroxyalkanoates) (PHAs). PHAs are polymeric materials naturally produced by many bacteria and some archaea, which can be processed into a number of forms suitable for packaging, coatings, and biomedical applications (Nitschke et al. 2011). The general structure of PHAs is given below in Figure 2.2. PHAs are produced commercially through bacterial fermentation, although quantities are somewhat limited due to increased production expenses compared to conventional plastics, and the lack of high-value applications (Chen 2009, Akaraonye et al. 2010, Kunasundari and Sudesh 2011). Manufacturers have been able to decrease expenses to some degree by utilising cheaper foodstocks for metabolism by bacteria; PHAs can be produced from waste materials such as whey, wheat and rice bran, molasses, vegetable oil, and even carbon dioxide (Akaraonye et al. 2010), however the main prohibitive expense remains the extraction procedure for recovery of the polymer (Kunasundari and Sudesh 2011). Extraction methods include solvent extraction, chemical digestion, enzymatic treatment, mechanical disruption, supercritical fluid disruption, flotation, gamma irradiation, and two-phase systems, however as of yet no sufficiently inexpensive extraction technique has been developed to allow PHAs to truly compete with conventional plastics in the market (Kunasundari and Sudesh 2011).

![General molecular structure of PHAs.](image)

**Figure 2.3.** General molecular structure of PHAs. R side chains consist of alkyl groups up to 13 carbon atoms long, and the number of consecutive CH₂ groups in the polymer backbone (x) ranges from 1 to 4.
Despite the significant work that has been conducted into the development of biodegradable polymers, it seems unlikely that any polymer that undergoes significant environmental degradation will ever be able to compete with conventional synthetic plastics in terms of material properties and widespread application. The very reason conventional plastics are so popular across a wide range of applications is the fact that they are so physically and chemically stable, which is also the cause for their lack of degradability. Therefore any attempt to increase biodegradability will likely compromise the physical properties of the material to some degree. There is however potential for compromise between degradability and durability on an intended application basis.

2.3. Plastic disposal methods

Currently, there are three main methods for plastic waste handling: burying in landfill, incineration, and recycling (Zhang et al. 2004). Each has its own inherent limitations.

2.3.1. Landfill

The first drawback associated with disposal of plastic waste is the fact that landfill facilities occupy space that could be utilised for more productive means such as agriculture (Zhang et al. 2004). This is compounded by the slow degradability of most plastics, as this means the occupied land is unavailable for long periods of time. Plastic components of landfill waste have been shown to persist for more than 20 years (Tansel and Yildiz 2011). This is due to the limited availability of oxygen in landfills; the surrounding environment is essentially anaerobic (Massardier-Nageotte et al. 2006, Tollner et al. 2011). As discussed in Section 2.2.5, the limited degradation that is experienced by many plastics is largely due to thermooxidative degradation (Andrady 2011), and the anaerobic conditions in landfills only serves to further limit degradation rates.

Plastic debris in landfill also acts as a source for a number of secondary environmental pollutants (Zhang et al. 2004). Pollutants of note include volatile
organics such as benzene, toluene, xylenes, ethyl benzenes and trimethyl benzenes released both as gases and contained in leachate (Urase et al. 2008), and endocrine disrupting compounds, in particular BPA (Svenson et al. 2009, Tsuchida et al. 2011, Xu et al. 2011a). In addition to its endocrine disruption properties, BPA released from plastics in landfill has also been shown to lead to an increase in production of hydrogen sulphide by sulphate-reducing bacteria in soil populations (Tsuchida et al. 2011). High concentrations of hydrogen sulphide are potentially lethal (Tsuchida et al. 2011).

2.3.2. Incineration

Another technique routinely used for disposal of plastic waste is incineration (Zhang et al. 2004). Plastic incineration overcomes some of the limitations placed on landfill in that it does not require any significant space, and there is even the capability for energy recovery in the form of heat (Sinha et al. 2010), however there is a significant trade-off. Incineration of plastics leads to the formation of numerous harmful compounds, most of which is released to the atmosphere (Zhang et al. 2004). PAHs, PCB, heavy metals, toxic carbon- and oxygen-based free radicals, not to mention significant quantities of greenhouse gases, especially carbon dioxide are all produced and released when plastics are incinerated (Simoneit et al. 2005, Valavanidis et al. 2008, Astrup et al. 2009, Khoo and Tan 2010, Shen et al. 2010). The significant environmental drawbacks of plastic disposal via both landfill and incineration led to the development of plastic recycling process.

2.3.3. Recycling

There are two approaches currently widely used for the recycling of PET: chemical and mechanical processing (Awaja and Pavel 2005). Chemical processing of PET is performed by carrying out chemolysis with one of a number of compounds, resulting in depolymerisation of the plastic (Awaja and Pavel 2005). Depolymerisation can be carried out by hydrolysis (using water), methanolysis (methanol), glycolysis (EG), or aminolysis (methylamine, e.g.) (Awaja and Pavel 2005, Sinha et al. 2010). Each results in different monomer units that can be recovered, but all of the monomers can be used as polymerisation material to produce new plastic. Mechanical processing, however, is
generally preferred to chemolysis, because even though it is a complicated process, it has the advantage of being much more cost effective (Awaja and Pavel 2005).

Mechanical recycling of PET consists of several individual steps (Awaja and Pavel 2005). The first of which is the removal of as much contaminating material as possible. The minimum requirements for PET flakes to undergo mechanolytic recycling are listed in Table 2.3. There are a number of contaminants that can significantly inhibit the recycling process and result in severe deterioration of the recovered plastic (Awaja and Pavel 2005). These contaminants will be discussed later in this section. The first step in contamination removal is sorting of plastic waste in order to separate the PET from other plastics. It is of particular importance that PVC be removed from the PET to be recycled, and this is typically done manually (Awaja and Pavel 2005). After sorting the PET is ground into flakes and washed, either using 2% NaOH and detergent at 80 ºC followed by rinsing in cold water, or using tetrachloroethylene (Awaja and Pavel 2005). After washing, the PET must be dried, usually under desiccation at ~170 ºC for six hours (Awaja and Pavel 2005). Washed and dried flakes are then ready to be melted down and extruded into new forms, however it is at this step that the real limitations of mechanolysis arise. Contaminants still reside within the PET flakes, and under high extrusion temperatures they lead to hydrolytic and thermal degradation, and subsequent decreases in both the molecular weight and intrinsic viscosity of the plastic (Awaja and Pavel 2005).
Table 2.3. Minimum requirements of PET flakes to undergo mechanical recycling.

<table>
<thead>
<tr>
<th>Property</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flake size</td>
<td>0.4 – 8 mm i.d.</td>
</tr>
<tr>
<td>Melting temperature</td>
<td>&gt;240 °C</td>
</tr>
<tr>
<td>Viscosity [n]</td>
<td>&gt;0.7 dl g⁻¹</td>
</tr>
<tr>
<td>Water content</td>
<td>&lt;0.02 %</td>
</tr>
<tr>
<td>Dye content</td>
<td>&lt;10 ppm</td>
</tr>
<tr>
<td>PVC content</td>
<td>&lt;50 ppm</td>
</tr>
<tr>
<td>Polyolefin content</td>
<td>&lt;10 ppm</td>
</tr>
<tr>
<td>Metal content</td>
<td>&lt;3 ppm</td>
</tr>
<tr>
<td>Yellowing index*</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

*Yellowing index is a measure of the tendency of polymers to turn yellow through photodegradation

There are a number of contaminants that can significantly limit the effectiveness of PET recycling processes. The first class of contaminants is acids, and acid-producing contaminants, for example PVC, which produces hydrochloric acid (Paci and La Mantia 1999, Awaja and Pavel 2005). Acids act as catalysts for chain cleavage reactions (Cardi et al. 1993, Paci and La Mantia 1998). Similarly, elevated water content can lead to chain breakage through hydrolysis. Most water contamination arises from the washing process, and can be removed by proper drying (Awaja and Pavel 2005). Dyes and colouring agents can lead to undesirable colours in recycled plastic (Awaja and Pavel 2005). Finally, contaminants such as acetaldehyde (a natural degradation product of PET) and other contaminants arising from misuse of PET by consumers (such as storage of fuel, pesticides, other dangerous materials) are potential health hazards in recycled PET products (Villain et al. 1995, Demertzis et al. 1997).

While recycling processes address the environmental shortcomings of both landfill and incineration, it is a relatively expensive and inefficient process (Yamada-Onodera et al. 2001, Zhang et al. 2004). The presence of additives and impurities can complicate the recycling procedure and decrease both the yield and quality of the recovered product (Zhang et al. 2004, Awaja and Pavel 2005).
2.3.4. Biodegradation

It is generally accepted that less than 20% of microorganisms are known to man (Muyzer et al. 1993, Iranzo et al. 2001). This reason alone provides compelling evidence that there is a vast, largely untapped reservoir of metabolic pathways to be investigated for biotechnological applications. Bacteria, in particular, are diverse in metabolic activity, and highly adaptable (Iranzo et al. 2001, Synnes 2007), which makes them excellent sources of biotechnologically useful products.

The metabolic diversity of bacteria makes them a useful resource for remediation of pollution in the environment (Iranzo et al. 2001). Bacteria have been utilised in the clean-up of oil spills (Head and Swannell 1999, Piedad Diaz et al. 2002, Rosa and Triguis 2007, Hazen et al. 2010), polychlorinated biphenyls (PCBs) (Luigi et al. 2007), and heavy metals such as arsenic, mercury, cadmium and lead (Takeuchi et al. 2007, De et al. 2008). In fact, there are sufficient examples to suggest there are few if any substances that cannot be used at least in part by microbes for metabolic activities (Iranzo et al. 2001). Biodegradation is an attractive alternative to current practices for waste disposal, as it is generally a cheaper process, potentially much more efficient, and does not produce secondary pollutants such as those associated with incineration and landfill (Head and Swannell 1999, Pieper and Reineke 2000, Iranzo et al. 2001, Luigi et al. 2007, Takeuchi et al. 2007). In some cases, it may even be possible to obtain useful end products with economic benefit from bacterial metabolism of pollutants, for example ethanol for use in biofuels (Iranzo et al. 2001).

Bioremediation of hydrocarbons, crude oil for example, poses a number of practical difficulties. Bacteria prefer aqueous nutrients (Iranzo et al. 2001), and hydrocarbons are often immiscible with water. Also, hydrocarbons are largely deficient in certain essential elements, namely nitrogen, potassium and phosphorus (Iranzo et al. 2001, Rosa and Triguis 2007). It has been shown in the literature that manually adding these elements in the form of fertiliser or ammonium sulphate can significantly aid the degradation of hydrocarbons by bacteria (Rosa and Triguis 2007). Hydrocarbons also actively interfere with cell membranes, accumulating within and disrupting the phospholipid bilayer (Pieper and Reineke 2000), however some bacteria have even been isolated that resist organic solvents (Pieper and Reineke 2000).
Despite the difficulties and limitations that have been encountered in previous attempts, the potential environmental and economic benefits that would be associated with a viable protocol for plastic biodegradation make the pursuit of this kind of plastic disposal technique a worthwhile one. Couple this with the sheer adaptational potential of bacteria, and not only do you have a worthy cause, but an achievable one. But to maximise the chances of developing an efficient, green plastic waste disposal technique, a detailed understanding of the biodegradation processes need to be attained, and to this end there are two separate components that need to be analysed: i) the bacteria capable of plastic biodegradation and their metabolic pathways and ii) the changes they induce in the chemical and physical properties of the polymer in question.

2.4. Surface characterisation techniques

In order to trace plastic biodegradation, alterations made to the polymer being degraded need to be monitored. As interactions between the material and potential degrading bacteria occur almost exclusively on the outer surface, techniques that characterise material properties at the surface will provide the most informative data. Techniques for physical and chemical characterisation of surfaces are discussed below.

2.4.1. Atomic force microscopy

Atomic Force Microscopy (AFM) is a surface scanning analytical technique which allows for the measurement and mapping of the topographical features of a sample (Binnig et al. 1986, Dorobantu and Gray 2010). A sharp tip on a flexible cantilever is scanned across the sample surface while the interactions between the tip and the surface monitored by measuring the deflections of a laser that reflected off of the back of the cantilever (Fig. 2.3), enabling construction of a three-dimensional representation (Müller and Dufrène 2008). AFM can be performed in a variety of media, including liquids such as buffers, at nanometre resolution, which make it excellent for use in cell biology applications (Müller and Dufrène 2008, Dorobantu and Gray 2010, Liu and Wang 2010). There are two standard modes of AFM scanning: contact mode, where the tip is in constant contact with the surface and the piezoelectric scanner maintains constant force between the tip and the surface, and semi-contact mode (also known as
‘tapping’ mode), in which a driving voltage is applied to cause the tip to oscillate, and the amplitude and phase difference between the driving voltage and tip oscillation is monitored (Dorobantu and Gray 2010, Liu and Wang 2010). Tapping mode is generally preferred in microbiological contexts, as lateral shear forces are minimised; contact mode imaging can result in sweeping weakly adsorbed cells and molecules from the surface (Fritz et al. 1995, Engel et al. 1999).

![Diagram of atomic force microscope](image)

**Figure 2.4.** Technical configuration of an atomic force microscope. A laser is reflected off the back of a flexible cantilever onto a position sensitive detector. As the tip interacts with the sample the cantilever bends, causing deflection of the laser to different sections of the detector.

The apparent topography of any surface, and the classification of its structure both depend on the scale upon which they are examined. For example, consider the surface of a melon. At first glance, the melon appears to be approximately spherical; this is the melon’s shape, or form, and would typically be measured in centimetres. Closer
examination of the melon rind reveals numerous bumps and/or cracks on the surface; this level of topography is referred to as surface waviness and is measured in millimetres. Detailed surface analysis of a single bump on the micron scale reveals yet another distinct topography, classified as surface roughness (Fig. 2.4). The thresholds between these three levels of surface structure are somewhat arbitrary and subjective depending on context (Thomas 1998), however when investigating the interactions between cells and material surfaces we are primarily concerned with micron and sub-micron levels of roughness.

Figure 2.5. Levels of topography. Topography can vary significantly depending on the scale of analysis.

In order for adequate characterization and comparison of roughness on any scale, surface topographical data needs to be summarized into one or more quantifiable parameters. A set of 14 roughness parameters was proposed by Stout et al. (Stout et al.
for comprehensive roughness analysis, primarily intended for engineering applications, referred to throughout the literature as the Birmingham 14. These parameters, among others, will be addressed in this section.

A three dimensional surface can be considered to be composed of a number of discrete line profiles; indeed, this is in fact the method by which most analysis techniques reconstruct a surface profile. In turn a profile can be considered to be analogous to a sinusoidal wave, which can be adequately described in terms of just two properties, its wavelength and amplitude (Thomas 1998). Generally, roughness parameters are indicative of one or the other, however there are some that incorporate both. Additionally, while many parameters for describing the roughness of a profile have an equivalent counterpart for describing three dimensional surfaces, some do not, and some parameters exclusively describe surfaces. It should be noted that in most cases the conventional annotation for roughness parameters describing profiles is the letter $R$, followed by an identifying subscript, e.g. $R_a$, while surface parameters begin with the letter $S$ (Stout et al. 1993). For the purposes of this review, most annotations will be restricted to profile parameters, and the reader should assume the given descriptions also apply to their surface parameter counterparts, unless otherwise stated.

Amplitude parameters are those that are calculated based on the height values of a given profile or surface, i.e. the ‘amplitude’ of the sinusoidal wave representing a topographical profile. The most commonly cited amplitude parameters describe either the range or distribution of heights. The maximum roughness ($R_{\text{max}}$, also referred to as $R_t$) is the vertical distance between the highest and lowest points on a topographical profile or surface. Sometimes instead of reporting $R_{\text{max}}$, authors may divide it into two components: $R_p$, the distance from the mean line or plane to the maximum height value, and $R_m$, the distance between the mean line and the lowest height value (Thomas 1998). These three parameters, however, provide minimal characterization of a profile or surface, and may not be truly representative of the data anyway as they are significantly affected by outliers such as surface damage or contamination. The ten point average roughness $R_z$ was developed in response to this, which is calculated as the difference between the average of the five highest peaks and the average of the five lowest valleys (Brune et al. 1997, Thomas 1998). While this does improve the representativeness of the statistic, it does raise the question of how to define and distinguish between individual peaks and valleys, which will be discussed later.
The parameters discussed thus far are all extreme value parameters. However those parameters that describe the distribution of height values are generally more representative of the topographical amplitude. The average roughness, $R_a$, is the average deviation of the height values from the mean line/plane, and similarly the RMS roughness, $R_q$ is root-mean-square deviation from the mean line/plane, i.e. the standard deviation. These two parameters are by far the most cited statistics for the description of roughness in microbiological papers (Švorčík et al. 2007, Kuo et al. 2008, Mitik-Dineva et al. 2008, Lee et al. 2010, Truong et al. 2010, Joergensen et al. 2011, Kim et al. 2011a, Li et al. 2011, Park and Jeong 2011, Samyn et al. 2011, Santra et al. 2011), and they are essentially interchangeable. For an ideal sinusoidal profile, the ratio of $R_a:R_q$ is equal to $2^{3/2} \pi$ (Thomas 1998). In practice this is not always the case, and $R_q$ is more susceptible to outliers (Thomas 1998). Statisticians generally prefer the use of $R_q$ as standard deviation is considered more meaningful than average deviation, however in practical use there is essentially no difference.

Unfortunately, in most biological papers, characterization of roughness is limited to the presentation of $R_a$ and/or $R_q$ (An et al. 1995, Boulangé-Petermann et al. 1997, Li and Logan 2004). However it is common for two surfaces with clearly different structures to have similar values for these two parameters (Fig. 2.5). Ideally, to resolve this one or more spatial parameters would be cited, but there are amplitude parameters commonly available in roughness analysis software that give at least an indication of the spatial variation in height. The skewness, $R_{sk}$, and kurtosis, $R_{ku}$, of the distribution of height values are two such parameters (Stout et al. 1993, Brune et al. 1997, Thomas 1998). Profiles with positive skewness tend toward having higher peaks with shallower, broader valleys, while negative skewness indicates smaller peaks with deep and narrow valleys (Brune et al. 1997). Kurtosis is a measure of the ‘sharpness’ of the height distribution, and although it can also be an indication of the sharpness of the peaks in a profile or surface, it has found little practical use (Thomas 1998). Even though both $R_{sk}$ and $R_{ku}$ can be indicative of spatial variations in height, they are considered amplitude parameters as they are calculated based solely on the height distribution.

The final group of amplitude parameters that will be discussed here are those that are based on bearing area. Bearing area is the area a surface occupies in a single plane at a given height $h$ above the mean plane, and is often expressed as a fraction of the projected area, i.e. the bearing area ratio $t_p$ (Abbott and Firestone 1933, Thomas 1998).
The bearing ratio can be used to find a further three roughness parameters, namely the core roughness, $R_k$, peak height, $R_{pk}$, and valley depth, $R_{vk}$ (Schneider et al. 1988, Thomas 1998). First, the height is plotted as a function of the bearing area. The tangent with the minimum slope within the middle 40% of the bearing ratios is then extended in both directions until it intersects $t_p = 0$ and $t_p = 1$. The height difference between these two intercepts is the core roughness. To find $R_{pk}$, a right-angled triangle is constructed with an area equal to the area enclosed by the curve, the height axis and the upper limit of $R_k$. The peak height is equal to the length of the vertical edge of the triangle, when the right angle is placed on the intersection of the tangent and the height axis, and the lower right corner touches the curve. Valley depth is found by a similar method, at the lower end of the curve, substituting $t_p = 1$ for the height axis. A graphical representation of the calculation of $t_p$, $R_k$, $R_{pk}$ and $R_{vk}$ is presented in Figure 2.6. These parameters are able to distinguish between different surfaces with similar average roughness, however they have a significant drawback in that they are quite convoluted, and do not directly relate to the structures on the surface.

*Figure 2.6. An example of the limitations of $R_a$ for roughness characterization. The two sinusoidal profiles presented above have clearly different structures, however the value of $R_a$ for each is identical.*
Figure 2.7. Calculation of bearing ratio, \( t_p \), core roughness, \( R_k \), peak height, \( R_{pk} \), and valley depth, \( R_{vk} \).

While amplitude parameters are certainly mandatory when characterizing roughness and surface topography, they give adequate description of only the z-dimension. For comprehensive characterization there also needs to be some analysis of the spatial variations in roughness. A relatively simple, and easy to interpret example is summit density, \( S_{ds} \) (Stout et al. 1993). The summit density is simply defined as the number of summits per unit area on a surface, and a corresponding profile parameter
could be easily calculated as the number of peaks per unit length. However calculation of this parameter (and several others) can be significantly complicated by the definition of peaks/summits. Most commonly a peak is defined as any point that is higher than its nearest neighbours, i.e. the two points on either side in a profile, and the surrounding four or eight points on a surface (Greenwood 1984, Stout et al. 1993, Thomas 1998). The major drawback of this method is the tendency to overestimate the number of peaks, particularly on very smooth samples where noise can have a greater influence. An alternative method was described for use in calculating the mean spacing between peaks, where a peak was only included in the analysis provided it was more than 1% of \( R_{\text{max}} \) higher than the preceding valley (Brune et al. 1997).

The periodicity of a surface or profile can be assessed by the construction of one (or more) of several related statistical functions. These functions measure how the correlation of the heights of two points in the profile or surface varies with the distance between them (Brune et al. 1997, Thomas 1998). For example, the autocovariance function (ACVF) is given by:

\[
R(\tau) = \frac{1}{L - \tau} \int_{0}^{L-\tau} z(x)z(x + \tau) \, dx
\]

where \( L \) is the length of a profile, \( \tau \) is the separation interval between two points, and \( z \) is the height of a given point (Bennett and Mattsson 1989, Thomas 1998). The ACVF is usually normalized and expressed as the autocorrelation function (ACF) by dividing \( R(\tau) \) by the variance of the height distribution, i.e. \( R_{q}^{2} \) (Peklenik 1967, Thomas 1998, Zemek 2010). The ACF and ACVF both tend toward 0 as \( \tau \) increases, with relatively random surfaces decaying more rapidly than those with well-defined structure. This enables them to be characterized by a single value, the correlation length, which is defined as the value of \( \tau \) at which the ACF decays to a given fraction (1/10 and 1/e are sometimes used) of its original value (Thomas 1998). Points that are further apart than the correlation length are considered statistically independent (Thomas 1998).

Another function based on the ACVF is the power spectral density (PSD) function. The PSD is the Fourier transform of the ACVF (Bendat and Piersol 1966, Thomas 1998, Zemek 2010). Its units are height squared per unit frequency, and it is a function of frequency (Brune et al. 1997, Thomas 1998). A PSD of a single line profile generally appears as nothing more than a noisy line, however averaging several profile PSDs
often reveals a number of peaks in the function (Brune et al. 1997). These peaks indicate the frequencies (and wavelengths, as \( f = \frac{1}{\lambda} \)) which have the highest power, or amplitude. The PSD, ACF and ACVF are all particularly useful for identifying patterns that may be obscured by other surface features, and each can be plotted in three dimensions.

Three of the Birmingham 14 parameters are calculated based on the correlation and spectral functions, i.e. the fastest decay autocorrelation length, \( S_{al} \), the texture aspect ratio, \( S_{tr} \), and the texture direction, \( S_{td} \) (Stout et al. 1993). The fastest decay autocorrelation length is the shortest length required for the three-dimensional ACF to decay to 0.2. The texture aspect ratio is also calculated from the 3-D ACF; \( S_{tr} = S_{al}/\text{the longest correlation length} \). The texture aspect ratio describes the ratio of the shortest repeating pattern to the longest repeating pattern on the surface, and is always less than or equal to 1 (Tsukada and Sasajima 1983, Stout et al. 1993). The texture direction is defined as the angle between the major direction of the surface texture and the y-axis of the sampled area, and can be calculated from the 3-D PSD (Stout et al. 1993). However, as this parameter is entirely dependent on the orientation of the sampled field, it is of very limited use for characterization or comparison of surface nanoarchitecture.

A few roughness parameters exist which combine aspects of both amplitudinal and spatial characteristics. The average slope, \( \Delta_a \), and the RMS slope, \( \Delta_q \) are two of these, and both are relatively simple to calculate (Spragg and Whitehouse 1974, Whitehouse 1994, Brune et al. 1997, Thomas 1998, Zemek 2010). They are equivalent to \( R_a \) and \( R_q \) respectively, but calculated based on the derivative of the height profile. The average and RMS wavelengths, \( \lambda_a \) and \( \lambda_q \) respectively, can easily be found from their corresponding slopes, according to \( \lambda_a = \frac{2\pi R_a}{\Delta_a} \) and \( \lambda_q = \frac{2\pi R_q}{\Delta_q} \) (Spragg and Whitehouse 1974, Whitehouse 1994, Brune et al. 1997, Thomas 1998). Two further members of the Birmingham 14 are classified as hybrid parameters: the mean summit curvature, \( S_{sc} \), and the developed area ratio, \( S_{dr} \) (Stout et al. 1993). The mean summit curvature is defined as the average of the principle curvatures of each of the summits on a surface, and as such requires the summits be previously defined. The developed area ratio is calculated as the ratio of the surface area to the projected surface area, and it fluctuates with both amplitude and spatial variations.

Requirements for the adequate characterization of roughness depend greatly on the specific application, however for determining a minimum standard for use in
microbiology it is useful to start with the Birmingham 14. The original 14 parameters chosen by Stout et al. were RMS roughness, $S_q$, ten point average roughness, $S_z$, skewness, $S_{sk}$, kurtosis, $S_{ku}$, summit density, $S_{ds}$, texture aspect ratio, $S_{tr}$, texture direction, $S_{td}$, fastest decay autocorrelation length, $S_{al}$, RMS slope, $S_{Δq}$, mean summit curvature, $S_{sc}$, developed area ratio, $S_{dr}$, and three additional parameters not discussed here (Stout et al. 1993). These three additional parameters have been developed primarily for engineering applications, and have no significant relevance to microbiology. Any minimum set of parameters should include descriptors of both the amplitudinal and spatial properties, e.g. $S_q$ and $S_{ds}$, in order to give an indication of the vertical and the horizontal dimensions of the surface. The developed area ratio should also be included in microbiological contexts, as the substratum contact area available to cells plays a key role in governing their adhesion. Based on this, it is recommended that when describing roughness in microbiological research, the roughness parameters $S_q$, $S_{ds}$ and $S_{dr}$ be reported as the absolute minimum standard. Quotation of $S_z$, $S_{sk}$ and $S_{tr}$ is also encouraged, in addition to any relevant application specific parameters, to complement the minimum set.

2.4.2. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) is an analytical technique for determination of the surface chemistry of a material based on the ionisation energies of the atoms on or near the material surface (Hollander and Jolly 1970). The material in question is irradiated with monochromatic x-rays in a vacuum, which causes electrons in the material to escape from their orbitals. Electrons that are near the surface of the material are released into the vacuum where a detector simultaneously counts them and measures their kinetic energy. A spectrum is then constructed by plotting the number of electrons (often measured in counts per second) against their kinetic energy, or ‘binding’ energy. Different elements produce different characteristic peaks in the spectrum, and different chemical configurations of the atoms produce subtle differences in binding energies (Hollander and Jolly 1970).

XPS is typically performed by first scanning a wide range of binding energies, to first determine the elements present on the samples surface (Leone et al. 2006, Amor et al. 2009). Once the specific elements have been identified, narrow range, high
resolution spectra are recorded to identify the contributing chemical species in a given elemental peak of interest. Generally the different contributing components produce significantly overlapping peaks that together form a characteristic peak of the element in question, and in order to be resolved they need to be mathematically determined using a peak-fitting algorithm (Salvi et al. 1995, Hesse and Denecke 2011).

XPS is an excellent tool for the study of surface chemistry, as it has a very low depth of analysis; the data obtained are usually representative of no more than the first 10 nm of the sample surface (Gentle and Barnes 2005, Ivanova et al. 2010a). The major drawbacks associated with the technique lie within the limited lateral spatial resolution (Blomfield 2005), and the complication of spectra to arising from atmospheric contamination of samples (Johansson and Campbell 2004).

2.4.3. Infrared spectroscopy and Raman spectroscopy

An alternative chemical characterisation technique is infrared spectroscopy. Light from the infrared region of the electromagnetic spectrum is shone onto or through a sample, and the decrease in the energy of the beam due to interaction with the sample is measured as a function of the wavelength of the light (Coates 2000, Menzies et al. 2010). Absorption occurs when the frequency of the incident light matches one of the vibrational energies of a chemical bond in the sample. The result is a spectrum that contains absorption peaks that are characteristic of the vibrational energies of the chemical bonds in the sample. The major advantage of infrared spectroscopy is its non-destructive nature (Whelan et al. 2011). Additionally, by performing IR measurements in attenuated total reflection (ATR) mode, the analysis depth can be limited to just the first 1 to 2 µm, causing the generated signal to be more representative of the surface without the obscurement by the bulk material, as is the case in standard transmission mode. Regardless of whether ATR or transmission mode is used, two-dimensional spectral maps with micron-scale spatial resolution can be generated by collecting individual spectra in a grid or array, and integrating the area under a characteristic peak of interest (Croxford et al. 2011, St John et al. 2011). The spatial resolution can be further improved if a Synchrotron is used as the radiation source (Croxford et al. 2011, St John et al. 2011).
Similarly to infrared spectroscopy, Raman spectroscopy relies on the interaction of light with chemical bonds. Raman spectroscopy employs visible, near-infrared or near-ultraviolet light which is shone on the sample (Hartschuh et al. 2003, Sandt et al. 2009). The photons of the incoming light excite electrons in the sample causing them to move into higher orbitals. These electrons then drop back into lower orbitals, however they may not drop into the same orbital from which they originated, resulting in the emission of a photon of a different energy level to the excitation light (Dietzek et al. 2010). These photons are the measured signal in Raman spectroscopy. Like infrared spectroscopy, Raman spectroscopy is non-destructive and applicable to chemical mapping, and the two techniques are complementary to each other (Pappas et al. 2000, Ivleva et al. 2009).

2.5. Bacteria

While characterisation of the PET surfaces and determination of the biodegradation mechanisms is of great importance to the current project, another significant aspect lies in the identification and characterisation of the bacterial communities that have potential PET-degrading ability. There are a number of approaches that have been developed for taxonomical characterisation of bacteria.

2.5.1. Bacterial taxonomy

The traditional methods for classification of bacteria into species via observation of morphology and other phenotypic traits have some limitations. Firstly, there can be a great deal of ambiguity when classifying bacteria by characteristics such as morphology; there really is not much that can be deduced about a particular bacterium by observing its morphology (Olsen et al. 1986). Secondly, classification by phenotypic traits does not necessarily reflect genetic relatedness or evolutionary pathways (Joyce et al. 2002). One of the major reasons for this is bacteria undergo a significant amount of lateral transfer of genetic material, which can easily introduce new traits to a specific bacterium (Joyce et al. 2002). Another, practical difficulty in classifying organisms in this way is that it requires a pure culture of the microorganism in question to properly distinguish it from others (Amann et al. 1995). The vast majority of microorganisms are currently unable to be cultured properly in the laboratory (Olsen et al. 1986, Muyzer et
al. 1993, Ravenschlag et al. 2001, Harris et al. 2004, Carrigg et al. 2007, Horisawa et al. 2008, Malik et al. 2008). Despite these limitations, however, phenotypic classification is a useful approach for rapid and easy grouping of bacteria, provided that the validity of those groups is confirmed by other, more reliable techniques. It is for this reason that molecular methods of classification based on genetic analysis have been developed.

2.5.2. Molecular classification techniques

Molecular methods of characterisation focus on the comparison of various homologous molecules common to all species. Some methods have been developed which compare homologous proteins in order to determine genetic relatedness, however these are not ideal as there can be a large amount of variability even between homologous proteins, especially those from eukaryotes compared with those from prokaryotes (Olsen et al. 1986). Much more success has been experienced using nucleic acids, in particular sequence comparison of ribosomal RNA (rRNA) or genes encoding for rRNA (Olsen et al. 1986, Amann et al. 1995). Ribosomal RNA and DNA have at least five major advantages that make them particularly useful for taxonomic classification of bacteria:

i. rRNA is homologous among all organisms (Olsen et al. 1986)
ii. rRNA is extremely well conserved from species to species (Olsen et al. 1986, Malik et al. 2008)
iii. rRNA and rDNA are readily isolated (Olsen et al. 1986)
iv. Enough data can be generated to draw statistically significant conclusions (Olsen et al. 1986)
v. rRNA genes generally are not transferred laterally, which is very useful for tracing evolutionary pathways (Olsen et al. 1986).

The 5S and 16S ribosomal subunits are the most commonly employed (Olsen et al. 1986, Amann et al. 1995). The 5S subunit is particularly easy to sequence, however it is too small to provide useful information for phylogenetic classification (Olsen et al. 1986, Amann et al. 1995). On the other hand, the 16S subunit provides ample information for phylogenetic classification, and can be sequenced with relative ease (Olsen et al. 1986, Burgess et al. 1993, Amann et al. 1995). Also, by incorporating
polymerase chain reaction (PCR) protocols into molecular classification methods time consuming and labour-intensive techniques such as cloning into plasmids can be avoided (Olsen et al. 1986, Amann et al. 1995, Carrigg et al. 2007). In addition to this, PCR techniques have increased sensitivity in comparison to plate count techniques (Devos et al. 2005), and are able to identify species that constitute as little as 1% of the microbial population (Muyzer et al. 1993). The highly conserved nature of the 16S gene allows for PCR amplification of the 16S rDNA of an entire microbial population using a single pair of universal primers (Muyzer et al. 1993). The resultant mixture of amplified DNA can be separated according to base composition via denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993, Carrigg et al. 2007). The resulting set of bands is characteristic of the microbial population, with each band representing a discrete species (Muyzer et al. 1993). There are however some limitations associated with DGGE; slight sequence variation within a species may result in multiple bands that correspond to a single species (Carrigg et al. 2007), and the amplified products of more than one bacterial species may have identical mobility (Carrigg et al. 2007, Malik et al. 2008).

Other useful applications of PCR include reverse-transcription PCR and real-time PCR. Reverse transcription PCR uses RNA as the initial template, and includes an initial transcription using reverse transcriptase, providing information on gene expression (Amann et al. 1995). Real-time PCR is a useful tool for quantification of microbial community constituents (Heid et al. 1996, Carrigg et al. 2007, Malik et al. 2008, De Gregoris et al. 2011). A fluorescent dye is incorporated into the amplification reaction that intercalates double stranded DNA. By measuring the increase in fluorescence over time and comparing it to a standard curve of known quantities of DNA, the original concentration of DNA before amplification can be determined (Heid et al. 1996). Real-time PCR is fast, sensitive, accurate, and can be automated, but does not provide information on gene expression (Heid et al. 1996).

Alternative characterisation methods have also been developed. Fluorescent in situ hybridisation (FISH) involves the addition of a fluorescently labelled oligonucleotide probe complimentary to rRNA to microorganisms as they occur naturally in the environment, i.e. without any cultivation required (Amann et al. 1995, Malik et al. 2008). Use of in situ hybridisation techniques allows for the detection of individual cells and observations of morphology and spatial distribution (Amann et al. 1995, Malik et
There are disadvantages associated with FISH; as it is often used to detect low numbers of cells, the signal generated is often of low intensity, and it is often difficult to find an individual cell on the microscope stage (Amann et al. 1995). Also, signal intensity does not entirely correlate with cell numbers, as rRNA copies vary between species, faster growing species tending to have far more, resulting in increased signal strength (Amann et al. 1995).

Microarrays are a powerful tool that can be used for comparing genomic composition, and subsequently genetic relatedness (Joyce et al. 2002). Microarrays screen for the presence of many genes at once via immobilised probes (Joyce et al. 2002). This genomic-scale analysis along with high throughput are the main advantages of microarrays, but as they rely on complimentary probes, it can be difficult to obtain useful information on bacteria that have not previously had their genomes sequenced (Joyce et al. 2002). Also, while microarrays are very useful when analysing pure cultures, environmental samples introduce problems in specificity, sensitivity and quantification (Joyce et al. 2002).

2.5.3. Bacterial attachment and biofilm formation

Bacterial interactions with PET are limited almost entirely to the material surface. As such, these interactions need to be characterised, and the methods of cell adhesion and colonisation investigated. One of the primary modes of bacterial colonisation of surfaces is the formation of biofilms. Biofilms are microbial communities enclosed in a matrix of extracellular polymeric substances (EPS), adhered to a biological or non-biological surface (Armstrong et al. 2001, Hall-Stoodley et al. 2004, Türetgen and Cotuk 2007). Biofilms are a common and ancient style of bacterial growth, with fossil evidence of biofilms dating back approximately 3.25 billion years (Hall-Stoodley et al. 2004). They provide a number of advantages for the bacteria they contain. Biofilm formation provides protection for microorganisms from hostile environments, be it extreme conditions such as high temperatures, or immune response from an infected host (Yan et al. 2002, Hall-Stoodley et al. 2004, Palmer et al. 2007, Türetgen and Cotuk 2007). Biofilms also allow for better access to nutrients, as potentially nourishing organic material tends to accumulate on surfaces (Amann et al. 1995, Hall-Stoodley et al. 2004, Palmer et al. 2007). The formation of channels also allows the bulk liquid to
infuse a biofilm and improve the exchange of nutrients and waste products (Hall-Stoodley et al. 2004). The close proximity in which cells are kept promotes genetic exchange (Palmer et al. 2007) as well as the development of cell-cell communication systems (Hall-Stoodley et al. 2004). Finally, detachment of a biofilm (or pieces thereof) aids in the colonisation of new areas and ecological niches (Hall-Stoodley et al. 2004, Landini et al. 2010, Nijland et al. 2010).

Figure 2.8. Life-cycle of a biofilm

The adhesion potential between a bacterial cell and a substratum surface is governed by two general factors: the physico-chemical properties of the bacterium, and the physico-chemical properties of the substratum (Blenkinsopp and Costerton 1991, An and Friedman 1998, Bos et al. 1999b, Burgess et al. 2003, Ubbink and Schär-Zammaretti 2007, Busscher et al. 2010). While substantial research has been conducted investigating the effects of substratum surface chemistry on bacterial adhesion, the recent trend has been toward analysing the role of surface topography (Bos et al. 1999b, Katsikogianni et al. 2008, Katsikogianni and Missirlis 2010, Ploux et al. 2010, Bazaka et al. 2011, Parreira et al. 2011). The main reason for this shift of focus arises from inconsistencies in colonization rates on chemically similar surfaces; two samples with virtually identical surface chemistry may exhibit vastly different cell adhesion profiles due to differences in surface architecture and roughness.
Chapter 3: Materials and Methods
3.1. Overview

The nature of this work requires the implementation of many different analytical techniques from multiple disciplines, ranging from molecular biology to surface chemistry. Experiments have focussed on the characterisation of the bacterial strains and PET polymers, as well as the interactions between these two entities.

3.2. PET sources

PET samples were obtained from two separate sources. The first source was standard PET water bottles (Mount Franklin Spring Water, 400 mL, Coca-Cola Amatil, Australia), commonly sold at many retail stores in Australia. PET bottles were cut into approximately 1 cm$^2$ pieces, and sterilised by placing them in 70 % ethanol for 20 minutes before use. The second sample type was Mylar® PET films, kindly supplied by the Australian office of DuPont Teijin Films, Melbourne. Mylar® films were prepared and sterilised in the same manner as the PET bottles.

3.3. Enrichment conditions

Two separate experiments were performed, following the same general procedure, to enrich environmental populations of bacteria selecting for PET degrading strains. Environmental seawater samples were collected from Port Phillip Bay, Melbourne, at St. Kilda Beach, on December 12, 2007, and December 17, 2008. Samples were collected from the first metre of the water surface, into sterile glass bottles, and were used on the day of collection. Enrichment culture vessels (pre-sterilised conical flasks, 200 mL) were prepared by adding 100 mL of the collected seawater, and supplying the naturally present bacterial communities with approximately five grams of PET pieces (either bottle pieces or films), as their sole carbon and energy source. Several culture conditions were tested between the two enrichment experiments, shown in Table 3.1. Cultures were incubated statically at room temperature for up to a year. In addition to the two separate enrichment experiments, two quantification/characterisation experiments were performed in order to measure the biodegradation rate and determine the mechanisms of biodegradation. Individual PET pieces were weighed before and after incubation to detect any loss of mass. The latter two experiments also tested a
number of culture conditions and chemical supplements (Table 3.1) for their ability to enhance biodegradation.

**Table 3.1.** The range of experimental conditions used for enrichment and quantification/characterisation experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Bacterial culture</th>
<th>Type of water</th>
<th>PET sample</th>
<th>Light/Dark*</th>
<th>Additives/ supplements†</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrichment 1</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>Bottle pieces</td>
<td>Light</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Enrichment 1</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>None (Control)</td>
<td>Light</td>
<td>None</td>
<td>1</td>
</tr>
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<td>Enrichment 2</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>Films</td>
<td>Light</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Enrichment 2</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Films</td>
<td>Light</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Enrichment 2</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Films</td>
<td>Light</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Enrichment 2</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>Bottle pieces</td>
<td>Light</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Enrichment 2</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>Films</td>
<td>Dark</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Enrichment 2</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>None (Control)</td>
<td>Light</td>
<td>None</td>
<td>3</td>
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<tr>
<td>Quantification/characterisation</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>None (Control)</td>
<td>5</td>
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<tr>
<td>Quantification/characterisation</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃, KH₂PO₄</td>
<td>5</td>
</tr>
<tr>
<td>Quantification/characterisation</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>FeCl₂</td>
<td>5</td>
</tr>
<tr>
<td>Quantification/characterisation</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>Tween 20</td>
<td>5</td>
</tr>
<tr>
<td>Quantification/characterisation</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃, KH₂PO₄, FeCl₂, Tween 20</td>
<td>5</td>
</tr>
<tr>
<td>Quantification/characterisation</td>
<td><em>Thalassospira sp. H94</em></td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>None (Control)</td>
<td>5</td>
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<tr>
<td>Quantification/characterisation</td>
<td><em>Thalassospira sp. H94</em></td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃, KH₂PO₄</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td>Organism</td>
<td>Medium</td>
<td>Incubation</td>
<td>Light Source</td>
<td>Supplement</td>
<td>Plate Type</td>
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<tr>
<td>Quantification /characterisation</td>
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<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>FeCl₂</td>
<td>5</td>
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<tr>
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<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>Tween 20</td>
<td>5</td>
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<tr>
<td>Quantification /characterisation</td>
<td><em>Thalassospira sp.</em> H94</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃ KH₂PO₄ FeCl₂ Tween 20</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td><em>Thalassospira tepidiphila 1-1B</em>ᵀ</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>None (Control)</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td><em>Thalassospira tepidiphila 1-1B</em>ᵀ</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃ KH₂PO₄</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td><em>Thalassospira tepidiphila 1-1B</em>ᵀ</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>FeCl₂</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td><em>Thalassospira tepidiphila 1-1B</em>ᵀ</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>Tween 20</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td><em>Thalassospira tepidiphila 1-1B</em>ᵀ</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃ KH₂PO₄ FeCl₂ Tween 20</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td><em>Thalassospira /Alteromonas consortium</em> ‡</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>None (Control)</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td><em>Thalassospira /Alteromonas consortium</em> ‡</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃ KH₂PO₄</td>
<td>5</td>
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<td>Quantification /characterisation</td>
<td><em>Thalassospira /Alteromonas consortium</em> ‡</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>FeCl₂</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td><em>Thalassospira /Alteromonas consortium</em> ‡</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>Tween 20</td>
<td>5</td>
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<td>Quantification /characterisation</td>
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<td>Sterilised seawater</td>
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<td>Light</td>
<td>NaNO₃ KH₂PO₄ FeCl₂ Tween 20</td>
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</tr>
<tr>
<td>Quantification /characterisation</td>
<td>Enrichment culture§</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>None (Control)</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td>Enrichment culture§</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃ KH₂PO₄</td>
<td>5</td>
</tr>
<tr>
<td>Quantification /characterisation</td>
<td>Enrichment culture§</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>FeCl₂</td>
<td>5</td>
</tr>
<tr>
<td>Optimisation</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------</td>
<td>----------</td>
<td>-------------------------</td>
<td>-------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Optimisation</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Optimisation</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Dark</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Optimisation</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Dark</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Optimisation</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃ KH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>Optimisation</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>NaNO₃ KH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>Optimisation</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>PET pre-treated with SDS</td>
<td></td>
</tr>
<tr>
<td>Optimisation</td>
<td>None (Control)</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>PET pre-treated with SDS</td>
<td></td>
</tr>
<tr>
<td>Optimisation</td>
<td>Pseudoalteromonas citrea NCMB 1889†</td>
<td>Sterilised seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Optimisation</td>
<td>Environmental community</td>
<td>Seawater</td>
<td>Single film, (1 × 3 cm)</td>
<td>Light</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

*Light/Dark refers to sample incubation in the presence/absence of light, respectively.

†Concentration of additives were as follows: sodium nitrate – 0.4 µM, potassium dihydrogen phosphate – 0.04 µM, iron (II) chloride – 100 µM, Tween 20 – 0.04 % (v/v)

‡*Thalassospira/Alteromonas* consortium consisted of a mixture of *Thalassospira* sp. H88, *Thalassospira* sp. H93, *Thalassospira* sp. H94, *Alteromonas* sp. H86,
Alteromonas sp. H89 and Alteromonas sp. H91, in a ratio of 10:10:10:1:1:1, at a final OD600 = 0.3.

‡ Tubes were inoculated with 100 µL of enrichment culture taken from one of the light exposed PET + seawater replicates in enrichment experiment 2.

‖ PET samples pre-treated with sodium dodecylsulfate (SDS) were cleaned by sonication in 70 % EtOH, rinsed, dried, and soaked in a solution of 2 g/L SDS for 24 hours.

3.4. Bacterial recovery

Subsamples of 30 µL volume were aseptically taken from incubation vessels after gentle swirling and spread on marine agar 2216 (Difco, BD, U.S.A.), or oligotrophic marine agar at 0.1 × normal working concentration. A similar procedure was followed for recovery of strains attached to plastic pieces: a single piece of PET was removed from the incubation vessels and vortexed for 30 seconds with 3 mL of phosphate buffered saline. Thirty µL samples were taken from the resulting mixture. Three replicate plates were performed for each growth medium for each incubation vessel. Plates were incubated for up to 72 hours at 25 °C, before the resulting colonies were counted and grouped according to morphology. Multiple representatives of each phenotype were streaked on the same medium from which they were taken and incubated at 25 °C to assess purity, before stock cultures were prepared by inoculating 500 µL of marine broth 2216 (Difco, BD, U.S.A.) supplemented with 20 % glycerol, and storing at –80 °C until required.

3.5. 16S rRNA gene sequencing

Pure cultures of each strain to be sequenced were grown in 3 mL of marine broth. The cells were then centrifuged (12000 rpm for 3 minutes) and the genomic DNA was extracted using Wizard SV genomic DNA extraction kit (Promega, U.S.A.). Approximately 880 bp of the 16S rRNA genes for each strain was amplified via polymerase chain reaction (PCR) using the primer pair (sequences shown in Table 3.2) 27F (Chun and Goodfellow 1995) and 907R (Tait et al. 2007), and a temperature program consisting of an initial denaturation step at 95 °C for 4 min, then 30
amplification cycles of 94 °C for 30 s, 52 °C for 40 s and 72 °C for 70 s, and a final extension step of 72 °C for 20 min. The resulting 16S rRNA gene fragments were sequenced at the Australian Genome Research Facility (AGRF) using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Melbourne) and capillary electrophoresis. Amplification and sequencing primers were purchased from Invitrogen (U.S.A.). Based on the degree of similarity of each the returned sequences to their nearest match, obtained via BLAST analysis, four strains were selected for full 16S rRNA gene sequencing based on their likelihood of being novel species. Full gene sequences (~1500 bp) were obtained by amplifying the first ~880 bp and last ~900 bp (~280 bp overlap), using the above amplification conditions for the former and the following conditions for the latter: primer pair 518F (Tait et al. 2007) and 1482R (Tait et al. 2007) and temperature program denaturation – 95 °C for 15 min, amplification – 30 cycles of 94 °C for 1 min, 55 °C – 1 min and 72 °C – 2 min, and final extension – 72 °C for 10 min. Amplification products were again sequenced at AGRF, and reconstructed using BioEdit software (Hall 1999). The 16S rRNA nucleotide sequences were deposited to the GenBank DNA database.

**Table 3.2. Nucleotide sequences of primers used for 16S rDNA amplification**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F</td>
<td>5′-AGAGTTTGATCCTGGCTCAG-3′</td>
</tr>
<tr>
<td>907R</td>
<td>5′-CCGTCAATTCMTTTGAGTTT-3′</td>
</tr>
<tr>
<td>518F</td>
<td>5′-CCAGCAGCCGCGTAATACG-3′</td>
</tr>
<tr>
<td>1482R</td>
<td>5′-GGTTACCTTGTACGACTT-3′</td>
</tr>
</tbody>
</table>

**3.6. Phylogenetic analysis**

For each sequence obtained, 30-50 of the most similar sequences from the EzTaxon database of type strains (Chun et al. 2007) were retrieved and aligned using ClustalW2 (Larkin et al. 2007). Alignments were then manually checked and sequences trimmed using BioEdit (Hall 1999). Neighbour-joining phylogenetic trees were constructed using
the software available as part of PHYLIP (Phylogeny Inference Package) (Felsenstein 1989). Large trees were first constructed including 100+ different taxonomic strains, before smaller trees were constructed by the same method using approximately 10 of the nearest phylogenetic neighbours identified by the larger trees. One thousand replicates were generated for bootstrap analysis using the SEQBOOT program, distance matrices calculated using DNADIST, neighbour joining analysis performed by NEIGHBOUR using the Kimura 2-parameter algorithm, and replicate trees condensed using CONSENSE, all of which are part of PHYLIP.

3.7. **Biochemical characterisation**

Phenotypic profiles were generated using Biolog GN2 microplates (Biolog, U.S.A.) and API ZYM test strips (bioMérieux, France). Biolog GN2 microplates test bacterial strains for oxidative utilisation of 95 different carbons sources. API ZYM strips test for activity of 18 separate enzymes, namely: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-B1-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and β-fucosidase. Both kits were utilised according to the manufacturer’s specifications.

3.8. **Bacterial strains**

Many different bacterial strains were employed throughout this work, including the type strains of all validly described species of *Alteromonas*. As so many of the strains to be described belong to the same genus, it is perhaps convenient to present them in two groups, i.e. *Alteromonas* spp., and other bacterial strains.

3.8.1. **Alteromonas** spp.

All nine validly described species of the genus *Alteromonas* were used in the course of this work. *Alteromonas* spp. belong to the class *Gammaproteobacteria*, order *Alteromonadales*, family *Alteromonadaceae*. They are Gram negative, rod-shaped,
chemoorganotrophic bacteria, and grow strictly in aerobic conditions. Cells are motile by means of single polar flagella. They do not produce spores or, in most cases, accumulate polyhydroxybutyrate, however many species produces buds and prosthecae. Most species were originally isolated from open sea or coastal waters (Baumann et al. 1972, Gauthier et al. 1995, Van Trappen et al. 2004a). Specific descriptions are given in Sections 3.8.1.1-9 below.

3.8.1.1. *A. addita R10SW13\(^T\)*

*A. addita* are rod-shaped cells 0.7 – 0.9 µm in diameter, originally isolated from a seawater sample. They can grow in 1 – 10 % (w/v) NaCl concentrations, between 4 – 37 °C and pH 6.0 – 10.0 (optimum 7.5 – 8.0). The DNA G + C content is 43.0 mol% (Ivanova et al. 2005a).

3.8.1.2. *A. genovensis LMG 24078\(^T\)*

*A. genovensis* was originally isolated from an electroactive, marine biofilm. Cells are 1.8 µm long and 0.9 µm wide, and produce buds and prosthecae. Growth conditions are 2 – 15 % (w/v) NaCl, 4 – 37 °C and pH 6.0 – 8.5 (optimum 7.0 – 8.0). The DNA G + C content is 44.5 mol% (Vandecandelaere et al. 2008).

3.8.1.3. *A. hispanica F-32\(^T\)*

*A. hispanica* was originally isolated from a hypersaline lagoon in Spain. Cells are 1.0 – 2.0 µm in length and 0.75 µm wide. Cells also produce buds and prosthecae, and accumulate polyhydroxybutyrate. Growth conditions are 7.5 – 15 % (w/v) NaCl (optimum 7.5 – 10 %), 4 – 40 °C (optimum 32 °C) and pH 5.0 – 10.0 (optimum 7.0 – 8.0). The DNA G + C content is 46.3 mol% (Martínez-Checa et al. 2005).

3.8.1.4. *A. litorea TF-22\(^T\)*

*A. litorea* was originally isolated from intertidal sediment. Cells are 2.0 – 4.0 µm in length and 0.9 – 1.2 µm wide. Growth conditions are 2 – 14 % (w/v) NaCl (optimum 2
– 5 %), 10 – 43 °C (optimum 30 – 37 °C) and pH 5.5 – 8.0 (optimum 7.0 – 8.0). The DNA G + C content is 46.0 mol% (Yoon et al. 2004).

3.8.1.5.  A. macleodii DSM 6062T

A. macleodii is the type species of the genera, and was originally isolated seawater samples. Cells are 2 – 3 µm in length and 0.7 – 1.0 µm wide, and produce buds and prosthecae. Growth conditions are 1 – 12 % (w/v) NaCl, and 12 – 45 °C. The DNA G + C content is 45.6 ± 0.8 mol% (Baumann et al. 1972, Gauthier et al. 1995, Vandecandelaere et al. 2008).

3.8.1.6.  A. marina SW-47T

A. marina was originally isolated from a seawater sample. Cells are 2.5 – 4.0 µm in length and 1.0 – 1.2 µm wide, and produce buds and prosthecae. Growth conditions are 2 – 15 % (w/v) NaCl (optimum 2 – 5 %), 4 – 44 °C (optimum 30 – 37 °C) and pH 5.0 – 8. (optimum 7.0 – 8.0). Their DNA G + C content is 45.0 mol% (Yoon et al. 2003a).

3.8.1.7.  A. simiduii BCRC 17572T

A. simiduii was originally isolated from a Taiwanese estuary, and possesses the ability to reduce mercury (Hg²⁺ → Hg⁰). Cells are 1.2 – 2.5 µm in length and 0.4 – 0.8 µm wide. Cells also produce buds and prosthecae, and accumulate polyhydroxybutyrate. Growth conditions are 0.5 – 13 % (w/v) NaCl (optimum 2 – 4 %), 15 – 40 °C (optimum 30 °C) and pH 6.0 – 9.0. Their DNA G + C content is 45.3 mol% (Chiu et al. 2007).

3.8.1.8.  A. stellipolaris LMG 21861T

A. stellipolaris was originally isolated from an Antarctic seawater sample. Cells are 2.0 – 7.0 µm in length and 0.4 µm wide, and produce buds and prosthecae. Growth conditions are 1 – 10 % (w/v) NaCl, 5 – 37 °C and pH 6.0 – 9.0 (optimum 7.0 – 8.5). The DNA G + C content is 44.0 ± 1.0 mol% (Van Trappen et al. 2004a).
3.8.1.9. *A. tagae BCRC 17571\textsuperscript{T}*

*A. tagae* was originally isolated from a Taiwanese estuary, and possesses the ability to reduce mercury (Hg\textsuperscript{2+} \rightarrow Hg\textsuperscript{0}). Cells are 1.2 – 2.5 µm in length and 0.5 – 0.9 µm wide. Cells also produce buds and prosthecae, and accumulate polyhydroxybutyrate. Growth conditions are 0.5 – 13 % (w/v) NaCl (optimum 2 – 4 %), 15 – 40 °C (optimum 30 °C) and pH 6.0 – 9.0. Their DNA G + C content is 43.1 mol% (Chiu *et al.* 2007).

3.8.1.10. *Alteromonas sp. H86*

The bacterial strain designated H86 was isolated from the first enrichment experiment after six months of incubation. Colonies of H86 grown on marine agar after two days were white, opaque, convex and circular, 2 – 3 mm in diameter. Partial sequencing of the 16S rRNA gene and subsequent BLAST analysis identified this strain as being genetically most closely related to species of *Alteromonas*.

3.8.1.11. *Alteromonas sp. H89*

The bacterial strain designated H89 was isolated from the first enrichment experiment after six months of incubation. Colonies of H89 grown on marine agar after two days were pale yellow, opaque, convex and circular, 2 – 3 mm in diameter. Partial sequencing of the 16S rRNA gene and subsequent BLAST analysis identified this strain as being genetically most closely related to species of *Alteromonas*.

3.8.1.12. *Alteromonas sp. H91*

The bacterial strain designated H91 was isolated from the first enrichment experiment after six months of incubation. Colonies of H91 grown on marine agar after two days were yellow, opaque, convex and circular, 2 – 3 mm in diameter. Partial sequencing of the 16S rRNA gene and subsequent BLAST analysis identified this strain as being genetically most closely related to species of *Alteromonas*.
3.8.2. Other bacterial strains

A number of additional bacterial strains were also employed during this work. Most of these additional strains belong to either the class *Alphaproteobacteria* or the class *Gammaproteobacteria*.

3.8.2.1. *Celeribacter neptunius H14*\(^T\)

*Celeribacter neptunius* is an Alphaproteobacterium belonging to the family *Rhodobacteraceae*, first isolated during the course of this work. *C. neptunius* represents a novel genus and species not previously described. They are Gram-negative, rod-shaped cells, 0.8 – 1.8 \(\mu\)m in length and 0.4 – 0.9 \(\mu\)m wide. They are chemoorganotrophic, facultative anaerobes, and are motile by means of single polar or subpolar flagella. Requires 1 – 8 % NaCl, temperatures of 5 – 35 °C (optimum 25 °C) and pH ranging between 4.0 – 10.0 (optimum 7.5 – 8.0) for growth to occur. The DNA G + C content is 59.1 mol% (Ivanova *et al.* 2010b).

3.8.2.2. *Glaciecola polaris LMG 21857*\(^T\)

*Glaciecola polaris* is a Gammaproteobacterium belonging to the family *Alteromonadaceae*. They are Gram-negative, rod-shaped cells, 2 – 3 \(\mu\)m in length and 0.4 \(\mu\)m wide. They are chemoheterotrophic, strict aerobes, and are motile by means of polar or sub-polar flagella. Cells produce prosthecae and buds, and can tolerate up to 10 % (w/v) NaCl. Growth occurs at temperatures between 5 – 30 °C. The DNA G + C content is 44.0 mol% (Van Trappen *et al.* 2004b).

3.8.2.3. *Idiomarina baltica LMG 21691*\(^T\)

*Idiomarina baltica* is a Gammaproteobacterium belonging to the family *Idiomarinaceae*. They are Gram-negative, rod-shaped cells, 0.7 – 1.6 \(\mu\)m in length and 0.4 – 0.7 \(\mu\)m wide. They are chemoheterotrophic, strict aerobes, and are motile by means of a single polar flagellum. Salt is required for growth, with optimum concentrations between 3 – 6 % (w/v). Growth occurs at temperatures between 8 – 46
48

°C, with optimum temperatures at 30 – 40 °C. The DNA G + C content is 49.7 mol% (Brettar et al. 2003).

3.8.2.4. *Jannaschia rubra* 4SM3<sup>T</sup>

*Jannaschia rubra* is an Alphaproteobacterium belonging to the family *Rhodobacteraceae*. They are Gram-negative, rod-shaped cells, 1.0 – 2.0 µm in length and 0.5 µm wide. They are chemoorganotrophic, strict aerobes, and are motile by means of a multiple monopolar flagella. Cells require sodium and magnesium, and grow at marine salt concentrations between 0.34 – 9 % (w/v). Optimum incubation temperature for growth is 4 – 25 °C. The DNA G + C content is 64.6 % (Macián et al. 2005).

3.8.2.5. *Marinobacter litoralis* SW-45<sup>T</sup>

*Marinobacter litoralis* is a Gammaproteobacterium belonging to the family *Alteromonadaceae*. They are Gram-negative, rod-shaped cells, 1.5 – 3.0 µm in length and 0.5 – 0.8 µm wide. They are chemoorganotrophic, strict aerobes, and are motile by means of a single polar flagellum. Growth conditions are 0.5 – 18 % (w/v) NaCl (optimum 2 – 7 %), pH 7.0 – 8.5, and temperatures between 4 – 46 °C (optimum 30 – 37 °C). The DNA G + C content is 55 mol% (Gauthier et al. 1992, Yoon et al. 2003b).

3.8.2.6. *Nautella italica* LMG 24365<sup>T</sup>

*Nautella italica* is an Alphaproteobacterium belonging to the family *Rhodobacteraceae*, originally isolated from a marine electroactive biofilm. They are Gram-negative, rod-shaped cells, 1.5 – 2.1 µm in length and 0.7 – 0.11 µm wide. They are strict aerobes, and are motile by means of a single polar flagellum. The cells accumulate poly-β-hydroxybutyrate. Salt is required for growth, with optimum concentrations between 1 – 5 % (w/v). Growth occurs at pH 5.5 – 9.0, and temperatures between 4 – 45 °C, with optimum pH and temperatures of 6.5 – 8.0 and 20 – 28 °C. The DNA G + C content is 61.0 ± 0.8 mol% (Vandecandelaere et al. 2009).
3.8.2.7. *Nereida ignava* 2SM4ᵀ

*Nereida ignava* is an Alphaproteobacterium belonging to the family *Rhodobacteraceae*. They are Gram-negative, coccoid to elongated rod-shaped cells, 1 – 3 µm in length and 0.2 – 0.3 µm wide. They are chemooorganotrophic, strict aerobes, and are non-motile. Cells can grow in marine salt concentrations between 1.36 – 8 % (w/v), and at temperatures of 13 °C and 28 °C. The DNA G + C content is 56 mol% (Pujalte et al. 2005).

3.8.2.8. *Oceanicola marinus* AZO-Cᵀ

*Oceanicola marinus* is an Alphaproteobacterium belonging to the family *Rhodobacteraceae*. They are Gram-negative, rod-shaped cells, 0.9 – 1.0 µm in length and 0.5 µm wide. They are chemoheterotrophic, facultative anaerobes, and are non-motile. Growth occurs at NaCl concentrations between 2 – 8 %, pH 6 – 9 and temperatures between 4 – 42 °C, with optimum conditions of 3 – 5 % NaCl, pH 7 and 28 – 35 °C. The DNA G + C content is 70.9 % (Lin et al. 2007).

3.8.2.9. *Pseudoalteromonas citrea* NCMB 1889ᵀ

*Pseudoalteromonas citrea* is one of several species of *Gammaproteobacteria* that were originally classified as members of the genus *Alteromonas*, but later reclassified as *Pseudoalteromonas* (Gauthier 1977, Gauthier et al. 1995). They are Gram-negative, rod-shaped cells, 1 – 1.5 µm in length and 0.5 – 0.8 µm wide. They are chemoorganotrophic, strict aerobes, and are motile by means of a single polar flagellum. Cells produce non-carotenoid pigments that range from pale yellow to orange to brown, and do not produce endospores or capsules. Sodium ions are required for growth. Optimum growth occurs at concentrations between 0.5 – 0.6 M, pH 6 – 12 and at a temperature of 28 °C, but growth can proceed from 4 – 40 °C. The DNA G + C content ranges between 38.9 – 44.7%. Additionally, some strains are capable of producing autotoxic, polyanionic antibiotics (Gauthier 1977, Gauthier et al. 1995, Ivanova et al. 1998).
3.8.2.10. **Pseudoalteromonas ruthenica KMM 300^T**

*Pseudoalteromonas ruthenica* is a Gammaproteobacterium belonging to the family *Pseudoalteromonadaceae*. They are Gram-negative, rod-shaped cells, 0.7 – 0.9 µm in diameter. They are chemoorganotrophic, strict aerobes, and are motile by means of a single polar flagellum. Requires 1 – 9 % (w/v) NaCl, pH 6.0 – 10.0 (optimum 7.5 – 8.0), and temperatures between 10 – 35 °C (optimum 25 – 30 °C) for growth. The DNA G + C content is 48.4 – 48.9 mol% (Ivanova et al. 2002b).

3.8.2.11. **Pseudoalteromonas translucida KMM 520^T**

*Pseudoalteromonas translucida* is a Gammaproteobacterium belonging to the family *Pseudoalteromonadaceae*. They are Gram-negative, rod-shaped cells, 0.7 – 0.9 µm in diameter. They are chemoorganotrophic, strict aerobes, and are motile by means of multiple flagella on both poles. Requires 1 – 6 % (w/v) NaCl, and grows at pH 6.0 – 10.0 (optimum 7.5 – 8.0), and temperatures between 4 – 30 °C (optimum 25 °C). The DNA G + C content is 46.3 % (Ivanova et al. 2002a).

3.8.2.12. **Pseudoruegeria aquimaris SW-255^T**

*Pseudoruegeria aquimaris* is an Alphaproteobacterium belonging to the family *Rhodobacteraceae*. They are Gram-negative, rod-shaped cells, 1 – 8 µm in length and 0.3 – 0.8 µm wide. They are strict aerobes, and are non-motile. Cells require NaCl for growth, and can tolerate up to 8 % (w/v). Growth occurs at temperatures of 15 °C and 49 °C, and optimum pH is 7 – 8. The DNA G + C content is 67.0 mol% (Yoon et al. 2007).

3.8.2.13. **Roseisalinus antarcticus EL-88^T**

*Roseisalinus antarcticus* is an Alphaproteobacterium belonging to the family *Rhodobacteraceae*, isolated from a hypersaline lake in Antarctica. They are motile, Gram-negative, rod-shaped cells, 2.18 – 4.20 µm in length and 0.90 – 1.02 µm wide. They are heterotrophic and aerobic to microaerophilic. Cells produce bacteriochlorophyll a, and require artificial seawater for cultivation; NaCl alone was
insufficient. Optimum growth conditions were pH 7.0 – 7.8, temperatures of 16 – 26 °C and artificial seawater concentrations of 50 – 90 ‰. The DNA G + C content is 67 mol% (Labrenz et al. 2005).

3.8.2.14. **Shewanella japonica KMM 3299T**

*Shewanella japonica* is a Gammaproteobacterium belonging to the family *Shewanellaceae*. They are Gram-negative, rod-shaped cells, 1 – 2 µm in length and 0.6 – 0.8 µm wide. They are heterotrophic, facultative anaerobes, and are motile by means of a single polar flagellum. Does not require sodium ions for growth, but can tolerate up to 3 % (w/v) NaCl. Growth occurs at pH 6.0 – 9.0 (optimum 7.5), and temperatures between 10 – 37 °C (optimum 20 – 25 °C). The DNA G + C content is 43 – 44 mol% (Ivanova et al. 2001).

3.8.2.15. **Shimia marina CL-TA03T**

*Shimia marina* is an Alphaproteobacterium belonging to the family *Rhodobacteraceae*. Cells are Gram-negative rods, 0.8 – 3.6 µm in length and 0.3 – 0.6 µm wide. They are heterotrophic, strict aerobes, and are motile by means of multiple monopolar flagella. Sea salt is required for growth, with optimum concentrations between 3 – 7 % (w/v). Growth occurs at pH 6 – 10, and temperatures between 15 – 35 °C, with optimum temperatures at 30 – 35 °C. The DNA G + C content is 57.2 % (Choi and Cho 2006).

3.8.2.16. **Thalassobacter arenae GA2-M15T**

*Thalassobacter arenae* is an Alphaproteobacterium belonging to the family *Rhodobacteraceae*. They are Gram-negative, rod-shaped cells, 1.2 – 2.4 µm in length and 0.7 – 1.2 µm wide. They are chemoorganotrophic, strict aerobes, and are motile by means of polar flagella. The cells accumulate polyhydroxybutyrate. Salt is required for growth, within the range of 0.34 – 10 % (w/v). Growth occurs at pH 6 – 9, and temperatures between 5 – 35 °C, with optimum pH and temperature of 7 and 30 °C, respectively. The DNA G + C content is 56.0 % (Kim et al. 2009).
3.8.2.17. *Thalassospira tepidiphila* 1-1B<sup>T</sup>

*Thalassospira tepidiphila* is an Alphaproteobacterium belonging to the family *Rhodospirillaceae*, originally isolated from oil-contaminated seawater. They are Gram-negative, vibroid/spiral shaped cells, 0.9 – 2.0 µm in length and 0.17 – 0.33 µm wide. The genus is composed of chemoorganotrophic aerobes (López-López *et al.* 2002). *T. tepidiphila* is motile by means of a single polar flagellum. Cells are capable of degrading polycyclic aromatic hydrocarbons. Growth occurs at NaCl concentrations between 2 – 14 %, pH 5 – 10 and temperatures between 8 – 45 °C, with optimum conditions of 3 % NaCl, pH 7 and 43 °C. The DNA G + C content is 55.1 ± 0.4 % (Kodama *et al.* 2008).

3.8.2.18. *Thalassospira* sp. H88

The bacterial strain designated H88 was isolated from the first enrichment experiment after six months of incubation. Colonies of H88 grown on marine agar after two days were pale yellow, opaque, convex and circular, 1 – 2 mm in diameter. Partial sequencing of the 16S rRNA gene and subsequent BLAST analysis identified this strain as being genetically most closely related to species of *Thalassospira*.

3.8.2.19. *Thalassospira* sp. H93

The bacterial strain designated H93 was isolated from the first enrichment experiment after six months of incubation. Colonies of H93 grown on marine agar after two days were white/colourless, transparent, convex and circular, <1 mm in diameter. Partial sequencing of the 16S rRNA gene and subsequent BLAST analysis identified this strain as being genetically most closely related to species of *Thalassospira*.

3.8.2.20. *Thalassospira* sp. H94

The bacterial strain designated H94 was isolated from the first enrichment experiment after six months of incubation. Colonies of H94 grown on marine agar after two days were white/colourless, transparent, convex and circular, <1 mm in diameter.
Partial sequencing of the 16S rRNA gene and subsequent BLAST analysis identified this strain as being genetically most closely related to species of *Thalassospira*.

### 3.9. Biofilm characterisation using confocal laser scanning microscopy

In order to assess bacterial attachment and growth, confocal laser scanning microscopy (CLSM) was performed. Biofilms were imaged using a FluoView™ FV1000 spectroscopic confocal system (Olympus, Japan), which included an inverted microscope system Olympus IX81 with 20×, 40× (oil) and 100× (oil) UIS objectives, multi Ar and HeNe lasers (458 nm, 488 nm, 515 nm, 543 nm and 633 nm wavelengths) and a transmitted differential interference contract attachment and CCD camera (Cool View FDI, U.K.). For visualisation, biofilms were stained with two fluorescent dyes: an AlexaFluor 488-conjugated concanavalin A (Invitrogen, U.S.A.), and Vybrant CFDA SE Cell Tracer kit (Invitrogen, U.S.A.), according to the manufacturer’s protocols. Concanavalin A is a lectin that binds to a number of polysaccharides, including common exopolysaccharides produced by many bacteria. Thus, conjugation of concanavalin A with a fluorescent dye is an effective technique for labelling of the biofilm matrix. The excitation and emission maxima of AlexaFluor 488 are 495 nm and 519 nm respectively. The Vybrant CFDA SE Cell Tracer kit contains carboxyfluorescein diacetate succinimidyl ester, which is colourless and does not fluoresce. However, when this compound is taken up via passive diffusion into viable, active cells it is metabolised by cell esterases, resulting in the formation of highly fluorescent carboxyfluorescein succinimidyl ester which binds to amine groups within the cell, causing it to be retained. The end result is fluorescent labelling of all viable cells. The excitation and emission maxima of carboxyfluorescein succinimidyl ester are 492 nm and 517 nm respectively.

### 3.10. Scanning electron microscopy

*Celeribacter neptunius* H14ᵀ cells were immobilised for observation of morphology by scanning electron microscopy (SEM). Cells were grown overnight at 25 °C, and 1 mL of the resulting culture was subsequently incubated on aluminium discs for 24 h at 25 °C. After incubation, discs were rinsed with distilled water and air dried, before
coating with a thin layer of gold using a Dynavac CS300 unit. Electron micrographs were obtained using a field emission scanning electron microscope (FESEM, Supra 40 VP, Carl-Zeiss, Germany) under 2000×, 5000×, 10000×, 15000×, 70000× and 100000× magnification.

3.11. Surface characterisation

Bacterial recovery and characterisation only accounts for half of the required experimental work of this project. In order to measure and assess the degradation effect bacteria have on PET surfaces, certain surface analytical tools needed to be employed. The physico-chemical characterisation techniques used to analyse the PET surfaces are described below.

3.11.1. Atomic force microscopy

Unless otherwise indicated, AFM analysis conditions were as follows. AFM scans were performed in air at ambient conditions using an Innova scanning probe microscope (Veeco, Bruker, U.S.A.) in tapping mode. Phosphorus-doped silicon cantilevers (MPP-31120-10, Veeco, Bruker) with a spring constant of 0.9 N/m, tip radius of curvature of 8 nm and a resonance frequency of ~20 kHz were utilized for surface imaging. Scanning was performed perpendicular to the cantilever axis at a speed of 1 Hz. The resulting topographical data were processed with first order horizontal and vertical levelling before performing roughness analysis. Topographical analysis was performed by importing the resulting AFM data files into the software Nanoscope (v1.10, Veeco) and selecting the roughness tool. The entire scan field was encompassed in the roughness calculations. The roughness parameters included the average roughness ($S_a$), root-mean-square (rms) roughness ($S_q$), maximum height difference ($S_{max}$), skewness ($S_{sk}$), kurtosis ($S_{ku}$) and developed surface area ($S_{da}$). To minimize the effect of noise on peak counts, surfaces were smoothed using a convolution algorithm, and the minimum threshold for definition of peaks was set at 10% of the average roughness above the mean height. Processed data files were exported to Avizo® (Visualization Sciences Group, France) for generation of three-dimensional surface profiles.
3.11.2. Surface wettability and surface free energy

The wettability and free energy of PET surfaces were determined using the sessile drop technique and the Lewis acid/base method (Van Oss et al. 1986, Crawford et al. 1987, Van Oss et al. 1988, Guy et al. 1996, Öner and McCarthy 2000). Contact angles of three diagnostic liquids (water, formamide and diiodomethane) were measured using an FTA1000 instrument (First Ten Angstroms, U.S.A.) on PET surfaces and bacterial lawns. Bacterial lawns were prepared by filtering 100 mL of bacterial culture grown overnight in marine broth, through cellulose acetate filters with a pore diameter of 0.2 µm. The resulting bacterial lawns were allowed to air-dry for approximately 30 minutes before performing contact angle experiments. Once a minimum of three repeated contact angle measurements had been taken for each diagnostic liquid for a given PET sample or bacterial lawn, the surface free energy was calculated based on the results, using the instrument software.

3.11.3. X-ray photoelectron spectroscopy

The surface chemistry of the PET plastic was analysed using an Axis Ultra x-ray photoelectron spectrometer (Kratos Analytical Ltd., UK) fitted with a monochromatic x-ray source (Al Kα, hv = 1486.6 eV) operating at 150W. The Au 4f7/2 photoelectron peak with binding energy of 83.98 eV was used to calibrate the spectrometer energy scale. Survey scans were first performed from 0 to 1400 eV, and the relative atomic concentrations of the elements detected were calculated from the areas under the peaks in the spectrum. High resolution scans were then performed across the peaks corresponding to C1s, O1s, N1s, Cl2p and S2p binding energies using scanning ranges of no more than 20 eV. Peaks in the high resolution spectra were fitted to Gaussian-Lorentzian components with subtraction of a linear background using Kratos Vision II software.

3.11.4. Infrared microspectroscopy

The spatial distribution of chemical functionalities present across selected areas of the PET samples was mapped by FTIR microspectroscopy at the Australian Synchrotron on the Infrared Microspectroscopy beamline. Samples were scanned in transmission mode and in
attenuated total reflection (ATR) mode over several scanned areas of approximately 50 µm × 50 µm, using a Bruker Hyperion 2000 FTIR microscope, (Bruker Optic GmbH, Germany). The microscope was equipped with a 36× (0.5 numerical aperture) reflecting objective and condenser, a narrow-band mercury cadmium telluride detector and a Bruker V80v FTIR spectrometer. Infrared map data sets were constructed by collecting square arrays of infrared absorbance spectra at spatial intervals of 3 µm. Maps of chemical functional groups were generated using the instrument software (OPUS v6.5, Bruker, Germany) by integrating the area under each absorption curve of a given array of infrared spectra, specifying the start and end wavenumber from which both the integration and a straight baseline were taken.
Chapter 4: Mechanisms of PET biodegradation
4.1. Overview

Interactions between PET and bacteria are localised to the interface between the two. Accordingly, it is expected that any degradative effect that bacteria are able to exert upon PET polymers occurs on the surface of the plastic. This chapter will present an analysis of the physical and chemical changes of PET surfaces induced by bacteria, and attempt to elucidate possible degradation mechanisms.

4.2. Long-term PET degradation studies – Enrichment 1

PET biodegradation is known to proceed exceedingly slowly in the environment. As such, allowances were made during the initial enrichment culture experiments to facilitate long periods of incubation. The data presented in Sections 4.2.1 and 4.2.2 were obtained after incubation periods of six and ten months, respectively.

4.2.1. PET degradation after six months

4.2.1.1. Topographical analysis

After six months of incubation in enrichment culture, individual PET pieces were aseptically removed from the enrichment vessels and subjected to topographical and chemical analysis. Topographical analysis was performed using a Solver P7LS scanning probe microscope (NT-MDT, Netherlands), fitted with carbon ‘whisker’ silicon cantilevers (NSC05, NT-MDT, Netherlands) with a spring constant of 11 N m\(^{-1}\), in semi-contact mode. Typical topographical images of control (undegraded PET samples) and degraded surfaces are shown in Figure 4.1, along with single line profiles extracted from the positions indicated on two-dimensional maps.

While both surfaces were in general quite smooth, there is quite an obvious difference observable between the two. The degraded surface lacks the particle-like features that are present on control surface, and appears to be somewhat smoother overall. This fact was confirmed by statistical roughness analysis (Table 4.1).
Figure 4.1. Two dimensional representations of control and degraded PET surface topography, with single line profiles of the indicated positions.

Table 4.1. Comparative roughness analysis of control and degraded PET surfaces.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Scanned area</th>
<th>$S_a$ (nm)</th>
<th>$S_q$ (nm)</th>
<th>$S_{max}$ (nm)</th>
<th>$S_{sk}$</th>
<th>$S_{ku}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 μm × 10 μm</td>
<td>3.29</td>
<td>4.9</td>
<td>64.4</td>
<td>2.46</td>
<td>13.2</td>
</tr>
<tr>
<td>Degraded</td>
<td>10 μm × 10 μm</td>
<td>1.81</td>
<td>2.68</td>
<td>79.8</td>
<td>4.92</td>
<td>87.4</td>
</tr>
</tbody>
</table>
The decreased average roughness, $S_a$, and RMS roughness, $S_q$, of the degraded sample relative to that of the control sample is a good indication that the degraded sample has in fact become smoother. The maximum roughness, $S_{\text{max}}$, appears contradictory as it is higher for the degraded sample, however as $S_{\text{max}}$ describes the entire range of height values of a surface it can be substantially affected by the presence of outliers, as is the case here. The increased skewness, $S_{sk}$, and kurtosis, $S_{ku}$, of the degraded sample indicate that the shape and spatial distribution of the peaks has also altered. Higher $S_{sk}$ values indicate a tendency toward high, narrow peaks and shallow, broader valleys, while large kurtosis values indicate that the peaks are relatively sharp.

The decreased number and size of peaks, combined with increased sharpness of the remaining peaks may indicate that the protrusions on the surface of PET are degraded preferentially by bacteria. This is likely due to the larger exposed surface area of these sections, which makes them more readily available to bacterial cells.

4.2.1.2. Chemical analysis

Chemical analysis of the control and two separate degraded PET samples was performed using XPS, as described in Section 3.11.3. Survey scans were first performed on each sample (Figs 4.2 – 4.4) to determine the elemental composition of each, summarized in Table 4.2. High-resolution scans were then performed of the binding energy ranges containing the major elements detected on the two surfaces, and Gaussian-Lorentzian components were fitted to the resulting spectra to identify the contributing chemical species (Figs 4.5 – 4.7). Components contributing to the C$_{1s}$, O$_{1s}$, N$_{1s}$ and S$_{2p}$ peaks are compared in Table 4.3.

The elemental composition of each of the PET samples was dominated by carbon and oxygen, which together comprised more than 97% of the surface in each case. This is unsurprising, as PET polymer chains are composed of only these two elements (and hydrogen, which cannot be detected by XPS). However the ratio of carbon to oxygen is not as expected; based on the stoichiometry of the PET molecule the ratio of C:O should be 5:2, but is in fact is above 3:1 for each of the samples. This is likely due to adsorption of various organic contaminants on the sample surfaces, which is a common, and difficult to resolve problem when performing XPS analysis (Henderson 1998).
Figure 4.2. XPS survey spectrum of a control PET surface.

Figure 4.3. XPS survey spectrum of degraded PET surface 1.
Generally speaking, the elemental composition of each of the degraded samples remained relatively unchanged in comparison to control sample. There were some small fluctuations in the proportions of many of the detected elements, however in most cases the two degraded samples deviated from the elemental composition of the control in different ways, indicating either a) that the two degraded samples were being modified in different ways, or b) that the elemental fluctuations were within the ranges of natural elemental variation for PET surfaces immersed in seawater for six months. The
degraded samples did however show consistent increases in both nitrogen and sulphur content, both of which are elements commonly cycled by bacteria (Gonzalez et al. 1999, Aminot and Kérouel 2004, Simjouw et al. 2004, Vila-Costa et al. 2006). This may or may not indicate bacterial modifications of the polymer, as the measured increases were quite small.

**Figure 4.5.** High-resolution spectra of the C\(_{1s}\), O\(_{1s}\), N\(_{1s}\) and S\(_{2p}\) peaks of a control PET surface as determined by XPS, with fitted components.
Figure 4.6. High-resolution spectra of the C\textsubscript{1s}, O\textsubscript{1s}, N\textsubscript{1s} and S\textsubscript{2p} peaks of degraded PET sample 1 as determined by XPS, with fitted components.
Figure 4.7. High-resolution spectra of the C\textsubscript{1s}, O\textsubscript{1s}, N\textsubscript{1s} and S\textsubscript{2p} peaks of degraded PET sample 1 as determined by XPS, with fitted components.

Table 4.3. Chemical component contributions to the C\textsubscript{1s}, O\textsubscript{1s}, N\textsubscript{1s} and S\textsubscript{2p} peaks of control and degraded PET surfaces.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Component</th>
<th>Control PET</th>
<th>Degraded PET 1</th>
<th>Degraded PET 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{1s}</td>
<td>Aromatic C</td>
<td>71.1</td>
<td>62.7</td>
<td>71.2</td>
</tr>
<tr>
<td></td>
<td>C–O</td>
<td>16.9</td>
<td>23.0</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>O=C–O</td>
<td>11.9</td>
<td>14.5</td>
<td>11.9</td>
</tr>
<tr>
<td>O\textsubscript{1s}</td>
<td>O=C</td>
<td>56.8</td>
<td>52.8</td>
<td>54.1</td>
</tr>
<tr>
<td></td>
<td>O=C</td>
<td>43.2</td>
<td>47.2</td>
<td>45.9</td>
</tr>
<tr>
<td>N\textsubscript{1s}</td>
<td>N=C</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S\textsubscript{2p}</td>
<td>Component 1*</td>
<td>–</td>
<td>66.8</td>
<td>66.7</td>
</tr>
<tr>
<td></td>
<td>Component 2*</td>
<td>–</td>
<td>33.2</td>
<td>33.3</td>
</tr>
</tbody>
</table>

*Component 1 and 2 refer to unidentified peak components
Similarly to the elemental composition of the PET samples degraded over six months, the contributing molecular species of the C$_{1s}$, O$_{1s}$, N$_{1s}$ and S$_{2p}$ peaks are largely inconclusive, with one possible exception. The ratio between the carbonyl oxygen atom and the oxygen heteroatom incorporated into the polymer backbone has shifted slightly, with both degraded samples tending slightly more towards the latter. The carbonyl group may act as a nucleophile, leading to formation of new carbon – oxygen bonds with small organic molecules.

It should be noted that the components of the S$_{2p}$ peak could not be conclusively identified; the binding energies of each component correlated poorly with known sulphur-species binding energies. Additionally, while the data analysis software calculated two individual components to be contributing to the S$_{2p}$ peak, the peak is quite small and the spectrum is relatively noisy, which may lead to false identification.

4.2.2. PET degradation after ten months

4.2.2.1. Topographical analysis

AFM scans of a PET surface degraded over a period of 10 months were performed under the same conditions described in Section 4.2.1.1. Two different size scanning areas are presented in 2D and 3D form, i.e. a 10 μm × 10 μm field (Fig 4.8. A, C) and a 5 μm × 5 μm field (Fig 4.8. B, D). Performing roughness calculations for multiple different scanning areas of the same surface allows for a more comprehensive analysis of the surface, as surface topography is dependent on scale.

Compared to the degraded PET sample after six months, the 10 month-degraded samples became even smoother, with all calculated roughness parameters decreasing (Table 4.4). This suggests a progression of the ‘peak erosion’ scenario put forward in Section 4.2.1.1. The taller protrusions on the PET surface have been worn down further, decreasing the overall height of the peaks, as indicated by the decreases in $S_a$, $S_q$ and $S_{max}$. Skewness and kurtosis of the 10 month sample decreased, after the initial increase observed after six months. Erosion of the sides of the taller surface features would result in an initial increase in peak sharpness, however at some point the feature would become exceedingly thin and fragile and may be easily broken off, resulting in a small ‘stub’ being left behind on the surface. This would be observable by roughness analysis.
as decreases in typical height parameters (i.e. $S_a$, $S_q$) and decreased $S_{sk}$ and $S_{ku}$, which is consistent with the current data.

![Figure 4.8. Two dimensional representations of AFM scans of 10 μm × 10 μm (A) and 5 μm × 5 μm (B) areas of PET degraded over a period of 10 months, and corresponding three-dimensional constructions (C, D respectively).]

**Table 4.4.** Roughness analysis of different size fields of view of PET samples degraded over ten months

<table>
<thead>
<tr>
<th>Scanned area</th>
<th>$S_a$ (nm)</th>
<th>$S_q$ (nm)</th>
<th>$S_{max}$ (nm)</th>
<th>$S_{sk}$</th>
<th>$S_{ku}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μm × 10 μm</td>
<td>1.20</td>
<td>1.86</td>
<td>35.0</td>
<td>2.65</td>
<td>20.8</td>
</tr>
<tr>
<td>5 μm × 5 μm</td>
<td>0.84</td>
<td>1.15</td>
<td>18.8</td>
<td>1.64</td>
<td>14.5</td>
</tr>
</tbody>
</table>
4.2.2.2. Chemical analysis

Chemical analysis of the 10 month degraded PET samples was performed using XPS, as described in Section 3.12.3. Survey scans detected the presence of some ions not detected on the six month degraded samples (Fig. 4.9). In addition to this, some interesting changes in elemental proportions were observed (Table 4.5).

![XPS survey spectrum of a PET surface degraded over a period of 10 months.](image)

**Figure 4.9.** XPS survey spectrum of a PET surface degraded over a period of 10 months.

**Table 4.5.** Elemental composition of a PET sample degraded over 10 months.

<table>
<thead>
<tr>
<th>Element</th>
<th>Relative atomic concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>72.9</td>
</tr>
<tr>
<td>Oxygen</td>
<td>21.5</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>2.6</td>
</tr>
<tr>
<td>Sulphur</td>
<td>0.3</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.9</td>
</tr>
<tr>
<td>Potassium</td>
<td>1.4</td>
</tr>
<tr>
<td>Chlorine</td>
<td>0.4</td>
</tr>
</tbody>
</table>
The most significant change in elemental composition observed is that of nitrogen. The proportion of nitrogen present on the surface of the 10 month sample is more than double the proportion of nitrogen on the six month sample. The increase in nitrogen that occurred during the additional four months far exceeds that of the first six, which suggests that it is not simply a result of adsorption or absorption processes that may occur naturally in seawater. Such processes would be fastest immediately upon immersion, and would progressively slow as the system neared equilibrium. Rather, it is more likely that the process is mediated by bacteria, or more specifically bacterial enzymes which are able to work with increasing efficiency due to positive feedback mechanisms, e.g. a polymer chain may protrude slightly further from the bulk material in the immediate area surrounding an introduced functionalisation, facilitating further functionalisation and subsequently increased chain availability.

The carbon, oxygen and nitrogen components that make up the 10 month degraded PET surface are significantly more complex than that of six months samples (Fig. 4.10, Table 4.6). First of all, new components of all three elements were detected on the 10 month samples which were not previously detected. New chemical species detected were alkyl carbon, carbonyl carbon (as opposed to the carbonyl carbon of the polyester backbone), an unidentified oxide (most likely $\text{SO}_3^2$), alcohol and/or ether and ammonium. In addition to the new chemical components, the proportions of the chemical groups that make up the polymer chain changed. The ratio of aromatic carbon : $\text{C}–\text{O}$ : $\text{O}=\text{C}–\text{O}$ (expected from stoichiometry to be 3:1:1) was approximately 5:2:1, and similarly the ratio of the two oxygen components (expected to be 1:1) was approximately 3:2.
Figure 4.10. High-resolution spectra of the $C_{1s}$, $O_{1s}$, $N_{1s}$, $S_{2p}$ and $Cl_{2p}$ peaks of a PET surface degraded over a period of 10 months, with fitted components.
Table 4.6. Chemical component contributions to the C\textsubscript{1s}, O\textsubscript{1s} and N\textsubscript{1s} peaks of a PET sample degraded over 10 months.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Component</th>
<th>Component contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{1s}</td>
<td>Aromatic C</td>
<td>53.1</td>
</tr>
<tr>
<td></td>
<td>C–C, C–H</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>C–O</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td>C=O</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>O=C–O</td>
<td>9.5</td>
</tr>
<tr>
<td>O\textsubscript{1s}</td>
<td>Oxide\textsuperscript{*}</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>O=C</td>
<td>45.3</td>
</tr>
<tr>
<td></td>
<td>C–O–R\textsuperscript{†}</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>O–C</td>
<td>28.7</td>
</tr>
<tr>
<td>N\textsubscript{1s}</td>
<td>N–C</td>
<td>82.6</td>
</tr>
<tr>
<td></td>
<td>N\textsuperscript{†}</td>
<td>17.4</td>
</tr>
</tbody>
</table>

\textsuperscript{*}This component was inconclusively identified, but is thought to be an oxide of some form, most likely sulphite.

\textsuperscript{†} C–O–R refers to an alcohol or ether, and is to be distinguished from O–C, which is the oxygen atom between two carbons in an ester bond, such as is part of the PET chain.

4.3. The role of light in bacterial degradation of PET

Long-term enrichment culture experiments suggested that bacteria do have the capability for modifying and degrading PET surfaces. However for any practical application to be possible, the efficiency of the process needs to be significantly improved. While the culture conditions of the original enrichment experiment provide selective pressure for organisms with the capability to utilise PET as an energy source, organisms which employ other strategies for obtaining energy may also receive a selective advantage. Two examples are photoautotrophic bacteria and chemolithotrophic bacteria. Chemolithotrophs obtain energy by reduction of various inorganic substrates (Madigan \textit{et al.} 2003), however in this instance are not of particular concern, as they are expected to deplete their energy sources relatively quickly, due to the confined nature of the experiment. Of greater concern are the photoautotrophs, which utilise light for
energy production (Madigan et al. 2003). In order to assess the role, if any, which photoautotrophic bacteria play in this particular enrichment culture, and to provide the true PET-degrading bacteria with an enhanced selective advantage, a second enrichment experiment was performed, which included an incubation condition that was maintained in the absence of light. Five different incubation conditions (including 2 controls) were prepared in triplicate: i) PET films in sterilised seawater, ii) PET films in sterile pure water (MilliQ), iii) PET films in seawater, iv) PET bottle pieces in seawater and v) PET films in seawater in the absence of light. The incubation period was eight months, with subsequent topographical and chemical characterisation.

4.3.1. Topographical analysis

AFM scans of degraded PET bottle pieces and PET films degraded in the presence and absence of light were performed after eight months of enrichment incubation. The degraded bottle sample showed a distinctly different topography, while the difference between the two films was more subtle (Fig. 4.11). The differences were all the samples were better defined and expounded by comparative roughness analysis (Table 4.7).
**Figure 4.11.** Three-dimensional reconstructions of approximately 10 μm × 10 μm scanning areas of a PET film incubated in seawater, b PET bottle piece incubated in seawater and c PET film incubated in seawater in dark conditions. All incubations lasted eight months.
Table 4.7. Comparative roughness analysis of PET samples degraded under various conditions over a period of eight months.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>$S_a$ (nm)</th>
<th>$S_q$ (nm)</th>
<th>$S_{max}$ (nm)</th>
<th>$S_{uk}$</th>
<th>$S_{ku}$</th>
<th>$S_{dr}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET film + Sterile seawater</td>
<td>3.69</td>
<td>4.75</td>
<td>38.1</td>
<td>0.568</td>
<td>4.02</td>
<td>100.03</td>
</tr>
<tr>
<td>PET film + MilliQ water</td>
<td>1.12</td>
<td>1.54</td>
<td>14.7</td>
<td>1.79</td>
<td>7.21</td>
<td>100.02</td>
</tr>
<tr>
<td>PET film + seawater</td>
<td>3.48</td>
<td>4.50</td>
<td>38.0</td>
<td>0.878</td>
<td>4.73</td>
<td>100.03</td>
</tr>
<tr>
<td>PET bottle + seawater</td>
<td>1.04</td>
<td>1.51</td>
<td>17.6</td>
<td>2.25</td>
<td>12.7</td>
<td>100.02</td>
</tr>
<tr>
<td>PET film + seawater (dark)</td>
<td>2.95</td>
<td>3.83</td>
<td>28.2</td>
<td>0.782</td>
<td>4.10</td>
<td>100.02</td>
</tr>
</tbody>
</table>

For the most part, PET films samples displayed higher amplitude roughness parameters than the bottle sample. The exception to this was the MilliQ water control, which remained particularly smooth. This suggests some ‘roughening’ effect exerted on the PET films by seawater that does not occur on plastic bottles. Despite this, there was a partial compensation observed particularly in the dark sample, which saw a decrease in $S_a$, $S_q$, and $S_{max}$, compared to that of the control seawater film. This suggests a scenario where upon immersion, PET film surfaces initially, and probably relatively quickly, increase in roughness, perhaps due to water absorption processes, before bacteria begin to erode the surface features. Erosion occurred to a greater degree in the dark conditions than in the light-exposed, which may indicate that inhibition of photoautotrophic growth does indeed provide PET-degrading organisms with additional selective advantage.

4.3.2. Chemical analysis

Chemical analysis was again performed using XPS. Survey scans of samples from each incubation condition are shown in Figure 4.12, and relative elemental concentration details are presented in Table 4.8.
**Figure 4.12.** XPS survey spectra of **a** PET film incubated in sterile seawater, **b** PET film incubated in sterile pure water, **c** PET film incubated in seawater, **d** PET bottle piece incubated in seawater and **e** PET film incubated in seawater in dark conditions. All incubations lasted for a period of eight months.

**Table 4.8.** Elemental composition of PET samples incubated over a period of eight months under different conditions.

<table>
<thead>
<tr>
<th>Element</th>
<th>PET film + sterile seawater</th>
<th>PET film + MilliQ water</th>
<th>PET film + seawater</th>
<th>PET bottle pieces + seawater</th>
<th>PET film + seawater (dark)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>73.1</td>
<td>75.1</td>
<td>73.2</td>
<td>72.5</td>
<td>74.7</td>
</tr>
<tr>
<td>Oxygen</td>
<td>25.1</td>
<td>23.9</td>
<td>25.4</td>
<td>25.2</td>
<td>22.3</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>1.5</td>
<td>0.7</td>
<td>1.1</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Sulphur</td>
<td>–</td>
<td>–</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Sodium</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.2</td>
</tr>
<tr>
<td>Silicon</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Tin</td>
<td>0.1</td>
<td>–</td>
<td>0.1</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Elemental analysis did not reveal many substantial changes in surface chemistry of the degraded samples, however there are two differences to note. The first is the presence of sulphur on the surfaces of PET samples from all three of the degradation conditions, while sulphur concentration on the control surfaces remained below the detectable level. This confirms that the appearance of sulphur on degraded samples is a result of bacterial modifications. The second point to note is the significant amount of sodium detected on the surface of the PET incubated in dark conditions, likely in the form of salt crystals.

The detection of silicon and tin on the surfaces of the PET samples was unexpected, and is unlikely that their presence is the result of any biological actions. However for certainty, both of these elements were included in chemical component analysis.

A number of revelations are made by chemical component analyses that are not apparent from elemental analysis (Figs 4.13 – 4.17, Table 4.9). First, the sulphur present on each of the degraded samples is in a single form, i.e. sulphate. Second, the silicon present on all samples was in oxide form, i.e. silica/sand. Strangely however, no oxide component was detected in the O\textsubscript{1s} peak of any sample except the dark condition sample. It may be that as silicon was detected in small amounts only just exceeding the detection limit, the corresponding contribution to the O\textsubscript{1s} peak may be overwhelmed by the more abundant oxygen components. Third, the tin detected on three of the samples was approximately equal proportions of Sn\textsuperscript{2+} and Sn\textsuperscript{4+} ionic species, and likely formed nitrides; the same three PET samples displayed nitride components of the N\textsubscript{1s} peak. Finally, the PET sample incubated under dark conditions showed an increase in the proportion of aromatic carbon relative to the other two carbon components that form the polymeric chains. This means that either new aromatic groups are being formed on the surface, or more likely, that oxygenated carbon species are being removed from the surface via an unspecified mechanism.
Figure 4.13. High-resolution spectra of the $C_{1s}$, $O_{1s}$, $N_{1s}$, $S_{2p}$, $Si_{2p}$ and $Sn_{3d}$ regions of the XPS survey spectrum of PET film incubated in sterile seawater for eight months.
Figure 4.14. High-resolution spectra of the $C_{1s}$, $O_{1s}$, $N_{1s}$, $S_{2p}$, $Si_{2p}$ and $Sn_{3d}$ regions of the XPS survey spectrum of PET film incubated in sterile pure water for eight months.
Figure 4.15. High-resolution spectra of the C$_{1s}$, O$_{1s}$, N$_{1s}$, S$_{2p}$, Si$_{2p}$ and Sn$_{3d}$ regions of the XPS survey spectrum of PET film incubated in seawater for eight months.
Figure 4.16. High-resolution spectra of the C_{1s}, O_{1s}, N_{1s}, S_{2p}, Si_{2p} and Sn_{3d} regions of the XPS survey spectrum of PET bottle pieces incubated in seawater for eight months.
Figure 4.17. High-resolution spectra of the C$_{1s}$, O$_{1s}$, N$_{1s}$, S$_{2p}$, Si$_{2p}$ and Sn$_{3d}$ regions of the XPS survey spectrum of PET film incubated in seawater in dark conditions for eight months.
### Table 4.9. Chemical component contributions to the $C_{1s}$, $O_{1s}$, $N_{1s}$, $S_{2p}$, $Si_{2p}$ and $Sn_{3d}$ peaks of PET samples degraded over eight months under various incubation conditions.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Component</th>
<th>PET film + sterile seawater</th>
<th>PET film + MilliQ water</th>
<th>PET film + seawater</th>
<th>PET bottle pieces + seawater</th>
<th>PET film + seawater (dark)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1s}$</td>
<td>$C_{Ar}$</td>
<td>60.7</td>
<td>62.7</td>
<td>60.9</td>
<td>64.9</td>
<td>70.1</td>
</tr>
<tr>
<td></td>
<td>$C–O$</td>
<td>22.3</td>
<td>21.6</td>
<td>22.2</td>
<td>20.3</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>$O=\cdot C–O$</td>
<td>16.8</td>
<td>15.7</td>
<td>16.6</td>
<td>13.5</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>$C=O$</td>
<td>0.2</td>
<td>–</td>
<td>0.3</td>
<td>1.3</td>
<td>2.2</td>
</tr>
<tr>
<td>$O_{1s}$</td>
<td>$O=\cdot C$</td>
<td>49.0</td>
<td>46.7</td>
<td>48.6</td>
<td>54.3</td>
<td>49.5</td>
</tr>
<tr>
<td></td>
<td>$O–C$</td>
<td>51.0</td>
<td>53.3</td>
<td>51.4</td>
<td>45.7</td>
<td>40.3</td>
</tr>
<tr>
<td></td>
<td>$O^2–/OH^–$</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>10.2</td>
</tr>
<tr>
<td>$N_{1s}$</td>
<td>$N–C$</td>
<td>83.8</td>
<td>73.1</td>
<td>83.1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$N^3–$</td>
<td>16.2</td>
<td>26.9</td>
<td>16.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$S_{2p}$</td>
<td>$SO_4$</td>
<td>–</td>
<td>–</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$Si_{2p}$</td>
<td>$Si–O$</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$Sn_{3d}$</td>
<td>$Sn^{2+}$</td>
<td>51.8</td>
<td>51.3</td>
<td>50.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>$Sn^{4+}$</td>
<td>48.2</td>
<td>48.7</td>
<td>49.7</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*The third oxygen component contributing to the $O_{1s}$ peak consists of oxide and/or hydroxide; distinction between the two could not be made conclusively.

#### 4.4. Degradation quantification and optimisation of conditions

The previous sections in this chapter are all devoted to qualitative characterisation of the PET biodegradation mechanisms of bacteria. In this section, the first attempt at quantification of the degradation rate is presented. In addition to simply measuring the rate of PET biodegradation, various bacterial cultures and chemical additives were tested for their ability to enhance the rate of degradation.
4.4.1. Measured loss of mass

Four different bacterial cultures were used, plus a sterile control. Two cultures were single species cultures: *Thalassospira* sp. H94, isolated from early enrichment experiments, and *Thalassospira tepidiphila* 1-1B\(^T\), a known degrader of polycyclic aromatic hydrocarbons (Kodama *et al.* 2008). The other two cultures were mixed species; one was a consortium of three *Thalassospira* spp. and three *Alteromonas* spp., all of which were isolated during early enrichment experiments, and the other was a sample of the entire bacterial community developed in an enrichment experiment, taken directly from the incubation flask. For each culture, four different combinations of additives were tested, as well as a control without any additives. The four additive combinations are as follows:

- Supplementation of the nitrogen and phosphorus reserves naturally present in seawater (designated as N/P samples). Previous studies have indicated that bacterial growth in hydrocarbon contaminated environments can be limited by low concentration of trace nutrients required for growth (Iranzo *et al.* 2001, Nikolopoulou and Kalogerakis 2008).
- Addition of Fe (II) to culture media. Detection of alkyl carbon presented in Section 4.2.2.2 suggested a possibility of aromatic ring-opening oxygenases, many of which require the presence of Fe\(^{2+}\) ions (Schlafl et al. 1994, Fukuhara *et al.* 2008).
- Addition of Tween 20 surfactant. Previous studies have suggested the idea that aromatic polyester degradation is limited by polymer chain mobility (Marten *et al.* 2003, Müller *et al.* 2005). By adding surfactants to the culture media it is hoped that chain mobility into the aqueous phase may be more favourable.
- Combination of all three conditions listed above.

Individual pieces of polymer film were weighed both before and after three months incubation in each of the above experimental conditions to trace any loss of mass (Fig. 4.18). Because of the nature of this kind of experiment, i.e. involving biological systems, relatively large error values were associated with the measured changes in mass of the PET films. Despite this, however, a significant loss of mass was recorded for films degraded by the enrichment community supplemented with additional nitrogen.
and phosphorus. On average, PET films degraded under these conditions lost 1.43% of their original mass.

**Figure 4.18.** Average loss of mass of PET measured after three months incubation in various culture conditions. The loss of mass associated with PET samples incubated in bacterial enrichment community culture supplemented with nitrogen and phosphorus was statistically significant.

4.4.2. **Topographical analysis**

To determine any qualitative differences in the mode of PET biodegradation when bacteria are supplemented with extra nitrogen and phosphorus, samples from the above experiment were subjected to topographical and chemical analysis. AFM scans were performed on PET samples that had been degraded by the bacterial enrichment community both with and without nitrogen and phosphorus supplementation and subsequently compared (Fig. 4.19).
Figure 4.19. Three-dimensional representations of AFM scan data on PET film samples degraded by the bacterial enrichment community a with, and b without supplementation with nitrogen and phosphorus. Films were degraded over a period of three months.

Comparison of the two surface topographies showed that architecture of both was quite similar. There is a small observable difference in the number and size of the major
peaks on the two surfaces; N/P supplementation appears to result in slightly smoother surfaces. Roughness analysis also appears to suggest this; samples degraded with nitrogen and phosphorus supplements had with slightly lower amplitude values (Table 4.10). The two samples appear to have been degraded via essentially the same mechanisms, although the degradation of the supplemented sample may be slightly more advanced. This supports the hypothesis that bacterial growth is limited by the availability of trace nutrients, and suggests that increasing the reserves of these nutrients helps to boost the metabolic activities of the bacterial community.

Table 4.10. Comparative surface roughness analysis of PET films degraded by the bacterial enrichment community with and without nitrogen and phosphorus supplementation.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>$S_a$ (nm)</th>
<th>$S_q$ (nm)</th>
<th>$S_{\text{max}}$ (nm)</th>
<th>$S_{sk}$</th>
<th>$S_{ku}$</th>
<th>$S_{dr}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additional N/P</td>
<td>2.58</td>
<td>3.96</td>
<td>47.92</td>
<td>2.4</td>
<td>8.08</td>
<td>100.68</td>
</tr>
<tr>
<td>N/P supplemented culture</td>
<td>2.17</td>
<td>3.48</td>
<td>53.08</td>
<td>2.67</td>
<td>10.9</td>
<td>100.47</td>
</tr>
</tbody>
</table>

4.4.3. Chemical analysis

In comparison to changes in topography, changes in the surface chemistry of the PET film degraded with nitrogen and phosphorus supplementation were more obvious (Figs 4.20, 4.21, Table 4.11). Perhaps the most interesting change observed was a shift in the ratio of carbon to oxygen, decreasing from 3.04:1 to 2.42:1. This corresponds to quite a substantial increase in the amount of oxygen on the surface, indicating that when supplemented with nitrogen and phosphorus, bacteria are able to oxidise the surface of PET at a faster rate.
**Figure 4.20.** XPS survey spectra of PET films degraded by a bacterial enrichment community without nitrogen and phosphorus supplementation.

**Figure 4.21.** XPS survey spectra of PET films degraded by a bacterial enrichment community with nitrogen and phosphorus supplementation.
Table 4.11. Elemental composition of PET films degraded by a bacterial enrichment community with and without nitrogen and phosphorus supplementation.

<table>
<thead>
<tr>
<th>Element</th>
<th>Relative atomic concentration (%)</th>
<th>No additional N/P</th>
<th>N/P supplemented culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td></td>
<td>73.8</td>
<td>68.8</td>
</tr>
<tr>
<td>Oxygen</td>
<td></td>
<td>24.3</td>
<td>28.4</td>
</tr>
<tr>
<td>Nitrogen</td>
<td></td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Sulphur</td>
<td>–</td>
<td>–</td>
<td>0.4</td>
</tr>
<tr>
<td>Sodium</td>
<td>–</td>
<td>–</td>
<td>0.3</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.2</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Silicon</td>
<td>0.3</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

This increased degree of oxidation was also observed in the chemical component analysis (Figs 4.22, 4.23, Table 4.12). PET films degraded by nitrogen and phosphorus supplemented cultures showed a shift toward a lower proportion of aromatic carbon and an increase in the amount carbon/oxygen single bonds. There was also a small increase in the incidence of carbonyl groups, supporting the observations in Section 4.4.2. that the degradation mechanisms in the two culture conditions are similar, and that the PET sample degradation in supplemented cultures was more advanced.
Figure 4.22. High resolution XPS spectra of PET films degraded by a bacterial enrichment community without nitrogen and phosphorus supplementation.

Figure 4.23. High resolution XPS spectra of PET films degraded by a bacterial enrichment community with nitrogen and phosphorus supplementation.
Table 4.12. Chemical component contributions to the C$_{1s}$ and O$_{1s}$ peaks of PET samples degraded by a bacterial enrichment culture with and without nitrogen and phosphorus supplementation.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Component</th>
<th>Component contribution (%)</th>
<th>No additional N/P</th>
<th>N/P supplemented culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_{1s}$</td>
<td>C$_{Ar}$</td>
<td>65.4</td>
<td>57.8</td>
<td></td>
</tr>
<tr>
<td>C=O</td>
<td>18.9</td>
<td>25.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O=C–O</td>
<td>14.8</td>
<td>14.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C=O</td>
<td>0.8</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_{1s}$</td>
<td>O=C</td>
<td>52.7</td>
<td>52.6</td>
<td></td>
</tr>
<tr>
<td>O–C</td>
<td>47.3</td>
<td>47.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.4.4. Determination of optimal degradation conditions

Based on the data presented throughout this chapter, an experiment was performed to attempt to find the incubation conditions that result in optimal PET biodegradation. In most of the conditions PET film samples were incubated in environmental seawater samples containing natural bacterial communities. Among the experimental conditions tested were degradation in dark conditions and in nitrogen/phosphorus supplemented culture, for reproducibility. Two new conditions were also tested: surfactant pre-treatment and degradation by pure culture of *Pseudoalteromonas citrea*. Pre-treating films with sodium dodecyl sulphate (SDS) surfactant was a variation on addition of Tween 20 from the previous section, and was intended to increase chain mobility. *P. citrea* is a known producer of biosurfactants, which may increase chain mobility, and also secretes a number of extracellular enzymes which help may facilitate degradation (Kalinovskaya et al. 2004). Films were degraded for one month, before AFM and infrared microspectroscopic analysis.

4.4.4.1. Topography

Three-dimensional representations of AFM scans for each of the experimental conditions are presented below in Figure 4.24. PET film surfaces of samples degraded
in the presence and absence of light both exhibited similar topographical patterns to those observed in previous experiments. PET degraded in nitrogen\phosphorus supplemented culture however resulted in much rougher surface topography than observed previously (Table 4.13). This is probably due to the differences in bacterial community composition, which may contain members that degrade PET by different mechanisms.

An interesting effect was observed on surfactant pre-treated films. Surfactant pre-
treatment resulted in a surface architecture that was not previously observed, which was relatively rough. Bacterial degradation of pre-treated films however, caused the surfaces to become very smooth. The increased surface area made available by surfactants may have allowed bacterial enzymes better access to the polymer chains, resulting in more effective peak erosion.

In addition to this, PET incubated in pure \textit{P. citrea} culture exhibited a similar structure to the surfactant control, indicating that the biosurfactants produced by the strain had a similar effect on the surface to SDS. It is unclear whether \textit{P. citrea} was capable of degrading PET, but at the very least co-culture of \textit{P. citrea} with a proven degrader could lead to more effective biodegradation.
Figure 4.24. Surface topography of PET incubated under various conditions over one month. Culture conditions were: a sterile seawater (control), b non-sterile seawater, c non-sterile seawater in dark conditions, d non-sterile seawater supplemented with nitrogen/phosphorus, e films pre-treated with SDS in sterile seawater, f SDS pre-treated films in non-sterile seawater and g pure culture of Pseudoalteromonas citrea.
Table 4.13. Comparative roughness analysis of PET films degraded over a period of one month in a variety of culture conditions.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>( S_a ) (nm)</th>
<th>( S_q ) (nm)</th>
<th>( S_{\text{max}} ) (nm)</th>
<th>( S_{sk} )</th>
<th>( S_{ku} )</th>
<th>( S_{dr} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile seawater</td>
<td>2.70</td>
<td>4.00</td>
<td>147.2</td>
<td>3.29</td>
<td>50.3</td>
<td>101.1</td>
</tr>
<tr>
<td>Non-sterile seawater</td>
<td>2.65</td>
<td>3.92</td>
<td>65.1</td>
<td>2.20</td>
<td>10.6</td>
<td>100.9</td>
</tr>
<tr>
<td>Non-sterile (dark)</td>
<td>2.41</td>
<td>3.54</td>
<td>109.4</td>
<td>1.99</td>
<td>9.94</td>
<td>100.9</td>
</tr>
<tr>
<td>Non-sterile, N/P</td>
<td>8.0</td>
<td>10.60</td>
<td>179.5</td>
<td>1.02</td>
<td>6.57</td>
<td>104.1</td>
</tr>
<tr>
<td>Supplemented</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterile, SDS pre-treatment</td>
<td>6.48</td>
<td>8.08</td>
<td>94.1</td>
<td>0.325</td>
<td>3.07</td>
<td>101.0</td>
</tr>
<tr>
<td>Non-sterile, SDS</td>
<td>1.70</td>
<td>2.51</td>
<td>99.8</td>
<td>2.96</td>
<td>36.7</td>
<td>100.7</td>
</tr>
<tr>
<td>pre-treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. citrea culture</td>
<td>7.10</td>
<td>8.90</td>
<td>115.2</td>
<td>0.351</td>
<td>3.49</td>
<td>102.2</td>
</tr>
</tbody>
</table>

4.4.4.2. Chemistry

A different approach was taken for the chemical characterisation of PET films in this optimisation experiment. Infrared microspectroscopy was performed on PET samples degraded in the presence and absence of light, at the infrared microspectroscopy beamline of the Australian Synchrotron. IR microspectroscopy allows for mapping of the spatial distribution of chemical groups, not possible using standard XPS.

Individual spectra were collected in attenuated total-reflection (ATR) mode at 3 µm intervals across approximately 50 µm × 50 µm, before the areas under characteristic peaks of the spectra were integrated and mapped according to percent variation, relative to the maximum intensity (Figs 4.25 – 4.27). Five peaks were chosen for integration, with corresponding wavenumbers of approximately 1710 cm\(^{-1}\), 1415 cm\(^{-1}\), 1345 cm\(^{-1}\), 1100 cm\(^{-1}\) and 1020 cm\(^{-1}\).

For the most part, each of the PET films showed a relatively even distribution of each of the functional groups mapped. However, a clear variation was observed in the intensity of the C–O–C stretching peak (1113 – 1074 cm\(^{-1}\)) of the sample degraded in the presence of light (Fig. 4.26). This peak forms a doublet with an adjacent peak at a
slightly lower wavenumber, therefore to ensure that the correct peak was analysed the ‘shoulder’ of the doublet at the higher wavenumber was integrated. The intensity of this peak indicates that this part of the molecule is in gauche conformation. PET molecules in crystalline sections tend to lay mostly in the one plane, in trans conformations (Zhu and Kelley 2005). Gauche conformations occur almost exclusively in amorphous regions, therefore integration of the C–O–C stretching peak is a good approximation of the level of crystallinity of the PET samples. The resulting map shows a boundary between two regions of differing crystallinity.

It should be noted that in the IR maps of the control PET surface, there are a number of atypically high intensity points just to the right of centre (Fig. 4.25). These are probably due to inconsistent degrees of contact between the surface and the ATR crystal. Some of the maps of the light-degraded and dark-degraded samples (Figs 4.26 and 4.27) have similar apparent outliers, however as these points aren’t observed across all maps for a given sample they are more likely to be truly representative of heterogeneity in the chemical composition of the surface. Inconsistent contact is usually an indication of microscale roughness on the sample surface.

Single point spectra were also acquired for each of the samples (Fig. 4.28). Generally speaking the spectra all have peaks in similar positions, and only their relative absorbances vary, indicating that each of the surfaces has quite similar chemistry. The spectrum of the PET film exposed to pure culture of P. citrea however was substantially more complex, which may be due to adsorption of one or more of the numerous extracellular substances secreted by the cells.
**Figure 4.25.** Spatial distribution of chemical groups on the surface of a control PET surface, generated by integration of selected peaks in the IR spectra. Maps are coloured according to variation in intensity, relative to the maximum.
Figure 4.26. Spatial distribution of chemical groups on the surface of a PET surface degraded by bacteria in the presence of light, generated by integration of selected peaks in the IR spectra. Maps are coloured according to variation in intensity, relative to the maximum.
Figure 4.27. Spatial distribution of chemical groups on the surface of a PET surface degraded by bacteria in dark conditions, generated by integration of selected peaks in the IR spectra. Maps are coloured according to variation in intensity, relative to the maximum.
Figure 4.28. Single point spectra of PET samples degraded under a variety of conditions.
4.5. Summary

Marine bacteria did appear to have the ability to degrade PET surfaces and utilise them as carbon and energy sources. Degradation appeared to occur preferentially at the higher, more exposed points on the PET surface. These points have more exposed surface area, which allows for increased polymer chain mobility and relative ease of access for bacterial enzymes. This mode of peak erosion resulted in surfaces which were measurably smoother.

The chemical functional groups on the surfaces of degraded PET samples did not qualitatively change much with respect to the control surfaces. However one functionality that was commonly detected only on degraded surfaces was non-ester, carbonyl groups. The major changes between degraded and control surfaces was the quantities and proportions, rather than types, of chemical groups. This is an indication that degradation of PET occurs by excision of small sections of the polymer chains.

The rate of PET biodegradation was shown to be quite slow, with the optimum rate calculated to be approximately 5 nm per exposed face per day. However strategies to increase this rate proved promising; supplementation of bacterial culture with nitrogen and phosphorus, enrichment culture in dark conditions, pre-treatment with surfactants and culture of biosurfactant producing bacteria all presenting favourable results.
Chapter 5: Taxonomic affiliation of bacteria associated with PET biodegradation
5.1. **Overview**

Bacterial recovery and subsequent phenotypic and genetic characterisation was performed in order to identify bacterial strains with highest PET-degrading potential. Samples of enrichment cultures were taken after approximately eight months incubation and spread on agar plates for bacterial recovery. The resulting colonies were counted and grouped according to colony morphology, before representative colonies were picked and spread on agar plates to obtain pure cultures. Once pure cultures were obtained taxonomic characterisation was possible.

5.2. **Colony morphology and abundance**

Bacteria were recovered from four different enrichment cultures: bacteria grown in seawater with PET films, bacteria grown in seawater with PET bottle pieces, bacteria grown in seawater with PET films in dark conditions and a control culture without any PET. Recovery was performed as described in Section 3.4. In total, across all four conditions, 23 different colony morphologies were recovered and identified, and each was assigned a letter (Table 5.1). The number of colony forming units of each phenotype in each culture was quantified (Fig. 5.1).

Several phenotypes appeared to show preference for one or more growth conditions. For example, phenotypes r, s, t, u, v and w were all only recovered from the enrichment culture maintained in dark conditions. Each of these groups of bacteria may have been unable to gain a selective advantage in light-exposed cultures, but in cultures where no light was available for photoautotrophic bacteria they were better able to proliferate. Phenotype j showed similar behaviour; although it was recovered from all cultures it was more abundant in the dark culture. This phenotype may have experienced the same selective pressures as the other dark-preferring strains, but was more robust and better able to compete with photoautotrophs.
Table 5.1. Morphological description of colonies recovered from enrichment cultures after eight months.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Colony morphological description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>~3 mm i.d., yellow, opaque, smooth, convex</td>
</tr>
<tr>
<td>b</td>
<td>~3 mm i.d., white, opaque, smooth, convex</td>
</tr>
<tr>
<td>c</td>
<td>~2 mm i.d., white, translucent</td>
</tr>
<tr>
<td>d</td>
<td>~1 mm i.d., white, opaque, smooth, convex</td>
</tr>
<tr>
<td>e</td>
<td>&lt;1 mm i.d., white/colourless</td>
</tr>
<tr>
<td>f</td>
<td>2-3 mm i.d., pale yellow/white, transparent edges</td>
</tr>
<tr>
<td>g</td>
<td>~1 mm i.d., orange/yellow, transparent</td>
</tr>
<tr>
<td>h</td>
<td>~2 mm i.d., pale brown, opaque</td>
</tr>
<tr>
<td>i</td>
<td>Fluffy/filamentous, fungus</td>
</tr>
<tr>
<td>j</td>
<td>~1 mm i.d., yellow, opaque</td>
</tr>
<tr>
<td>k</td>
<td>~3 mm i.d., pale pink/orange, opaque</td>
</tr>
<tr>
<td>l</td>
<td>~1 mm i.d., orange, transparent</td>
</tr>
<tr>
<td>m</td>
<td>1-2 mm i.d., white, opaque</td>
</tr>
<tr>
<td>n</td>
<td>~2 mm i.d., white, translucent centre with opaque edges</td>
</tr>
<tr>
<td>o</td>
<td>~1 mm i.d., red, opaque</td>
</tr>
<tr>
<td>p</td>
<td>&lt;1 mm i.d., yellow, transparent</td>
</tr>
<tr>
<td>q</td>
<td>~2 mm i.d., irregular shape, white opaque centre with translucent edges</td>
</tr>
<tr>
<td>r</td>
<td>~1 mm i.d., yellow, opaque with transparent edges</td>
</tr>
<tr>
<td>s</td>
<td>~1 mm i.d., pale yellow, transparent</td>
</tr>
<tr>
<td>t</td>
<td>1-2 mm i.d., yellow, transparent, possible agar digester</td>
</tr>
<tr>
<td>u</td>
<td>~2 mm i.d., yellow, opaque</td>
</tr>
<tr>
<td>v</td>
<td>1-2 mm i.d., orange, opaque</td>
</tr>
<tr>
<td>w</td>
<td>~1 mm i.d., white, opaque</td>
</tr>
</tbody>
</table>

Phenotypes f and h were both recovered from all three plastic containing cultures, but not from the control. This is an indication that strains from these two groups may be the most versatile plastic degraders recovered from this experiment. Phenotype h in particular may be effective at degrading PET, as its abundance between the three cultures was similar to that of phenotype j, i.e. it showed maximum abundance in dark conditions where competition from photoautotrophs is lowest. Other phenotypes with potential for PET degradation are phenotypes m, n, o, p and q.
Figure 5.1. Abundance of individual bacterial phenotypes recovered from four separate enrichment cultures after eight months.

Even after eight months with extremely limited nutrient supplies, there were still a number of bacterial strains that could be isolated from the control culture. These strains are likely the most robust and resilient members of the natural bacterial community normally present in seawater, and are simply able to endure the longest in nutrient deficient conditions. Interestingly, most phenotype groups that were isolated from the control culture were also isolated from cultures containing PET, but in greater numbers. Their metabolic activities appear to be stimulated somewhat in these culture conditions. Bacterial strains from these groups are probably ‘metabolic scavengers’ that feed off the various organic compounds produced by other members of the enrichment cultures. The primary producers in these cultures are the strains capable of obtaining carbon from the PET samples.

A summary of the phenotypic proportions of the bacterial communities in each of the enrichment cultures is presented in Figure 5.2. Each of the cultures containing PET is dominated by phenotype e, with relatively substantial contributions from phenotype d. Phenotype e consists of colonies that are very small and are therefore difficult to distinguish from one another, which may mean that this group is actually comprised of a
relatively large number of taxonomically different bacterial species. This may account in part for the dominance of this group. This is not to say however, that the various members of this phenotype are not PET degraders; as PET degradation occurs at a slow rate, the strains capable of degrading PET likely grow slowly, and favour relatively oligotrophic conditions.

![Pie chart comparison of bacterial phenotypes](image)

**Figure 5.2.** Proportions of bacterial phenotypes recovered from each of the four enrichment cultures.

The major component of the bacterial community in the control was phenotype a. This phenotype also contributed a sizable portion to the light-exposed culture with PET films. It is quite likely that members of this group possess photoautotrophic ability, as
they grew only in the presence of light, and the distinct lack of alternative energy sources in the control culture could provide an explanation for their proportional dominance. A comparison of the light-exposed and dark condition bacterial cultures is shown in Figure 5.3.

![Figure 5.3. Comparison of the proportions of bacterial phenotypes recovered from PET enrichment cultures in the presence and absence of light.]

5.3. **Bacterial taxonomy and phylogenetics**

Grouping bacterial isolates by colony morphology is a very broad and general classification system. For more accurate taxonomic assignment representative isolates form the major phenotypes and some of isolates of phenotypes in lower abundance were subjected to genetic analysis via partial 16S rRNA gene sequencing. The resulting sequences were compared against a database of 16S rRNA gene sequences of bacterial type strains to find their nearest taxonomic neighbour with the highest degree of 16S rRNA gene similarity (Tables 5.2 – 5.7).

5.3.1. **Sequence similarity of 16S rRNA genes**

Strains grouped under phenotype a consistently found to identify closest to species of *Erythrobacter*, from the class *α*-proteobacteria (Table 5.2). Several species of
*Erythrobacter*, and the closely related genera of *Erythromicrobium* and *Porphyrobacter* possess bacteriochlorophyll a, and are thus capable of photosynthetic growth (Yurkov et al. 1994, Denner et al. 2002, Hiraishi et al. 2002). This is consistent with the results of Section 5.2, in which isolates classified under phenotype a were found to favour conditions where photosynthetic ability would provide the most selective advantage.

**Table 5.2.** Taxonomic affiliations of isolates assigned to phenotype a.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Colony Phenotype</th>
<th>Sequence length (bp)</th>
<th>Nearest match</th>
<th>Similarity (%)</th>
<th>Phylogenetic class</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1a1</td>
<td>a</td>
<td>631</td>
<td><em>Erythrobacter pelagi</em></td>
<td>96.8</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>B2a1</td>
<td>a</td>
<td>760</td>
<td><em>Erythrobacter nanhaisediminis</em></td>
<td>96.2</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>B2a2</td>
<td>a</td>
<td>137</td>
<td><em>Erythrobacter seohaensis</em></td>
<td>94.1</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>B3a1</td>
<td>a</td>
<td>723</td>
<td><em>Erythrobacter citreus</em></td>
<td>97.5</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>B3a2</td>
<td>a</td>
<td>524</td>
<td><em>Erythrobacter seohaensis</em></td>
<td>60.3</td>
<td>α-proteobacteria</td>
</tr>
</tbody>
</table>

Strains grouped under phenotype b primarily identified to members of the class α-proteobacteria, with two strains belonging to γ-proteobacteria (Table 5.3). Five of these strains returned nearest matches to *Roseovarius nubinhibens*, and an additional strain matched to *Sulfitobacter litoralis*. Both of these species are α-proteobacteria that participate in sulphur cycling (González et al. 2003, Park et al. 2007). Sulphur was identified as potentially important for bacterial biodegradation of PET in Sections 4.2.1.2 and 4.3.2.

All strains assigned to phenotype c returned nearest matches to members of the class γ-proteobacteria (Table 5.4). Most of these were *Alteromonas macleodii*, however one strain was matched to each of the genera *Marinobacter* and *Glaciecola*. Species of *Alteromonas* produce and secrete considerable extracellular polysaccharide (Raguénes et al. 1996, Hayase et al. 2003, Raguénès et al. 2003, Martínez-Checa et al. 2005). These polysaccharides can be quite viscous, and provide good protection to cells as a
biofilm matrix material. Some *Alteromonas* strains also produce some extracellular enzymes (Ivanova et al. 2000, Ivanova et al. 2005a).

**Table 5.3. Taxonomic affiliations of isolates assigned to phenotype b.**

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Colony Phenotype</th>
<th>Sequence length (bp)</th>
<th>Nearest match</th>
<th>Similarity (%)</th>
<th>Phylogenetic class</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1b1</td>
<td>b</td>
<td>710</td>
<td><em>Thalassospirulacentensis</em></td>
<td>97.6</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>B1b2</td>
<td>b</td>
<td>516</td>
<td><em>Roseovarius nubinhibens</em></td>
<td>96.1</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>B2b1</td>
<td>b</td>
<td>438</td>
<td><em>Roseovarius nubinhibens</em></td>
<td>98.4</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>B2b2</td>
<td>b</td>
<td>833</td>
<td><em>Roseovarius nubinhibens</em></td>
<td>98.9</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>B3b1</td>
<td>b</td>
<td>834</td>
<td><em>Roseovarius nubinhibens</em></td>
<td>98.1</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>B3b2</td>
<td>b</td>
<td>837</td>
<td><em>Roseovarius nubinhibens</em></td>
<td>98.0</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>D2b1</td>
<td>b</td>
<td>148</td>
<td><em>Alteromonas addita</em></td>
<td>93.7</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>D3b1</td>
<td>b</td>
<td>630</td>
<td><em>Sulfitobacter litoralis</em></td>
<td>97.0</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>E1b1</td>
<td>b</td>
<td>359</td>
<td><em>Cobetia crustatorum</em></td>
<td>97.4</td>
<td>γ-proteobacteria</td>
</tr>
</tbody>
</table>
Table 5.4. Taxonomic affiliations of isolates assigned to phenotype c.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Colony Phenotype</th>
<th>Sequence length (bp)</th>
<th>Nearest match</th>
<th>Similarity (%)</th>
<th>Phylogenetic class</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3c1</td>
<td>c</td>
<td>337</td>
<td>Marinobacter sediminum</td>
<td>97.3</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>B1c2</td>
<td>c</td>
<td>637</td>
<td>Alteromonas macleodii</td>
<td>97.5</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>B2c1</td>
<td>c</td>
<td>787</td>
<td>Alteromonas macleodii</td>
<td>98.0</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>B2c2</td>
<td>c</td>
<td>885</td>
<td>Alteromonas macleodii</td>
<td>98.8</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>B3c1</td>
<td>c</td>
<td>791</td>
<td>Alteromonas macleodii</td>
<td>97.5</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>B3c2</td>
<td>c</td>
<td>900</td>
<td>Alteromonas macleodii</td>
<td>98.0</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>D3c1</td>
<td>c</td>
<td>480</td>
<td>Glaciecola lipolytica</td>
<td>89.6</td>
<td>γ-proteobacteria</td>
</tr>
</tbody>
</table>

Strains belonging to phenotype d mostly identified as γ-proteobacteria, with two strains of α-proteobacteria and a single strain of Actinobacteria (Table 5.5). The γ-proteobacteria strains came from two closely related genera in Alteromonas and Aestuariibacter. The two α-proteobacteria belong to the genus Thalassospira. To date only five species of Thalassospira have been validly described, and most of these are documented to degrade highly-hydrophobic aromatic hydrocarbons (López-López et al. 2002, Liu et al. 2007, Kodama et al. 2008, Zhao et al. 2010). This is of some significance, as PET polymer chains contain aromatic constituents.
Table 5.5. Taxonomic affiliations of isolates assigned to phenotype d.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Colony Phenotype</th>
<th>Sequence length (bp)</th>
<th>Nearest match</th>
<th>Similarity (%)</th>
<th>Phylogenetic class</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1d1</td>
<td>d</td>
<td>195</td>
<td><em>Alteromonas addita</em></td>
<td>86.3</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>A1d2</td>
<td>d</td>
<td>137</td>
<td><em>Alteromonas genovensis</em></td>
<td>84.3</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>A1d3</td>
<td>d</td>
<td>202</td>
<td><em>Alteromonas addita</em></td>
<td>89.4</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>A1d4</td>
<td>d</td>
<td>203</td>
<td><em>Alteromonas addita</em></td>
<td>87.6</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>A1d6</td>
<td>d</td>
<td>437</td>
<td><em>Aestuariibacter salexigens</em></td>
<td>92.0</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>D2d1</td>
<td>d</td>
<td>202</td>
<td><em>Thalassospira profundimaris</em></td>
<td>95.5</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>D2d3</td>
<td>d</td>
<td>795</td>
<td><em>Thalassospira tepidiphila</em></td>
<td>97.0</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>D3d1</td>
<td>d</td>
<td>401</td>
<td><em>Micrococcus yunnanensis</em></td>
<td>98.7</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>D3d3</td>
<td>d</td>
<td>437</td>
<td><em>Aestuariibacter halophilus</em></td>
<td>91.8</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>D3d4</td>
<td>d</td>
<td>198</td>
<td><em>Alteromonas addita</em></td>
<td>88.3</td>
<td>γ-proteobacteria</td>
</tr>
</tbody>
</table>

Bacterial strains classified under phenotype e were primarily from the classes α- and β-proteobacteria (Table 5.6). Among the β-proteobacteria, nearest matches were commonly species of *Limnobacter*. Two validly described species of *Limnobacter* currently exist: *L. thiooxidans* and *L. litoralis*. Both of these species are chemolithoheterotrophs that oxidise thiosulfate for energy (Spring et al. 2001, Lu et al. 2011). Another two strains from phenotype e were matched to the genus *Kordiimonas* from the class α-proteobacteria. Like *Limnobacter*, there are only two validly described species of *Kordiimonas* (Kwon et al. 2005, Xu et al. 2011b). The type species of *Kordiimonas, K. gwangyangensis*, is capable of degrading high molecular weight, polycyclic aromatic compounds (Kwon et al. 2005).
Table 5.6. Taxonomic affiliations of strains assigned to phenotype e.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Colony Phenotype</th>
<th>Sequence length (bp)</th>
<th>Nearest match</th>
<th>Similarity (%)</th>
<th>Phylogenetic class</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1e1</td>
<td>e</td>
<td>165</td>
<td><em>Limnobacter litoralis</em></td>
<td>91.1</td>
<td>β-proteobacteria</td>
</tr>
<tr>
<td>A1e2</td>
<td>e</td>
<td>146</td>
<td><em>Burkholderia andropogonis</em></td>
<td>92.5</td>
<td>β-proteobacteria</td>
</tr>
<tr>
<td>A1e3</td>
<td>e</td>
<td>142</td>
<td><em>Limnobacter thiooxidans</em></td>
<td>95.7</td>
<td>β-proteobacteria</td>
</tr>
<tr>
<td>A1e7</td>
<td>e</td>
<td>357</td>
<td><em>Aestuariibacter halophilus</em></td>
<td>92.2</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>A1e8</td>
<td>e</td>
<td>179</td>
<td><em>Limnobacter litoralis</em></td>
<td>87.8</td>
<td>β-proteobacteria</td>
</tr>
<tr>
<td>A1e9</td>
<td>e</td>
<td>296</td>
<td><em>Limnobacter thiooxidans</em></td>
<td>98.3</td>
<td>β-proteobacteria</td>
</tr>
<tr>
<td>A3e7</td>
<td>e</td>
<td>679</td>
<td><em>Erythrobacter nanhaisediminis</em></td>
<td>96.2</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>D2e1</td>
<td>e</td>
<td>643</td>
<td><em>Jannaschia cystaugens</em></td>
<td>94.8</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>D2e2</td>
<td>e</td>
<td>639</td>
<td><em>Oceanicola nitratireducens</em></td>
<td>93.2</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>D3e1</td>
<td>e</td>
<td>555</td>
<td><em>Methylonatrum kenyense</em></td>
<td>87.5</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>D3e2</td>
<td>e</td>
<td>603</td>
<td><em>Kordiimonas lacus</em></td>
<td>95.3</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>D3e3</td>
<td>e</td>
<td>277</td>
<td><em>Kordiimonas lacus</em></td>
<td>93.1</td>
<td>α-proteobacteria</td>
</tr>
</tbody>
</table>

Table 5.7 summarises the nearest match found for each of the strains with less abundant phenotypes. Many of these strains returned similar matches to other strains from the major phenotypes. Three strains belonging to phenotype f are of note, as they each returned particularly low percentage similarity to *Alteromonas macleodii* (strains A3f2, A3f3, A3f4). The widely accepted 16S gene similarity threshold for description of new species is 97% (Stackebrandt *et al.* 2002, Tindall *et al.* 2010), which suggests that there is a high probability that these three strains belong to distinct, undescribed taxonomic species.
Table 5.7. Taxonomic affiliation of strains assigned to low-abundance phenotypes.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Colony Phenotype</th>
<th>Sequence length (bp)</th>
<th>Nearest match</th>
<th>Similarity (%)</th>
<th>Phylogenetic class</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3f2</td>
<td>f</td>
<td>592</td>
<td>Alteromonas macleodii</td>
<td>72.0</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>A3f3</td>
<td>f</td>
<td>608</td>
<td>Alteromonas macleodii</td>
<td>76.2</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>A3f4</td>
<td>f</td>
<td>722</td>
<td>Alteromonas macleodii</td>
<td>79.0</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>D3f1</td>
<td>f</td>
<td>320</td>
<td>Micrococcus yunnanensis</td>
<td>97.0</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>A1g1</td>
<td>g</td>
<td>558</td>
<td>Maribacter forsetii</td>
<td>97.2</td>
<td>Flavobacteriia</td>
</tr>
<tr>
<td>D2g1</td>
<td>g</td>
<td>321</td>
<td>Micrococcus yunnanensis</td>
<td>99.3</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>D2g2</td>
<td>g</td>
<td>638</td>
<td>Erythrobacter citreus</td>
<td>97.5</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>A1h1</td>
<td>h</td>
<td>198</td>
<td>Thalassospira profundimaris</td>
<td>94.4</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>D3h1</td>
<td>h</td>
<td>147</td>
<td>Lentibacter algarum</td>
<td>96.8</td>
<td>α-proteobacteria</td>
</tr>
<tr>
<td>D3j1</td>
<td>j</td>
<td>557</td>
<td>Micrococcus yunnanensis</td>
<td>99.4</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>D3j2</td>
<td>j</td>
<td>299</td>
<td>Micrococcus endophyticicus</td>
<td>97.6</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>D3j3</td>
<td>j</td>
<td>440</td>
<td>Micrococcus yunnanensis</td>
<td>99.5</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>D2m1</td>
<td>m</td>
<td>481</td>
<td>Marinobacter sediminum</td>
<td>97.3</td>
<td>γ-proteobacteria</td>
</tr>
<tr>
<td>D2o1</td>
<td>o</td>
<td>678</td>
<td>Arthrobacter agilis</td>
<td>96.6</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>D3p1</td>
<td>p</td>
<td>263</td>
<td>Erythrobacter citreus</td>
<td>98.5</td>
<td>α-proteobacteria</td>
</tr>
</tbody>
</table>

5.3.2. Phylogenetic analysis

Based on the 16S rRNA gene similarity results shown in Section 5.3.1, phylogenetic trees for each class of bacteria isolated were constructed using
approximately 10 of the closest matches for each strain. Subtrees were then extracted showing the phylogenetic positions of each of the identified strains (Figs 5.4 – 5.12).

Figure 5.4 shows the phylogenetic position of strain D3b1 amongst its nearest neighbours in the class α-proteobacteria. This strain forms quite a stable cluster with other species of Sulfitobacter, with a bootstrap percentage of 61 and agreement between the three difference algorithms.

**Figure 5.4.** Subtree showing the phylogenetic position of one isolated strain, extracted from a larger tree containing 125 members of the class α-proteobacteria. Numbers at nodes indicate the bootstrap percentage, and the symbols + and X denote agreement by maximum likelihood and maximum parsimony algorithms, respectively.

The phylogenetic positions of eight isolated strains of α-proteobacteria are presented in Figure 5.5. Five of these strains were classified to the one phenotype, and are shown to be phylogenetically closely related. These five strains form a relatively
stable cluster with members of *Nautella*, *Roseovarius*, *Lentibacter* and *Ruegeria*. Three more strains form an unstable cluster with *Leisingera nanhaiensis*, *Jannaschia cystaugens* and *Thalassobius aestuarii*.

**Figure 5.5.** Subtree showing the phylogenetic position of eight isolated strains, extracted from a larger tree containing 125 members of the class α-proteobacteria. Numbers at nodes indicate the bootstrap percentage, and the symbols + and X denote agreement by maximum likelihood and maximum parsimony algorithms, respectively.

The phylogenetic positions of a further 14 strains belonging to *α-proteobacteria* are shown in Figure 5.6. Four of these strains form a very stable cluster with species of *Thalassospira*. A further two form a stable cluster with the genus *Kordiimonas*. The remaining eight strains form a somewhat stable cluster (bootstrap = 98%) that contains members of *Citromicrobium*, *Erythrobacter*, *Altererythrobacter*, *Novosphingobium*, *Sphingomonas* and *Porphyromonas*. These genera belong to the order *Sphingomonadales*, which is a complex group of closely related strains.
Figure 5.6. Subtree showing the phylogenetic position of fourteen isolated strains, extracted from a larger tree containing 125 members of the class α-proteobacteria.
Numbers at nodes indicate the bootstrap percentage, and the symbols + and X denote agreement by maximum likelihood and maximum parsimony algorithms, respectively.

The five isolated strains of \( \beta \)-proteobacteria cluster together in between species of \textit{Limnobacter} and \textit{Chromobacterium} (Fig. 5.7). All but one of these strains returned a 16S rRNA gene similarity below 97\%, which indicates a high probability of these strains belonging to distinct species. As a group they may form a distinct taxonomic lineage at the genus level, representing a phylogenetic link between \textit{Limnobacter} and \textit{Chromobacterium}.

**Figure 5.7.** Subtree showing the phylogenetic position of five isolated strains, extracted from a larger tree containing 34 members of the class \( \beta \)-proteobacteria. Numbers at nodes indicate the bootstrap percentage, and the symbols + and X denote agreement by maximum likelihood and maximum parsimony algorithms, respectively.

The phylogenetic positions of fourteen strains of \( \gamma \)-proteobacteria are presented in Figure 5.8. The isolates shown in this tree grouped quite strongly according to phenotype. Five isolates from phenotype c grouped well with members of \textit{Alteromonas},
while three isolates each from phenotypes d and f formed small clusters near, but separate from the *Alteromonas* group. A further three strains formed a stable cluster with relatively long evolutionary length branches. Many of these isolates are good candidates for description of novel species, especially the group of phenotype f strains (16S rRNA gene similarity: 72 – 79%).

**Figure 5.8.** Subtree showing the phylogenetic position of fourteen isolated strains, extracted from a larger tree containing 98 members of the class γ-proteobacteria. Numbers at nodes indicate the bootstrap percentage, and the symbols + and X denote agreement by maximum likelihood and maximum parsimony algorithms, respectively.
Each of the isolates whose phylogenetic positions are shown in Figure 5.9 formed stable clusters with their nearest neighbours. Two strains formed a stable cluster with members of the genus *Marinobacter*. One strain formed a stable cluster with two species of *Cobetia*, and another strain made a stable cluster with *Congregibacter litoralis*.
The four isolates whose phylogenetic positions are indicated in Figure 5.10 formed a stable cluster with the genus *Aestuariibacter*. These four strains are highly likely to be one or more novel species of *Aestuariibacter*, due to the stability of the cluster coupled with low 16S rRNA gene similarity; all four strains showed less than 93% similarity to their nearest matches.

![Figure 5.10. Subtree showing the phylogenetic position of four isolated strains, extracted from a larger tree containing 98 members of the class γ-proteobacteria. Numbers at nodes indicate the bootstrap percentage, and the symbols + and X denote agreement by maximum likelihood and maximum parsimony algorithms, respectively.](image)

Most of the clusters formed by the *Actinobacteria* isolates lacked stability (Fig 5.11). One isolate grouped well with most of the species of *Arthrobacter*, although there was poor agreement between phylogenetic algorithms. Four strains did however cluster stably with *Micrococcus luteus* and *Micrococcus yunnanensis*. 
Figure 5.11. Subtree showing the phylogenetic position of one isolated strain, extracted from a larger tree containing 20 members of the class Flavobacteriia. Numbers at nodes indicate the bootstrap percentage, and the symbols + and X denote agreement by maximum likelihood and maximum parsimony algorithms, respectively.

A single strain amongst those isolated and identified belonged to the class Flavobacteriia, in the genus Maribacter. Phylogenetic analysis revealed that it clustered very well with other species of Maribacter (Fig. 5.11).
Figure 5.11. Subtree showing the phylogenetic position of one isolated strain, extracted from a larger tree containing 20 members of the class Flavobacteriia. Numbers at nodes indicate the bootstrap percentage, and the symbols + and X denote agreement by maximum likelihood and maximum parsimony algorithms, respectively.

5.4. Celeribacter neptunius H14T

During the course of initial bacterial enrichment experiments, one bacterial isolate with the designated strain number H14 was found to belong to the Roseobacter lineage of α-proteobacteria. This strain was equally distantly related from three separate taxonomic genera, i.e. Nautella, Roseovarius and Pseudoruegeria. After phylogenetic, biochemical and physiological characterisation, strain H14 was classified as a novel species of a previously undefined genus, and accordingly a taxonomic paper was published describing the strain as Celeribacter neptunius H14T (Ivanova et al. 2010b).

Cell size and morphology were determined using scanning electron microscopy (Fig. 5.13). Cells were grown in marine broth liquid culture to OD₆₀₀ ≈ 0.3. One millilitre of this culture was then incubated for 24 hours on aluminium disks at a temperature of 25 °C. After incubation, disks were rinsed gently with sterile MilliQ water, air dried, and coated with a layer of gold using a Dynavac CS300 unit. Electron micrographs were obtained using a field emission scanning electron microscope (FESEM, Supra 40 VP, Carl-Zeiss, Germany) at 2000×, 5000×, 10000×, 15000×, 70000× and 100000× magnifications.
Figure 5.13. Scanning electron micrographs of Celeribacter neptunius H14\textsuperscript{T} at magnifications of 10000× and 100000× (inset).

Taxonomic analysis was performed by 16S rRNA gene sequencing and construction of phylogenetic trees. The sequence was compared with three gene sequence databases (University of Nice BLAST server, http://bioinfo.unice.fr/blast, DDBJ gene sequence database, http://blast.ddbj.nig.ac.jp/top-e.html, and EzTaxon database, http://147.47.212.35:8080/) in order to retrieve the most similar sequences of validly described bacteria. The sequences were aligned using Clustal X 2.0 (Larkin \textit{et al.} 2007) and checked using SeaView (Galtier \textit{et al.} 1996). Trees were prepared using neighbour-joining, maximum likelihood and maximum parsimony algorithms. The nearest phylogenetic neighbour to \textit{C. neptunius} is \textit{Nautella italica}, which forms a cluster with bootstrap of only 77%. This level of relatedness is an indication, depending on biochemical profiles, that \textit{C. neptunius} belongs to a distinct genus.
Figure 5.14. Phylogenetic position of *C. neptunius*, based on 16S rRNA gene sequence analysis. Bootstrap percentages above 50% are shown at nodes. Reproduced with permission from Ivanova et al. 2010b.

Comprehensive biochemical analysis was performed to complement genetic analysis. Biochemical tests performed included: motility, oxidation/fermentation of glucose, Gram stain, reduction of nitrate/nitrite, oxidase activity, catalase activity, gelatinase activity, arginine dihydrolase activity, lysine decarboxylase activity, ornithine decarboxylase activity, poly-β-hydroxybutyrate production, acetoin production, sodium requirements, indole production, H₂S production, amylase activity, lipase activity and temperature range. *C. neptunius* was also tested for production of bacteriochlorophyll a and susceptibility to a number antibiotics, and was also subjected to phospholipid and fatty acid analysis. The results of biochemical characterisation are summarised in Table 5.8 and Table 5.9.
Table 5.8. Susceptibility of Celeribacter neptunius H14<sup>T</sup> to various antibiotics.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
<th>Susceptibility of C. neptunius H14&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>10 U</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>100 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>15 µg per disc</td>
<td>Resistant</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>5 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>5 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Oleandomycin</td>
<td>15 µg per disc</td>
<td>Resistant</td>
</tr>
<tr>
<td>Cephazolin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>10 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Polymyxin</td>
<td>300 U</td>
<td>Resistant</td>
</tr>
<tr>
<td>Neomycin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>
Table 5.9. Comparison of biochemical characteristics of Celeribacter neptunius to those of closely related strains.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA G + C (mol%)</td>
<td>59</td>
<td>61</td>
<td>59-65</td>
<td>52-54</td>
<td>62-69</td>
<td>56</td>
<td>64-72</td>
<td>57</td>
<td>67</td>
<td>67</td>
<td>58-66</td>
<td>54-64</td>
<td>57</td>
<td>59</td>
<td>57-61</td>
</tr>
<tr>
<td>Bacteriochlorophyll a</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>v</td>
<td>v</td>
<td>+</td>
<td>v</td>
<td></td>
</tr>
<tr>
<td>Pigment</td>
<td>–</td>
<td>Beige</td>
<td>v</td>
<td>Brown</td>
<td>v, pink</td>
<td>–</td>
<td>Yellow</td>
<td>–</td>
<td>Yellow/ grey</td>
<td>Red</td>
<td>Pink/beige</td>
<td>v</td>
<td>Beige</td>
<td>Brown</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Temp. range (°C)</td>
<td>5-35</td>
<td>4-45</td>
<td>4-32</td>
<td>27-31</td>
<td>4-37</td>
<td>13-28</td>
<td>4-40</td>
<td>5-37</td>
<td>15-49</td>
<td>3-33</td>
<td>4-40</td>
<td>5-37</td>
<td>15-35</td>
<td>13-37</td>
<td>13-37</td>
</tr>
<tr>
<td>NaCl range (% w/v)</td>
<td>1-8</td>
<td>1-5</td>
<td>0.34-9</td>
<td>0-4</td>
<td>0-14</td>
<td>1.4-8</td>
<td>0-10</td>
<td>0-8</td>
<td>0-9</td>
<td>0.1-13</td>
<td>0-15</td>
<td>0.6-12</td>
<td>3-7</td>
<td>0.85-7</td>
<td>1-8</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>v</td>
<td>–</td>
<td>w</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Lipase production</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>v</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>+</td>
<td>v</td>
<td>–</td>
<td>+</td>
<td>v</td>
<td></td>
</tr>
<tr>
<td>Amylase production</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>v</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>v</td>
<td>w</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gelatinase production</td>
<td>v</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>v</td>
<td>v</td>
<td>+</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>α-glucose utilisation</td>
<td>+</td>
<td>–</td>
<td>v</td>
<td>–</td>
<td>+</td>
<td>w</td>
<td>v</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilisation</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>v</td>
<td>v</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>α-xylene utilisation</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>v</td>
<td>–</td>
<td>–</td>
<td>v</td>
<td></td>
</tr>
<tr>
<td>l-Arabinose utilisation</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>v</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Major polar lipids</td>
<td>PE, PG, PC, LPE, AL</td>
<td>PE, PG, PC, PE, AL</td>
<td>LPE, AL</td>
<td>PE, PC, AL</td>
<td>PE, PC, DPG</td>
<td>DPG, PC</td>
<td>PE, PL, GL</td>
<td>DPG, PC</td>
<td>PE, PC, AL</td>
<td>PE, PC, AL</td>
<td>PE, PC, AL</td>
<td>PE, PC, DPG</td>
<td>PE, PC, LPE, AL</td>
<td>PE, PC, DPG, AL</td>
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Taxa: 1, H 144-1; 2, Nautella italicc LMG 24365T (Vandecandelaere et al. 2009); 3, Jannaschia rubra 4SM3T (Macián et al. 2005); 4, Ketonoglucococcus (Urbence et al. 2001); 5, Loktanella (Ivanova et al. 2005b); 6, Nereida ignava 2SM4T (Pujalte et al. 2005); 7, Oceanicola marinus AZO-C7T (Cho and Giovannoni 2004); 8, Octadecabacter (Gosink et al. 1997); 9, Pseudoruergienia aquisimaris SW-255T (Yoon et al. 2007); 10, Roseaisalisnus antarcticus EL-888 (Labrenz et al. 2005); 11, Roseovarius (Labrenz et al. 1999, Boetcker et al. 2005, Yoon et al. 2008); 12, Ruegeria (Uchino et al. 1998, Arahal et al. 2005, Martens et al. 2006); 13, Shimia marina CL-TA03T (Choi and Cho 2006); 14, Thalassobacter arenae GA2-M15T (Kim et al. 2009); 15, Thalassobiis (Arahall et al. 2005). All species are catalase-positive. +, Positive; –, negative; w, weakly positive reaction; v, variable reaction depending on strain; ND, no data available; AL, unidentified aminolipid; LPE, lysophosphatidylethanolamine; DP, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol. Adapted from Ivanova et al. (2010)b.
Based on the results of genetic and biochemical analyses, it was determined that *Celeribacter neptunius* H14\(^T\) is representative of a novel taxonomic classification at the genus level, under the class *α*-proteobacteria, family *Rhodobacteraceae*. The cells are Gram-negative and rod-shaped, 0.8 – 1.8 µm in length and 0.4 – 0.9 µm wide. They are chemoorganotrophic, facultative anaerobes, and are motile by means of single polar or subpolar flagella. Requires 1 – 8 % NaCl, temperatures of 5 – 35 °C (optimum 25 °C) and pH ranging between 4.0 – 10.0 (optimum 7.5 – 8.0) for growth to occur. The DNA G + C content is 59.1 mol%.

### 5.5. *Alteromonas australica* H17

Another bacterial strain isolated from early enrichment experiments, designated H17, was subjected to comprehensive taxonomic analysis. Based on 16S rRNA gene sequence analysis it was determined that this strain belonged to the genus *Alteromonas*, of the class *γ*-proteobacteria. Based on comparisons of biochemical profiles of H17 with that of the type strains of each of the validly described species of the genus it is suggested that H17 represents a novel species of *Alteromonas*, for which the specific name *australica* is proposed.


The 16S rRNA gene sequence was amplified and sequenced by the Australian Genome Research Facility (AGRF Laboratories) (Brisbane, Australia). The EzTaxon server 2.0 at http://147.47.212.35:8080 was used to identify the closest phylogenetic neighbours (Chun *et al.* 2007). Phylogenetic analyses were performed as described elsewhere (Ivanova *et al.* 2004, Ivanova *et al.* 2005a), using three different taxonomic algorithms: neighbour-joining (NJ), maximum likelihood (ML) and maximum
parsimony (MP). All three algorithms are available as part of the PHYLIP software package (Felsenstein 1993). Bootstrap analysis was performed using 1000 replications. For the NJ analysis, the distance matrix was calculated based on Kimura's two-parameter method using the DNADIST program. A subtree was extracted from the resulting phylogenetic tree, which demonstrates the taxonomic position of strain H17 among the *Alteromonas* (Fig. 5.15). The cluster formed by this group is very stable.

**Figure 5.15.** Phylogenetic position of *A. australica*, based on 16S rRNA gene sequence analysis. Bootstrap percentages above 50% are shown at nodes. The symbols + and X indicate branch agreement by maximum likelihood and maximum parsimony algorithms respectively.

Biochemical analysis of *A. australica* included tests of oxidation/fermentation of glucose, denitrification, oxidase and catalase activity, gelatin liquefaction, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, sodium requirements, growth temperatures, indole and H2S production and the ability to hydrolyse starch, Tween-80 and casein. Susceptibility to various antibiotics was also tested. The results of biochemical analyses are summarised in Table 5.10 and Table 5.11.
Table 5.10. Comparison of biochemical characteristics of Alteromonas australica to that of other species of Alteromonas.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>H17</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production of pigments</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>10 °C</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>40 °C</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>Nitrate reduction to nitrite</td>
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<td>v(−)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Growth in NaCl at:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>15%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>–</td>
<td>v(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
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<td>Agar</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>v(+)</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>–</td>
<td>v (+)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>L-Lactate</td>
<td>–</td>
<td>v(+)</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>L-Xylose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose, maltose, acetate, glycerol</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>v</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G+C mol %</td>
<td>43</td>
<td>45-46</td>
<td>45</td>
<td>43</td>
<td>44-45</td>
<td>46</td>
<td>43</td>
<td>45</td>
<td>46</td>
<td>44.5</td>
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Taxa: H17, A. australica; 1, A. macleodii (Baumann et al. 1972); 2, A. simiduii (Chiu et al. 2007); 3, A. tagae (Chiu et al. 2007); 4, A. marina (Yoon et al. 2003a); 5. A. hispanica (Martinez-Checa et al. 2005); 6, A. addita (Ivanova et al. 2005a); 7, A. stellipolaris (Van Trappen et al. 2004a); 8, A. litorea (Yoon et al. 2004); 9, A. genovensis (Vandecandelaere et al. 2008). All species/strains are Gram-negative, motile, oxidase and catalase positive, negative for indole and H₂S production, grow at 3-6% NaCl, produce lipase (Tween 80); +, positive; −, negative; v, variable reaction; w, weak reaction.
Table 5.11. Susceptibility of *Alteromonas australica* H17 to various antibiotics.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
<th>Susceptibility of <em>C. neptunius</em> H14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levomycetin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>10 U</td>
<td>Resistant</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>30 µg per disc</td>
<td>Resistant</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>100 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>15 µg per disc</td>
<td>Resistant</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>5 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>5 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>30 µg per disc</td>
<td>Resistant</td>
</tr>
<tr>
<td>Oleandomycin</td>
<td>15 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Cephazolin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30 µg per disc</td>
<td>Resistant</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>10 µg per disc</td>
<td>Resistant</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15 µg per disc</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Polymyxin</td>
<td>300 U</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Neomycin</td>
<td>30 µg per disc</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

*Alteromonas australica* cells are Gram-negative and rod-shaped, about 0.7 – 0.9 µm in diameter. They are chemoorganotrophic aerobes, and are motile by means of single polar flagella. Requires 1 – 10 % NaCl, temperatures of 4 – 37 ºC and pH ranging between 6.0 – 10.0 (optimum 7.5 – 8.0) for growth to occur. The DNA G+C content is 59.1 mol%. Based on the results of genetic and biochemical analyses, it was determined that *Alteromonas australica* H17 is likely a novel species of the genus *Alteromonas*, under the class *γ*-proteobacteria, however due to the relatively high 16S rRNA gene similarity of this strain with other members of species of *Alteromonas*, further analysis may be required, in the form of DNA-DNA hybridisation or multi-locus sequence analysis.
5.6. Summary

A total of 23 different phenotypes classified according to colony morphology were isolated from three different enrichment cultures supplied with PET as the sole carbon and energy source, and a control culture without any PET. Members of several of these phenotypes have high potential for PET biodegradation. Six phenotypes were isolated only from the PET enrichment culture in dark conditions, where the selective advantage for PET degrading organisms was greatest. A further two phenotypes were recovered from each of the cultures supplied with PET, but not from the control.

The composition of the bacterial communities that developed in the enrichment cultures that were supplied with PET was very different to that of the control condition. Each of the three PET enrichment communities were dominated by phenotype e, with a significant contribution from phenotype d, while the phenotype a comprised the largest proportion of the community developed in the control. This indicates that phenotypes d and e have high potential for containing PET degrading organisms.

Based on partial sequencing of the 16S rRNA gene, isolates recovered from the enrichment cultures belonged to the taxonomic classes α-, β- and γ-proteobacteria, Actinobacteria and Flavobacteriia. In the cases of several of the recovered strains, their nearest validly described taxonomic relatives are known to be important sulphur cyclers, which is an indication that sulphur may be an important element in the degradation of PET. Several more strains identified most closely to the genera Thalassospira and Kordiimonas, both of which are comprised of bacterial species that are known to degrade polycyclic aromatic hydrocarbons.

In addition to this, two strains that were isolated from enrichment cultures have been comprehensively characterised. As a result of this work one of these strains, designated H14, has been identified as belonging to a previously undescribed genus, and was subsequently named Celeribacter neptunius H14T. The other is thought to be a new species from the genus Alteromonas, which is expected to be confirmed upon further genetic analysis.
Chapter 6: Design of surface topographies for enhancing cellular interactions with PET
6.1. Overview

Bacterial interactions with PET, or any material for that matter occur almost exclusive on the outer surface of said material. As such, it is crucial that bacterial cells have as unrestricted access as possible to PET surfaces in order for them to have maximal biodegradative effect. In this chapter, the role of substratum surface architecture on attachment and adhesion of bacterial cells to a variety of materials will be covered, with particular emphasis on determining which surface structures allow for the maximum level of cell attachment.

6.2. Thermodynamics of cell attachment on PET

In the general case, the law of free energy of adhesion states that:

\[ \Delta G_{adh} = \gamma_{S/B} - \gamma_{S/W} - \gamma_{B/W} \]

where \( \Delta G_{adh} \) is the free energy of adhesion, \( \gamma_{S/B} \) is the interfacial tension (IFT) between a substratum and a bacterial cell, \( \gamma_{S/W} \) is the IFT between the substratum and water, and \( \gamma_{B/W} \) is the IFT between the bacterium and water (Bos et al. 1999a). Bacterial adhesion is favoured more as \( \Delta G_{adh} \) decreases, i.e. the system equilibrium has minimal free energy. According to equation 6.2.1, \( \Delta G_{adh} \) is lowest when \( \gamma_{S/B} \) is small and/or one or both of \( \gamma_{S/W} \) and \( \gamma_{B/W} \) are large. In the case of the latter two terms, IFT between a surface and water is directly related to hydrophobicity; highly hydrophobic surfaces have high IFT with water. It follows that cell attachment is strongly favoured when both the cell and the substratum are highly hydrophobic.

As a material, PET is moderately hydrophobic (Fig. 6.1). It exhibits a static water contact angle of approximately 81°. According to commonly accepted thresholds of hydrophobicity, PET is actually classified as a hydrophilic material; surfaces with water contact angles below 90° are considered hydrophilic, those between 90° and 150° are hydrophobic, and surfaces with contact angles above 150° are superhydrophobic. However hydrophobicity is a relative concept, and the above definitions are arbitrary.
Figure 6.1. Static contact angle of a water droplet on a PET surface.

Static water contact angles were also measured on lawns of bacterial cells immobilised on cellulose acetate filter papers, in order to estimate cell hydrophobicity (Fig. 6.2). Cells belonged to six representative strains isolated from early enrichment experiments. Three strains were identified as members of *Alteromonas*, while a further three were identified as *Thalassospira* spp. Water contact angles measured on bacterial lawns ranged from approximately 42° for *Alteromonas* sp. H89, to approximately 66° for *Alteromonas* sp. H91. None of the strains proved to be significantly hydrophobic, and were in fact more hydrophilic than the PET surface.
Figure 6.2. Static water contact angles of droplets resting on bacterial lawns of six representative strains isolated from enrichment experiments.

As the hydrophobicity of PET and each of the bacterial strains tested is relatively mediocre, it is expected that the level of cell attachment on PET will not be especially high. To test this, confocal laser scanning microscopy was carried out on bacterial biofilms formed on PET samples during enrichment experiments. PET samples were aseptically removed and stained with two fluorescent dyes to visualise viable cells and EPS attached to the surface. The resulting three-dimensional reconstructions show that even after nine months, the cells attached to the PET surface are quite sparse (Fig. 6.3).
The amount of EPS on the surface is also quite limited, and does not form a complete layer.

Figure 6.3. Three-dimensional reconstruction of the bacterial biofilm formed on PET surfaces after nine months of enrichment incubation. Viable cells are stained red (bottom left) while exopolysaccharides, which form the biofilm matrix, are stained green (bottom right). The top image combines both channels and demonstrates the location of viable cells within the biofilm matrix.

The restricted cell attachment observed on PET surfaces may be a contributor to the inhibited rate of PET biodegradation, as plastic/bacteria interactions are primarily limited to the surface. It therefore stands to reason that if the affinity of bacteria for PET surfaces can be enhanced, then PET biodegradability may also increase. By investigating the effect that a diverse range of surfaces structures have on regulating bacterial adhesion, it is thought that the specific surface properties that most favour bacterial attachment can be identified. Once these specific properties are known they can be introduced to PET through manipulation of the polymer surface, potentially maximising biodegradation.
6.3. Superhydrophobic surfaces

According to the equation of free energy of adhesion (Eq. 6.2.1), attachment of bacterial cells will be favoured on increasingly hydrophobic surfaces. Because of this, surfaces that exhibit extremely high hydrophobicity were chosen as the starting point for cell-substratum interaction studies.

6.3.1. Bacterial attachment on superhydrophobic titanium surfaces

Among superhydrophobic surfaces, the lotus leaf is perhaps the best known example. Much research has been conducted investigating the superhydrophobicity and surface structure of lotus leaves (Barthlott and Neinhuis 1997, Marmur 2004, Sun et al. 2005, Zhang et al. 2006b, Bhushan et al. 2009, Koch et al. 2009, Wang et al. 2009, Zhang et al. 2009). A certain amount of work has also gone into developing techniques for fabrication of lotus structures from a variety of materials (Sun et al. 2005, Bhushan et al. 2009, Koch et al. 2009, Fadeeva et al. 2011). One of these artificial lotus surfaces was selected for investigation of cell adhesion.

The surface in question was fabricated and supplied by a research group at Laser Zentrum Hannover e.V. in Germany using femtosecond laser ablation to create dual-scale hierarchical structures on titanium based on those found on the surfaces of lotus leaves (Fadeeva et al. 2011). *Staphylococcus aureus* 65.8 was chosen as a model bacterium for adhesion studies, due to its well characterised nature. Cells were prepared by overnight culture in nutrient broth (Oxoid, U.K.) at 25 °C. The resulting logarithmic-phase cells were then harvested and resuspended in phosphate buffered saline to OD$_{600}$ = 0.3. Approximately five millilitres of this cell suspension was then placed on lotus-replica titanium samples and incubated at 25 °C for 18 hours, after which time the samples were rinsed with MilliQ water and air dried. Once samples were dry they were sputter-coated with gold using a Dynavac CS300 gold-sputterer for subsequent scanning electron microscopy.

Lotus-structured titanium surfaces proved to be relatively favourable for attachment of *S. aureus* cells (Fig. 6.4). Attachment was localised primarily to the grooves and crevices between the microscale features on the surface, and encroaching up the sides of the micro-features. On some parts of the surface the bacterial cells appeared to form a
‘bridge’ of sorts; suspended across the microscale crevices and anchored to the micro-features on either side (Fig 6.4).

Figure 6.4. *Staphylococcus aureus* cell attachment on titanium surfaces with lotus-inspired hierarchical surface features.

It is not immediately clear by what mechanism this ‘bacterial suspension bridge’ might form. However, a possible explanation lies within the accepted theories of roughness induced surface wettability. The Cassie-Baxter model of surface wettability states that the apparent contact angle of a water droplet resting on a surface is dependent on the proportion of air trapped within the topographical features of the surface (Cassie and Baxter 1944). Thus as the proportion of trapped air approaches 100%, the apparent water contact angle approaches the contact angle between water and air, i.e. 180°. The air trapped on the surface may also be the key to explaining the cell attachment profile of *S. aureus* on lotus-like titanium. Nano-sized bubbles become trapped within the nanoscale surface features, and provided they are not spaced too far apart may provide a very low friction surface for bacterial cells to slide across (Fig. 6.5). This would cause the cells to slide down the sides of the microscale features and accumulate in the crevices between them. At the bottom of the crevices the trapped nanobubbles are in proximity to each other close enough to coalesce and form micro-sized air pockets. The
cells, lacking significant mass are unable to cross the air/water interface at the bottom of the crevice, and begin to form a layer on the interface, building inwards from the intersection of the three air/water/titanium interfaces.

Figure 6.5. Schematic diagram of the hypothetical cause of cell accumulation and ‘bridge’ formation, based on retention of air bubbles within surface features.

To test this hypothesis, Raman microspectroscopy was performed on the lotus-like titanium surfaces immersed in water. Individual Raman spectra were recorded in a 50 × 50 grid, over 100 µm × 100 µm. A map was then generated based on the intensity of the water peak in the individual spectra, indicating the spatial distribution of air trapped on the surface (Fig. 6.6). By comparing to scanning electron micrographs taken at a similar magnification, it can be seen that the areas of lowest intensity, i.e. the regions that are occupied by air correspond very well with the pattern of crevices between the microscale surface features (Fig. 6.6). While the spatial resolution and sensitivity of the technique is not sufficient to provide conclusive evidence of the presence of nanobubbles on the titanium surface, larger bubbles located at the bottom of the surface crevices were detected, supporting the idea of nano- and micro-bubble directed cell attachment.
Figure 6.6. Scanning electron micrograph of lotus-like titanium surfaces (left) and Raman microspectroscopy map of the spatial distribution of air retained by the surface features (right). Raman map is coloured according to intensity of the water peak, with darker areas indicating larger amounts of air.

Time-resolved Raman microspectroscopy was also performed to track the proportion of air retained on the lotus-like titanium surface over time. Titanium surfaces were immersed in water for discrete time intervals (0, 10, 30 and 60 minutes) to determine whether or not the amount of air trapped by surface features remained constant. It was found that the amount of air in fact does not remain constant, and that the trapped air is released over time until the surface nears equilibrium with the surrounding environment (Fig. 6.7). After one hour immersed in water, the amount of air trapped on the surface of the lotus-like titanium decreased to 6% of the air on the surface immediately upon immersion. This has implications in cell attachment, because the greater the proportion of the surface occupied by air, the less surface area that is available for cell adhesion. Cell adhesion studies after, 0, 10, 30 and 60 minute incubations showed that this is indeed the case; the number of cells attached to the lotus titanium surfaces was very low initially, and increased substantially once the proportion of trapped air dropped below 40% of the initial value (Fig. 6.7).
Figure 6.7. Dynamics of cell adhesion and proportion of air trapped between surface features on lotus-structured titanium surfaces. Air retention is measured as a percentage of the amount of air present immediately upon immersion.

6.3.2. Bacterial attachment on superhydrophobic cicada wings

Another type of superhydrophobic surface with a surface structure very different to that of the lotus-like titanium was tested for its favourability to bacterial cell attachment. The surfaces of the wings of the Clanger cicada (*Psaltoda claripennis*) possess an array of hexagonally-spaced, spherically-capped, conical nanopillars, which help to impart superhydrophobicity (Fig. 6.8). The nanopillars are approximately 200 nm tall, 60 nm in diameter at cap and are spaced approximately 170 nm apart, centre to centre. This surface structure contributes significantly to the hydrophobicity of the surface; the nanopillars are quite effective at trapping air between them, increasing the water contact angle according to the Cassie-Baxter model of wettability. The surface chemistry of the wing also plays a role in determining the hydrophobicity. The wings are composed primarily of three general components: protein, chitin and wax (Vincent and Wegst 2004), however it is mainly cuticular wax that is present on the outer surface of the wing. Figure 6.9 shows chemical distribution maps based on the Amide II C=O stretching peak and the CH₂ stretching, which are representative of the protein and wax components of the wing. The relatively higher variation in the amount of wax on the
surface is due to the fact that is present as a thin layer across the surface, while protein comprises most of the bulk of the wing. Scans were performed in transmission mode, and thus there was a higher contribution to infrared absorption spectra from proteins.

Figure 6.8. Scanning electron micrograph of a cicada wing surface, titled at an angle of approximately 40°.

Figure 6.9. Cicada wing chemical distribution maps generated based on integrated areas of Infrared absorption spectra. Colour maps indicated the percentage variation of the integrated areas relative to the highest value obtained.
The net result of the surface structure and surface chemistry of the cicada wings, in terms of wettability, is a surface that is extremely hydrophobic. Spatial mapping of surface wettability of cicada wings showed that in some parts of the wing the water contact angle can be as high as 171°, while the average contact angle across the analysed area was 158.8° (Fig. 6.10). A small degree of spatial variation in surface hydrophobicity was observed on the cicada wing surface, which is likely due to the layer of cuticular wax being inconsistent and/or incomplete, however the structure of the surface ensures that the wing remains particularly hydrophobic.

![Wettability map of a cicada wing surface.](image)

**Figure 6.10. Wettability map of a cicada wing surface.**

Cell adhesion experiments on cicada wings using *Pseudomonas aeruginosa* ATCC 9027 showed that bacterial cells do have appreciable affinity for attaching to the surface. However the wing structures elicited an effect that would be extremely detrimental if they were to be applied for PET biodegradation: the cells were ruptured and killed by the nanopillar structures on the wing surface. For this reason, cicada wing nanopillar structures are not suitable for fabrication on PET, unless their bactericidal effect can be eliminated.
6.4. **Molecularly smooth surfaces**

Lotus-like titanium and cicada wings, like most superhydrophobic surfaces, have relatively high levels of roughness. Irrespective of hydrophobicity, rough surfaces are generally expected to show higher levels of cell attachment than smoother surfaces, due to the higher availability of anchor points, and through providing shelter from potential detaching forces, e.g. water turbulence (Rowan *et al.* 2002, Whitehead *et al.* 2005, Rozhok *et al.* 2006, Díaz *et al.* 2007). To contrast against the rough lotus-like titanium and cicada wings presented in Section 6.3, some exceptionally smooth, thin titanium films were analysed for their bacterial cell attachment properties. These films were smooth at a molecular level, and as such were expected to show very limited levels of bacterial adhesion.

Titanium films were fabricated using magnetron sputtering. Films were deposited using a Kurt J. Lesker CMS-18 magnetron sputtering system in DC mode, with the power set to 150 kW, argon gas pressure at 4 mTorr and base pressure below $5 \times 10^{-5}$ mTorr. By modulating the exposure time, films of 3 nm, 12 nm and 150 nm thicknesses were deposited on pre-cleaned silicon wafers. After fabrication, films of each thickness were analysed using atomic force microscopy to assess their roughness and topology. Roughness analysis showed that each film was sub-nanometrically smooth, with the 3 nm film presenting the lowest average roughness at 0.19 nm (Table 6.1). Titanium naturally forms an oxide layer on its outer surface when exposed to atmospheric conditions, and considering that the bond length in a molecule of titanium dioxide is similar in magnitude to the average roughness of the 3 nm film, i.e. approximately 1.6 Å (Ivanova *et al.* 2011), it can be said that this film is molecularly smooth. The films were moderately hydrophobic, displaying water contact angles within the range of 95 – 105°.
Table 6.1. Quantitative roughness analysis of titanium thin films deposited on silicon wafer substrata.

<table>
<thead>
<tr>
<th></th>
<th>$S_a$ (nm)</th>
<th>$S_q$ (nm)</th>
<th>$S_{max}$ (nm)</th>
<th>$S_{sk}$</th>
<th>$S_{ku}$</th>
<th>WCA* (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 nm Ti film</td>
<td>0.19</td>
<td>0.22</td>
<td>2.81</td>
<td>-0.09</td>
<td>3.47</td>
<td>96</td>
</tr>
<tr>
<td>12 nm Ti film</td>
<td>0.20</td>
<td>0.24</td>
<td>6.61</td>
<td>0.11</td>
<td>2.63</td>
<td>97</td>
</tr>
<tr>
<td>150 nm Ti film</td>
<td>0.66</td>
<td>0.83</td>
<td>7.59</td>
<td>0.63</td>
<td>3.79</td>
<td>104</td>
</tr>
</tbody>
</table>

*WCA – Water contact angle

Cell adhesion studies were carried out on the molecularly smooth titanium films using both *Staphylococcus aureus* CIP 65.8T and *Pseudomonas aeruginosa* ATCC 9027. *P. aeruginosa* behaved in a manner consistent with what is predicted by the literature, i.e. few *P. aeruginosa* cells were able to adhere to the molecularly smooth titanium surfaces (Figs 6.11, 6.12). However, the attachment profile of *S. aureus* was vastly different; many cells were able to attach to the smooth titanium surfaces in relatively high density (Figs 6.11, 6.12).
Figure 6.11. Reconstructions of biofilms produced by Staphylococcus aureus (left) and Pseudomonas aeruginosa (right) on the surfaces of 3 nm (top), 12 nm (middle) and 150 nm (bottom) thick titanium films deposited on silicon wafers, based on CLSM analysis. Red areas represent viable cells, and green areas represent the biofilm matrix.
Figure 6.12. Levels of Staphylococcus aureus and Pseudomonas aeruginosa cell retention on 3 nm, 12 nm and 150 nm titanium films sputtered on silicon substrata, expressed as a percentage of the total cells available for attachment in the bacterial suspensions.

Given that the titanium surfaces were consistent for both of the strains tested, the differences between the attachment profiles of S. aureus and P. aeruginosa are probably due to cell-specific properties. A likely contributing factor is cell rigidity. S. aureus has higher rigidity than P. aeruginosa, which arises from a combination of several different cell properties, including morphology, thickness of peptidoglycan layer and turgor pressure (Whatmore and Reed 1990, Arnoldi et al. 2000, Koch 2003, Harold 2007). P. aeruginosa cells are relatively soft and flexible, and when these cells are in contact with molecularly smooth surfaces the flexibility of the membrane allows the cell to spread its load across a greater area, which causes the contact points between the cell and the substratum to be limited to just the tip of the sub-nanometric surface features. The cells are easily detached as they can slide across the surface with little resistance. S. aureus on the other hand is more rigid and less deformable, so the total load of the cell is concentrated on a much smaller contact area. The sub-nanoscale features penetrate the cell wall slightly and limit lateral movement, decreasing the likelihood of cell detachment (Fig. 6.13).
6.5. **Summary**

As bacterial degradation of PET occurs exclusively at the polymer surface, it is important to characterise how bacterial cells interact with and attach to the plastic. Visualisation of biofilms grown on PET surfaces shows that bacterial attachment to PET surfaces is quite limited, which may be a contributing factor to the inherent resistance of the polymer to biodegradation. If PET surfaces could be modified to be friendlier towards bacterial attachment it may result in an increase in biodegradability.

Bacterial attachment on several substrata with interesting surface properties was assessed as potential sources of inspiration for design of PET surfaces. Two of the substrata were superhydrophobic: titanium with surface features based of the structure of the lotus leaf, and the wings of a species of cicada. The lotus-like titanium did show a relatively high level of retention of bacterial cells; however the spatial distribution of the attached cells appeared to be directed by air bubbles trapped on the surface. As the
air was gradually replaced by water the overall cell attachment levels increased. Application of this type of surface structure not only has the potential to boost the level of cell attachment on the surface, but may also be useful for directing cells to attach to specific regions.

Cicada wings also retained quite a large number of cells, however in this case the surface also proved lethal to the cells. Every *Pseudomonas aeruginosa* cell to attach to the surface was ruptured and killed by the nanopillar structures on the wing surface. This would be detrimental to degradation processes if it were applied to PET.

Molecularly smooth surfaces were also shown to direct bacterial attachment. The number of cells that could attach to the surfaces of molecularly smooth titanium films was found to be dependent on the bacterial species. Spherical *Staphylococcus aureus* cells adhered to the titanium films in much greater number than the rod-shaped *Pseudomonas aeruginosa*. It is thought that the different attachment profiles of the two bacteria are based on their structural rigidity. If this is the case, fabrication of PET films with very low roughness could lead to selective attachment of specific bacteria.
Chapter 7:  General discussion
7.1. Overview

Several studies have been conducted previously that investigate the potential of biodegradation of polymers, including PET, by microorganisms (Grima et al. 2000, Ishigaki et al. 2000, Müller et al. 2001, Yamada-Onodera et al. 2001, Bonhomme et al. 2003, Marten et al. 2003, Müller et al. 2005, Herzog et al. 2006, Marqués-Calvo et al. 2006, Mueller 2006, Artham and Doble 2009). In most cases, the level of polymer degradation that was achieved was too low to apply for polymer disposal on a commercial scale, and of the techniques that were able to achieve faster degradation rates, few if any could feasibly be implemented commercially due to poor cost-effectiveness. For example, Müller et al. (2005) measured up to 54% loss of mass of PET samples upon incubation in a 0.1 mg mL\(^{-1}\) solution of purified hydrolase from an actinomycete, *Thermobifida fusca*. The enzyme solution was completely refreshed every week. If such an approach were to be applied on a commercial scale, the expense of preparing and purifying such large amounts of the enzyme would prove inhibitory.

More recently, there has been a shift in the literature away from the development of techniques for biodegradation of resistant polymers, and towards the development of new, more readily degradable polymers (Chen 2009, Akaraonye et al. 2010, Chanprateep 2010, Nampoothiri et al. 2010, Verbeek and van den Berg 2010, Brandelero et al. 2011, Canché-Escamilla et al. 2011, Ghasemlou et al. 2011, Kim et al. 2011b, Kunasundari and Sudesh 2011, Liu et al. 2011, Neppalli et al. 2011, Puls et al. 2011, Raquez et al. 2011, Sui et al. 2011, Ye et al. 2011, Zhu et al. 2011). The major drawback to this approach is that the resistance of polymers like PET to degradation is inextricably linked to their stability and durability, which is one of the primary properties which makes them such popular materials for use in a wide range of applications. Replacement of these polymers with biodegradable materials in many cases may defeat the purpose of using polymers altogether.

For the most part, the limiting factors in PET biodegradation have not previously been determined, however the low polymer chain mobility due to the semi-crystalline nature of the polymer has been identified as a contributor to degradation resistance (Marten et al. 2003, Müller et al. 2005, Mueller 2006). Despite the fact that one of the barriers to PET degradation is known, there has been little attempt to circumvent it. In the previous chapters, it has been shown that marine bacteria do possess the ability to
degrade PET, and that some enhancement strategies do aid them in that cause. This chapter will summarise and discuss the results from the previous chapters, and identify the major barriers to PET biodegradation as well as the most effective strategies for developing an effective and efficient PET disposal technique.

7.2. Mechanisms of bacterial biodegradation of PET

Before one can realistically attempt to enhance the biodegradation of PET, something must be known about the way in which the polymer is degraded. By characterising the surface of PET samples that have been partially degraded and comparing them to control samples, the physical and chemical changes induced by the bacteria were determined, and are discussed below.

7.2.1. Physical mechanisms of PET biodegradation

It was consistently observed throughout the PET degradation experiments that the degraded surfaces were noticeably smoother than control surfaces. Rougher surface features, i.e. larger topographical peaks on the surfaces generally became smaller and fewer in number. Based on this observation a hypothesis is proposed for the physical mechanism of bacterial biodegradation of PET. The larger features on the PET surfaces have increased localised surface area relative to the rest of the sample. That makes these regions more available to bacteria, and also enables greater mobility of the polymer chains. Bacterial enzymes are thus better able to access the PET chains in order to induce cleavage of the molecules. Consequently the larger surface features are degraded preferentially, and are subsequently eroded away, resulting in a smoothening of the surface (Fig. 7.1).
7.2.2. **Chemical mechanisms of PET biodegradation**

Generally speaking, the surface chemistry of the degraded PET samples was very similar to the control samples. This may simply be due to the fact that as degradation occurs slowly, the changes in surface chemistry are quantitatively small, and the corresponding differences in the detected signal are somewhat overwhelmed by the normal surface signal. Alternatively, degradation of PET surfaces may occur without much need for surface functionalisation, and instead small sections of the polymer chains are simply excised from the bulk material. Both explanations are equally probable, and likely both contribute.

Excision of small sections of the polymer chains is a particularly likely scenario. The results presented in Chapter 4 show that on some samples, the amount of alcohol groups on the surface increased. This is an indication that hydrolysis occurred at the ester bonds in the polymer molecule, which results in alcohol and carboxylate groups.
functionalities on either side of the chain scission. Hydrolysis at two adjacent ester bonds would result in the extraction of a single molecule of ethylene glycol (Fig. 7.2), which could then be easily metabolised by bacterial cells. It is unlikely that the aromatic portion of the polymer chain could be excised in this manner, however, as the aromatic rings tend to stack quite tightly together, which is the primary cause of the limited chain mobility of PET (Marten et al. 2003, Müller et al. 2005).

Aside from hydrolysis, the carbonyl group is a potential target for chain breakage by other mechanisms. X-ray photoelectron spectroscopy data suggest that the enzyme-catalysed formation of amide bonds may displace the ester bond, resulting in chain breakage. Several degraded samples showed an increase in the amount of nitrogen on their surfaces, and in most cases this nitrogen was in the form of C–N groups. In addition to this, an extra component was consistently detected in O$_{1s}$ which is not normally a part of the PET molecule: a non-ester carbonyl group. This suggests a possible degradation mechanism in which the ester carbon atom acts as an electrophile for the lone pair of electrons on a nitrogen atom of another molecule. The nitrogen-containing molecule may be a protein, or perhaps a single amino acid, e.g. lysine. The free electrons form a new bond to the carbon atom, inducing a tetravalent transition.
state, before one of the oxygen atoms is displaced, breaking the polymer chain (Fig. 7.3).

Figure 7.3. Displacement of an ester bond in a PET molecule by formation of an amide bond to a nitrogen-containing molecule.

One further characteristic several of the degraded surfaces had in common was the presence of small amounts of sulphur, usually in the form of sulphate. Sulphur is required as a trace nutrient by most organisms, and many marine bacteria are important cyclers of sulphur. It is unclear whether or not sulphur serves a specific function in PET biodegradation, or if it just a requirement of the cells that are responsible, but detection of sulphur only on degraded sample surfaces is an indication that, one way or another, sulphur plays an important role in bacterial degradation of PET.
7.3. *Bacterial strains responsible for PET biodegradation*

Taxonomic affiliations of several members of the bacterial communities that developed in the bacterial enrichment cultures were determined, in order to identify which marine bacteria might be capable of degrading PET and to provide some clues as to how biodegradation might be enhanced. Over 100 bacterial isolates were recovered from four different enrichment culture conditions: enrichment culture in seawater supplied with PET films, enrichment culture in seawater supplied with PET bottle pieces, enrichment culture in seawater supplied with PET films incubated in dark conditions, and control cultures not containing any plastic. Bacteria were initially grouped according to colony morphology and enumerated by plate counts. Twenty-three different colony morphologies were identified across all of the cultures.

Each of the three cultures that were incubated in the presence of PET was dominated by members of the phenotypes d and e. Of the strains belonging to phenotype d that were identified most belonged to the genus *Alteromonas* of the class *γ*-proteobacteria, with a couple of strains of the closely related *Aestuariibacter*. Several species of *Alteromonas* are known to be producers of a number of extracellular enzymes (Ivanova *et al.* 2000, Ivanova *et al.* 2005a) that may be involved in PET biodegradation. Identified strains of phenotype e belonged to mostly *α* and *β*-proteobacteria. Two strains of this phenotype identified most closely with *Kordiimonas lacus*, which is a bacterium known to be able to degrade polycyclic aromatic hydrocarbons (Xu *et al.* 2011b). The ability to degrade aromatic molecules is a useful ability in PET biodegradation, as the polymer chains possess a relatively high density of aromatic groups. Several other strains across multiple phenotypes identified to the genus *Thalassospira* of the *α*-proteobacteria, which are also known aromatic compound degraders (López-López *et al.* 2002, Liu *et al.* 2007, Kodama *et al.* 2008, Zhao *et al.* 2010).

Phenotypes f and h were isolated in relatively low numbers; however both of these phenotypes were isolated only from each of the three cultures that contained PET, and not from the control. These two phenotypes likely represent the most robust degraders of PET, as they were able to compete with photosynthetic bacteria. Strains of these phenotypes were most closely related to *Alteromonas* (phenotype f), *Thalassospira* and *Lentibacter*. The *Alteromonas*-related strains in particular were quite distantly related,
returning partial 16S gene sequence similarity below 80%. Not only do these strains have high potential to be PET degrading organisms, they also have high probability for belonging to as yet undescribed species, and perhaps an undescribed genus.

The nearest taxonomic neighbours of several of the strains isolated are important sulphur cycling bacteria. Multiple strains, mostly from phenotype b, returned nearest matches to *Roseovarius nubinhibens*, which is capable of dimethylsulfoniopropionate and other related sulphur-containing molecules, while several other strains returned matches to *Sulfitobacter* and *Limnobacter*, which are capable of obtaining energy via oxidation of sulphide and thiosulphate respectively.

Incubation of PET samples in pure and mixed cultures of strains with high degradation potential such as *Thalassospira* and *Alteromonas* failed to produce any significant loss of plastic mass. This is strong indication that a commensal relationship is required for effective biodegradation of PET. The enrichment community taken straight from the culture flask was however capable of degrading PET. The culture likely contained a proportion of sulphur cycling bacteria (e.g. *Limnobacter*, which belonged to phenotype e, the dominating phenotype in each of the plastic containing cultures), which supplied the PET degrading strains with a source of utilisable sulphur, and in turn these strains produced additional nutrients for the sulphur-cyclers.

Even after eight months of incubation without a significant food source, a few bacteria were still able to be recovered from the control culture. These strains most likely represent the most durable and robust strains present in the initial seawater samples, and are simply able to exist for long periods with limited nutrients. Most of these strains actually grew better in the cultures that contained plastic, probably feeding of the metabolic by-products of other strains with PET-degrading or photosynthetic ability.

In this study, the contributions of non-cultivable bacteria to PET biodegradation were not assessed in detail. The reason for this is that for the eventual application of the knowledge obtained to plastic disposal procedures, the ability to culture adequate amounts of bacteria will be essential. If a non-cultivable bacterium were discovered to possess significant PET degradative ability, it would be extremely hard to grow enough of it for application on a commercial scale. For this reason it was decided to focus primarily on cultivable bacteria.
7.4. **Barriers to PET biodegradation**

From the results of this study it has been determined that marine bacteria do indeed possess the capability for biodegradation of PET. However, there are at least three barriers that limit the degree to which biodegradation occurs. These barriers will be addressed in this section.

One limiting factor in PET biodegradation that was identified previously in the literature is concerning the crystallinity of the polymer (Marten *et al.* 2003, Müller *et al.* 2005). PET has a tendency to orient primarily in the *trans* configuration near its surface, which favours increased crystallinity (Zhu and Kelley 2005). The infrared data presented in Section 4.4.4.2 showed that much of the surface of the PET samples was indeed in *trans* configuration. If the outer surface were to form a predominantly crystalline layer, then regardless of how much of the bulk material was in amorphous form the limitations imposed on chain mobility by the crystalline outer layer would prohibit bacteria causing significant biodegradation.

The results of topographical characterisation of PET surfaces presented in Chapter 4 indicated that biodegradation occurred preferentially on larger surfaces features and that subsequently degradation occurred fastest on rougher surfaces. However, upon degradation, the surfaces became smoother as the larger surface peaks were eroded away. Degradation of smoother surfaces occurred at a slower rate, thus biodegradation of PET may be a self-limiting process; i.e. degradation causes smoothening of the surface which in turn increases the resistance of the surface to further degradation.

The third barrier to PET biodegradation lies in its propensity to promote bacterial attachment on its surface. In Section 6.2 it was shown that as a material, PET is not a particularly favourable substratum for bacterial attachment. This likely arises from a combination of the mediocre hydrophobicity of the material and its roughness profile. As a result bacterial cells are not able to easily access the PET surface and must produce larger amounts of enzyme to achieve comparable levels of biodegradation.

7.5. **Strategies for optimisation of PET biodegradation**

Based on the identified barriers to PET biodegradation, a number of strategies can be developed in an attempt to enhance the biodegradability of the polymer. These
strategies can be grouped into two broad approaches: techniques aimed at enhancing the biodegradation potential of the bacterial cultures, and techniques for increasing the susceptibility of PET to biodegradation.

The first group of strategies mostly aims at increasing the size of the PET degrading community and increasing the proportion of PET degrading members of the bacterial community. Supplementation of the bacterial community with nitrogen and phosphorus proved effective at increasing PET biodegradation. The mechanisms of degradation appeared to remain unchanged when the bacterial cultures were supplied with these essential nutrients, indicating that the measured increase was simply a result of greater bacterial numbers.

Another strategy for improving PET biodegradation is to manipulate the culture conditions so that PET degrading bacteria have as much selective advantage as possible. The specific example from this study is culture in dark conditions; the absence of light limits the growth potential of photosynthetic organisms, reducing the competitive pressure on PET degraders. Over time the composition of the bacterial community shifts towards higher proportion of the bacterial strains which are capable of obtaining energy through degradation of PET. Culture in dark conditions has not been thoroughly explored in the literature, as one of the primary mechanisms of the limited amount of degradation experienced by PET in the environment is exposure to UV light. Researchers have been reluctant to eliminate one of the few stimuli which are known cause degradation of PET.

The other broad approach for enhancement of PET biodegradation surrounds manipulation of the polymer itself. When it comes to surface modification there are two properties which can be altered to attempt to increase PET degradation: the surface chemistry and topography. However changing the surface chemistry of the material is not always an acceptable approach, for example, many of the current applications of PET are in food packaging, and coating of the polymer with a given chemical may lead to its absorption into the contents. Changing the topography of PET is a more favourable alternative, as its effects on many of the material properties will be minimal.

The roughness of PET surfaces has been identified in this study as a contributor to the resistance of the polymer to biodegradation. When the surface is particularly smooth the rate of biodegradation is decreased. The obvious solution to this problem would be
to fabricate a rough topography on the surface of PET during manufacture. However a limitation arises once the bacteria have degraded the material sufficiently to erode the surface features, revealing a smooth surface. Treatment of PET samples with surfactants was shown in Chapter 4 to increase the surface roughness of the polymer, although the selection of the correct surfactant is imperative. The surfactant needs to be adequately non-toxic to the degrading bacteria so as not to be antibacterial, but at the same time not so biocompatible that the bacteria are able to utilise the surfactant for energy over the polymer. In addition to this, a one-time pre-treatment of the polymer will result in the same problem that would arise from fabrication of a rough surface, and multiple treatments would increase costs and decrease efficiency. A possible solution to this is to incorporate a bacterium into the degradation community that is capable of producing biosurfactants. This would result in a dynamic process in which the biosurfactant continuously induces a roughening effect, while the PET degrading organisms erode the peaks.

Another surface modification strategy for enhancing biodegradation is to fabricate surface topographies which promote bacterial attachment. Increasing the amount of cells that can attach to the surface will increase the availability of the surface to bacterial cell. The question remains however as to what surface features enable the highest level of cell attachment. Surface structures based on the topography of the lotus leaf were shown in Chapter 6 to be quite favourable for bacterial attachment, with *Staphylococcus aureus* cells found to attach in relatively high numbers. The lotus structures were also able to direct bacterial attachment to specific areas by retaining air bubbles between the surface features. Molecularly smooth titanium films were also shown to promote attachment of *S. aureus*. However the limitation of these surface topographies is that the adhesion levels of other cells, in particular more flexible rod-shaped cells was extremely limited. Therefore they may not be applicable for fabrication on PET to enhance biodegradation. Many of the potential candidates for PET biodegradation identified in Chapter 5 have rod-shaped morphologies, and may be repelled by molecularly smooth surfaces.

As was mentioned previously, any effects that a fabricated PET surface may have on the rate of biodegradation will only last until those surface features have been degraded. However at the least they may stimulate the metabolic pathways of the bacteria which are involved in biodegradation of the polymer. At any rate, the
fabrication techniques that have been developed to date are not yet capable of adequately producing and controlling nanoscale surface topographies. Nanoimprinting and mask-mould lithography techniques currently show the most potential for producing highly controlled nano-topographies (Zhang et al. 2006a, Xie et al. 2008, Hong et al. 2009, Koch et al. 2009, Kostovski et al. 2009), but both require more development for application in the fabrication of PET surfaces with enhanced biodegradability.
Chapter 8: Conclusions and future directions
8.1. Summary and conclusions

The ability and potential of marine bacteria to biodegrade PET was assessed via bacterial enrichment cultures selecting for bacteria that could utilise PET as an energy source. The surface modifications to the polymer induced by bacterial interactions were traced with a variety of analytical techniques, and at the same time the composition of the bacterial enrichment community was monitored. It was found that marine bacteria do indeed possess the capability for PET biodegradation.

Bacteria were shown to preferentially degrade larger features on the PET surfaces, and to favour rougher surfaces. The increased exposed surface area of these sections allowed bacterial cells better access to the polymer chains. Based on these observations a model for the physical mechanisms of biodegradation was proposed, in which the larger, more exposed features were degraded faster than other regions of the surfaces, leading to erosion of the surface peaks and overall smoothening of the surfaces. No physical mechanism of degradation relating the rate of biodegradation of PET to the roughness of the polymer surface has been proposed before.

Subtle changes in the chemistry of the PET surfaces led to the proposal of two potential chemical mechanisms of biodegradation. The first assumed that the minor chemical changes detected on the surface were somewhat incidental, and suggested that degradation occurred via hydrolysis of ester bonds and excision of small sections of the molecule. The second proposed mechanism accounted for some of the surface chemistry changes, and suggested the possibility of displacement of an alcohol from the ester bonds, via formation of an amide bond to a secondary substrate or protein.

The bacterial enrichment communities that developed in the presence of PET were found to be dominated by members of the classes \textit{α-proteobacteria} and \textit{γ-proteobacteria}. Consistently recovered isolates included strains of \textit{Alteromonas}, \textit{Thalassospira}, \textit{Roseovarius} and \textit{Limnobacter}. The mostly likely candidates to be PET degrading organisms were strains that returned nearest relatives to species of \textit{Thalassospira} and \textit{Kordiimonas}; members of both genera are known to be capable of utilisation of polycyclic aromatic hydrocarbons. Biodegradation however appeared to require commensalism between multiple bacterial species. Other extracellular enzyme producers such as \textit{Alteromonas}, and sulphur-cycling organisms such as \textit{Roseovarius} and \textit{Limnobacter} may play contributing roles.
A number of biodegradation enhancement strategies were proposed, based on either manipulation of the bacterial culture or the polymer itself. Bacterial manipulation strategies included techniques for increasing the size of the bacterial community and adjusting its composition towards higher proportions of polymer-degrading members. This was achieved primarily by manipulating culture conditions; supplementation of essential elemental nutrients nitrogen and phosphorus were shown to slightly increase the rate of biodegradation by enabling increase bacterial growth, and increasing the selective advantage of PET degrading organisms by inhibiting photosynthetic also proved effective.

Enhancement strategies based on manipulation of the polymer properties focussed on surface topography and roughness. Bacteria were shown to degrade rougher surfaces faster than smoother surfaces; however degradation resulted in smoothening of the surface, limiting the degree of degradation that could take place. Treatment of the polymer with sodium dodecyl sulphate surfactant increased the surface roughness of the material temporarily, before degradation caused the surface to become smoother again. Biosurfactants produced by the bacterial species *Pseudoalteromonas citrea* had a very similar surface roughening effect, and thus incorporation of this strain into the bacterial degradation community may help to enhance biodegradation through continuous exposure to surfactants.

Low cell attachment was identified as one of the barriers that inhibit biodegradation of PET. Fabrication of the surface topography of the polymer has the potential to boost the level of attachment cells can achieve, while not affecting the chemical properties of the surface. Lotus-inspired surface features and molecularly smooth surfaces both showed potential to enhance the level of cell attachment on PET; however fabrication techniques are not yet sufficiently developed to adequately reproduce nanoscale surface features.

8.2. Future directions

While the current work has provided a significant contribution to the fundamental understanding of the biodegradation mechanisms of PET, further work is still required to apply this knowledge on a commercial scale. Firstly, the strategies for biodegradation enhancement presented here represent a starting point for further development; however
in their current state they are not efficient enough to be commercially viable. Further work needs to be conducted, particularly in the investigation of additional surface topographies and their ability to promote cell adhesion, and in the development of fabrication techniques that can accurately and reliably produce nanoscale topographies to specification.

The other major area in which further study is required is in the cellular metabolism and regulation of the plastic degrading bacteria. The primary focus of this study was to characterise the physical and chemical changes made to PET surfaces in the process of biodegradation, and as such metabolism of bacteria was not explored. Proteomic and genetic analysis of PET degrading bacteria could provide further inspiration for biodegradation enhancement strategies.

8.3. Close

Development of biodegradation as a truly viable alternative plastic waste disposal protocol is extremely beneficial for the environment. Biodegradation is an environmentally friendly process, and has the potential to be much more efficient and cost-effective than recycling, increasing the incentive to move away from traditional, environmentally hazardous techniques.

The data presented here support the premise that biodegradation has the potential to become feasible for application of a large scale. Marine bacteria are indeed capable of degrading PET surfaces, and this work provides significant and novel contributions to the fundamental understanding of biodegradation mechanisms. For the first time a relationship between the rate of biodegradation of PET and its surface roughness has been identified, and a number of bacterial strains have been isolated and identified as having high potential as plastic degrading organisms. Also, several enhancement strategies for the biodegradation of PET were presented.
Bibliography


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