Characterisation of microbial colonies on COLORBOND® steel substrates

Nicole Alexandra Pianegonda
University of Wollongong

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Characterisation of Microbial Colonies on COLORBOND® Steel Substrates

Nicole Alexandra Pianegonda

Bachelor of Science Advanced (Honours I)

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School of Chemistry

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ABSTRACT

COLORBOND® Steel is a hugely successful, branded, coil-coated steel product used in a wide range of building application throughout Australia and South East Asia. Fungal growth on light coloured COLORBOND® Steel used in roofing and walling material is both unsightly and absorbs solar radiation, negatively impacting the roof and underlying structure. Up until now, however, the extent and behaviour of such fungal growth on these unique substrates has been poorly understood. Methods of assessing the phenomenon have, likewise, been lacking. Future development of strategies to prevent and mitigate infestation of these substrates is dependent upon the mechanism of infestation being understood. To this end, the work detailed in this thesis seeks to develop and enact various methods to detect, quantify and identify the problematic organisms, building a knowledge base for future work in this area.

In this study, over 200 test panels were deployed at each of two outdoor testing sites in Burrawang (Australia) and Kapar (Malaysia). A minimum of six panels were harvested monthly (later bi-monthly) over a two to three year period and examined using optical microscopy and mass spectrometry based lipid profiling.

Optical microscopy was able to be used to detect fungal spores adhered to the COLORBOND® Steel surface after as little four weeks of outdoor exposure. This method was also able to quantify fungal area coverage. Given the lack of aerial and penetrative growth occurring on this substrate with these organisms, this method of quantification was most useful.

Two further sets of test panels were exposed at the Burrawang site, were also
monitored monthly. The first series, to study temporal development of total solar reflectance and the second to enable a time-lapse study to allow study of growth rates, environmental influences, maturation of colonies, colony interactions and attachment to and detachment from the substrate.

Lipid profiling using electrospray ionisation tandem mass spectrometry was conducted on several species of laboratory grown filamentous fungus. Lipid head group scans were employed to target individual lipid classes and the ability to differentiate between these fungal species was assessed. Preliminary results suggested that differentiation was indeed possible.

Similar lipid profiling methods were attempted on fungal colonies grown outdoors on exposed COLORBOND® Steel test panels. The capabilities of two extraction methods, liquid extraction surface analysis and panel ‘swabbing’ were compared with reference to this substrate. However, issues were identified in comparing the lipid profiles of samples grown in various outdoor environments and, hence, in different environmental conditions. Lipid profiles are affected by growth conditions and the extent of this effect is not well understood.

The work detailed in this thesis paves the way for future studies concerning fungal proliferation on this unique substrate, an issue which is currently of substantial commercial interest. Intimate knowledge of the behaviour and nature of infesting colonies and the availability of established methods of uncovering such information forms a baseline of knowledge which is integral in informing and guiding the course of future work.
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<tbody>
<tr>
<td>ARC</td>
<td>Australian Research Council</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
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<tr>
<td>CHCl₃</td>
<td>chloroform</td>
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<tr>
<td>CID</td>
<td>collision induced dissociation</td>
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<td>CPL</td>
<td>coil processing line</td>
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<td>Da</td>
<td>Dalton</td>
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<td>desorption electrospray ionisation</td>
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<td>deoxyribonucleic acid</td>
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<td>EMS</td>
<td>enhanced mass spectrum</td>
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<td>FISH</td>
<td>fluorescence <em>in situ</em> hybridisation</td>
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<td>glycerophospholipid</td>
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<td>IPA</td>
<td>isopropyl alcohol</td>
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<td>ITS</td>
<td>internal transcribed spacer</td>
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<td>LESA</td>
<td>liquid extraction surface analysis</td>
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<td>MeOH</td>
<td>methanol</td>
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<td>MS</td>
<td>mass spectrometry</td>
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<td>MS/MS</td>
<td>tandem mass spectrometry</td>
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<td><em>m/z</em></td>
<td>mass-to-charge ratio</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NH₄OAc</td>
<td>ammonium acetate</td>
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<td>NLS</td>
<td>neutral loss scan</td>
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<td>PA</td>
<td>phosphatidic acid</td>
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<td>PC</td>
<td>phosphatidylcholine</td>
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<td>principle component analysis</td>
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<td>phosphatidylinositol</td>
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<td>precursor ion scan</td>
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<td>polyvinylidene fluoride</td>
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<td>peptone yeast glucose</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>S/N</td>
<td>signal-to-noise ratio</td>
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<tr>
<td>sp.</td>
<td>species</td>
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<tr>
<td>TSR</td>
<td>total solar reflectance</td>
</tr>
<tr>
<td>UNSW</td>
<td>University of New South Wales</td>
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<td>UoW</td>
<td>University of Wollongong</td>
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Chapter 1: INTRODUCTION
1.1 COLORBOND® Steel

The corrugated iron roof has been an iconic part of the Australian landscape for over a century. Simple to transport, store and install, such roofs were originally made from galvanized iron and were often post painted for aesthetic reasons, although this needed to be repeated regularly due to poor adherence of paint to the galvanized coating\(^1\). In the 1950s, technology to enable paint to be bound to a galvanized base was developed and this led to the first production of COLORBOND® Steel at Port Kembla, Australia, in 1966\(^1\).

Currently, COLORBOND® Steel is produced from metal coated steel strip using the generalised process depicted in Figure 1.1. During this process, the metal coil is unrolled, cleaned and pre-treated. It then passes through a set of rollers known as the ‘primer coater’ where it is coated in primer. The primer is cured as the strip passes through the primer curing oven. Once cured, the primed strip is passed through a second set of rollers, the ‘finish coater’, where it is coated in a ‘topcoat’ and ‘backer’ on the top and reverse side of the strip, respectively. Again, the strip passes through a
curing oven and is finally recoiled.

COLORBOND® Steel consists of a steel substrate, a zinc/aluminium layer, a primer and polyester paint top-coat and backer[3], as shown in Figure 1.2. This product is then formed into a variety of building applications including roofing, fencing, guttering and roller doors as well as in hot water systems and cool-room panels. The paint for this process is unique in that it needs to be easily applied, cured and recoiled and then must undergo roll forming, meaning that it needs to be hard yet flexible. This paint must also be durable enough outdoors to last in excess of 30 years on a roof[4].

Figure 1.2: A COLORBOND® Steel cross section showing the steel substrate coated in a Zn/Al layer, primer and polymeric topcoat and backer (not to scale).

COLORBOND® Steel is manufactured by BlueScope and is a high value add product for the company. Almost half of the new homes built in Australia feature COLORBOND® Steel roofs and 90 % of new homes include COLORBOND® Steel in some form[1]. Continued advances on coatings technology have entrenched COLORBOND® Steel as a high quality, attractive and functional building product.
1.2 Exposure testing of surface coatings

As part of the rigorous testing conducted on COLORBOND® Steel, samples are exposed outdoors in various locations. Exposure testing is designed to evaluate coatings performance in a way that accelerated or artificial weathering methods can only mimic and is a significant feature of BlueScope’s in-house assessment process. Overall, the purpose is to assess sample durability and corrosion resistance in a range of environmental conditions. Atmospheric classifications are defined in the Australia/New Zealand standard, ‘Guide to the protection of structural steel against atmospheric corrosion by the use of protective coatings’ (AS/NZS 2312)[5], as mild, moderate, tropical, industrial, marine and severe marine[6]. Severe marine sites are within 25 meters of breaking surf, marine is further removed from the coast, industrial sites feature airborne industrial pollutants and tropical sites are located within areas with a tropical climate. Locations within capital cities with very light industrial and marine influences are referred to as ‘moderate’ while sites unaffected by industrial and marine influences, such as rural sites, are classified as ‘mild’. ‘Mild’ exposure sites, have been shown to exhibit higher rates of microbial proliferation than those closer to the sea and industrialised areas. This is discussed further in Section 1.3.

The geographical locations of the exposure sites chosen vary in the intensity of their environmental parameters and are chosen to assess variables such as influence of climate and intensity of incident solar radiation on paint films, corrosivity of the site to the steel substrate and miscellaneous parameters including chemical pollutants and airborne particulates. Where corrosion performance is important, samples need to be tested in ‘marine’ or ‘severe marine’ sites where environmental factors exacerbate
metal corrosion. To assess durability, the samples are placed at sites in which aesthetics, for example gloss loss and colour change, are compromised by environmental conditions. Locations in which these parameters are tested are typically hot and have high incident solar radiation or atmospheric pollutants. A large part of BlueScope operations are in South East Asia and therefore, exposure sites are also maintained in Kapar (Malaysia) and three locations in China, that are classed as ‘tropical’ exposure sites\(^7\).

Conventionally, panels are mounted on open ‘normal’ racks, as shown in Figure 1.3 (a). These are constructed from steel frames with horizontally attached hard wood slats. Panels are fixed to the slats with rubber lined washers and stainless steel screws. Over the past 10 years, exposures have also been performed on so called ‘hot’ racks (Figure 1.3 (b)) which are designed to elevate the temperatures of the test panels, particularly dark coloured test panels, to temperatures closer to those they would encounter in service, on an insulated roof.
Figure 1.3: (a) The ‘normal’ exposure rack at Burrawang (Australia). Panels are secured onto the wooden slats using rubber washers, secured with screws. (b) The ‘hot’ rack at the Burrawang exposure site. The black backing elevates the temperature of the rack through absorption of infrared radiation.
Chapter 1: Introduction

1. 3 Microbial colonisation of COLORBOND® Steel

1.3.1 Australia

Since the 1980s, there has been concern over ‘darkening’ of COLORBOND® Steel, an effect which has been noticed in many locations in Australia and South East Asia. Several studies have found this to be the result of microbial growth on the COLORBOND® Steel surface[8-13]. In Australian environments, most cases of microbial darkening take one of three forms[14]: isolated darkening, occurring mostly along the ridge cap of the roof (Figure 1.4 (a)), ‘spotty’ darkening, lichen growth which occurs across the body of the roof (Figure 1.4 (b)) and overall darkening in which the microbial growth is fairly uniform across the structure, resulting in an overall colour change of the material (Figure 1.4 (c)).

Figure 1.4: The three most common forms of darkening of COLORBOND® Steel in Australia: (a) isolated darkening along the ridge cap of the roof (circled) (Warwick, QLD), (b) ‘spotty’ darkening caused by lichen (Clayfield, QLD) and (c) overall darkening, a uniform colour change across the surface of the roof (Camden, NSW).
Chapter 1: Introduction

The Australian environment spans a diverse range of environmental conditions with many highly built up areas falling into the environmental categories discussed in Section 1.2. Significant microbial proliferation on test panels has been observed at a ‘mild’ exposure site located at Burrawang, a rural, inland location in NSW. In contrast, marine and severe marine environments in Australia show relatively low levels of microbial growth. This relationship is shown in Figure 1.5 which shows identical test panels exposed at severe marine (a), mild (Burrawang) (b), marine (c) and industrial/marine (d) exposure sites. Minimal microbial darkening is seen on (a), (c) and (d) while (b) shows significant growth.

![Figure 1.5: A comparison of identical panels exposed at various Australian exposure sites: (a) severe marine, (b) mild, (c) marine, (d) industrial/marine. Lack of infestation is seen on sites (a) and (c) compared to (b). Colonies at site (d) are masked by industrial fallout but are significantly less than (b).](image-url)
1.3.2 **South East Asia**

In South East Asian environments, darkening caused by atmospheric pollution is an additional contributor to surface darkening. This darkening effect, known as tropical discolouration, occurs within the first few months of outdoor exposure. Since 2001, COLORBOND® Steel sold in South East Asia is coated post-painting with a treatment designed to prevent tropical discolouration. This product, known as ‘Clean COLORBOND®’, has been very effective at resisting tropical discolouration. However, discolouration caused by microbial growth persists and is suggested that the ‘clean’ coating may actually exacerbate the microbial growth\[15\]. This is demonstrated in Figure 1.6 which shows microscope images of two identical test panels exposed in Malaysia, (a) without and (b) with a ‘clean’ coating. While the background appears darker in (a), due to tropical discolouration, the microbial colonies on the clean treated sample (b) are larger. The relationship between darkening due to tropical discolouration and microbial darkening on panels exposed in Malaysia for between 12 and 73 months was investigated by Boge *et al.* (2007). By comparing the change in ‘lightness’ (ΔL), before and after bleaching the fungal growth, between 50 and 90 % of the ΔL was found to be attributable to fungal growth.
Figure 1.6: Test panels exposed in Malaysia for 12 months. (a) has no ‘clean’ treatment while (b) does. Adapted from Barker et al. (2009).
1.4 Impacts of microbial colonisation on thermal properties of coated steel roofs

Increasing awareness of, and initiatives to lessen, global warming have led to interest in solar reflective roofing materials[16]. Roofing material that has high solar reflectance in the wavelength range 0.3-2.5 µm and high thermal emittance in the wavelength range of 4 – 80 µm resists heating due to solar radiation and such materials are referred to as ‘cool roofs’[17]. Such materials insulate the structure[18], reducing heating and cooling costs throughout the lifetime of the structure[19] and reducing energy generation requirements[17]. In addition, cool roofs mitigate the urban heat island phenomenon which results in increased air temperatures in urbanised environments[16, 20].

Due to the advantages of energy efficient ‘cool roofs’, COLORBOND® Steel sold in Australia since 2008 has incorporated ‘Thermatech® solar reflectance technology’, a range of infrared reflecting pigments[21]. Such roofing materials have a higher total solar reflectance (TSR) compared to similar colours with standard pigments. COLORBOND® Steel incorporating Thermatech® is advertised to reduce annual cooling energy consumption by up to 20 %, with an average reduction of 5 %[21]. Similar efficiency estimates have been calculated based on roofing materials located in Europe[22] and North America[23].

The long term benefits of ‘cool roofs’ can be diminished by loss of solar reflectance in the first few years of service[24]. It is suggested that the main culprits for the loss of solar reflectance in roofing materials is due to dirt, dust and/or biomass accumulation[24]. In studies based in California (USA), Cheng et al. observed a 5 %
decrease in solar reflectance of white polyvinylidene fluoride (PVDF) coated steel roofing over a 3 year exposure period. This was correlated to a build-up of microbial biomass, the most significant of which were cyanobacteria and fungi\cite{19}. Subsequent studies on similar substrates calculated solar reflectance decrease to be between 4 and 23% in the first year\cite{24}.
1.5 Fungi

1.5.1 Characteristics and significance

True fungi can be defined as heterotrophic eukaryotes, generally with walls of B-glycan and chitin\textsuperscript{25}. Filamentous fungi colonise surfaces via a network of hyphae. Hyphae extend from a common growth base, or mycelium, and may then develop into fruiting bodies or reproductive asexual conidial hyphae (Figure 1.7 (a))\textsuperscript{26}. Conversely, yeasts, another form of fungi, propagate by ‘budding’, a process of producing separate cells through mitosis (Figure 1.7 (b)). Yeasts are hydrophilic and lack extracellular adhesive molecules such as mannoproteins and hydrophobins; amphiphilic molecules capable of forming strong cell–substrate bonds\textsuperscript{27}. As such, they are generally dislodged by water and do not generally colonise building materials, of which filamentous fungi are the main infesters\textsuperscript{28}.

Figure 1.7: (a) SEM image of yeast, \textit{Saccharomyces cerevisiae}\textsuperscript{29} and (b) Image of a filamentous fungus, \textit{Cladosporium carrioni}\textsuperscript{30}.
1.5.2 **Cell wall and membrane**

The fungal cell wall is mainly comprised of the polysaccharides chitin and glucan in addition to glycoproteins\[^{31}\]. Chitin contributes to structural integrity of the cell\[^{31}\] and comprises approximately 1 – 2% of the cell wall dry mass of yeast fungi\[^{32}\] and 10 – 20% of filamentous fungi\[^{33}\]. The majority of the cell wall is made of glycan, with glycoproteins comprising the rest\[^{31}\]. Chitin links the cell wall to the plasma membrane\[^{34}\].

The cell membrane consists of a lipid bilayer\[^{35, 36}\] of which phospholipids are the major component, followed by sterols. It also contains carbohydrates and steryl esters\[^{37-39}\]. This membrane allows the uptake of nutrients, enzyme anchoring and signal transduction\[^{35}\].

1.5.3 **Fungal infestation of surfaces**

1.5.3.1 Deposition

Deposition of fungal material is hypothesised to occur via aerosol spore transport. Most fungal spores can readily become airborne\[^{40}\] and it is estimated that 1-5% of aerosol particle mass in the outdoor environment is fungal spores\[^{41}\]. Spores of *Cladosporium* sp. (species) are the most abundant airborne spores in many parts of the world\[^{42, 43}\]. *Alternaria* sp.\[^{44}\] is also an ubiquitous genus worldwide. Studies of fungal diversity in air have found filamentous fungi *Alternaria* sp. and *Cladosporium* sp. in significantly higher abundance in summer and autumn whilst other filamentous fungi, *Blumeria* sp. and *Penicillium* sp. for example, are in higher abundance in spring and winter\[^{41}\] (Mainz, Germany) while samples gathered in autumn (Tulsa,
USA), showed an abundance of *Cladosporium, Alternaria, Pithomyces* and *Curvularia* spores\textsuperscript{[42]}.

1.5.3.2 Adhesion and germination

Once a spore comes in contact with an appropriate surface, it excretes an adhesive substance in order to bind to the substrate\textsuperscript{[45]}. The composition of these substances vary significantly between species although they are typically a mixture of water-insoluble glycoproteins and other biomolecules such as lipids and polysaccharides\textsuperscript{[46]}. The environmental conditions required to instigate adhesion also vary between species\textsuperscript{[45]}.

Following adhesion, germination occurs with the formation of a ‘germ tube’. The environmental conditions required for successful germination vary between species and are influenced by a range of factors including nutrient availability, hydration, temperature and surface stimuli\textsuperscript{[45]}. The extension of this germ tube is influenced by a number of environmental factors such as the hardness, topography and hydrophobicity of the substrate in addition to biological signalling molecules. If the germ tube detects favourable environmental conditions, it will develop into an appressorium\textsuperscript{[45]}, which is designed to penetrate the substrate. Several examples of appressorium development on germ tubes are indicated by arrows in Figure 1.8.

Infection of plants by fungal appressoria has been reviewed\textsuperscript{[47]}. Some studies suggest hard inert substrates such as glass result in delayed\textsuperscript{[48]} appressoria formation although some species, such as *A. alternata*, are thought to develop appressoria regardless of surface topography\textsuperscript{[49]}.
Figure 1.8: A light micrograph showing growth of *Alternaria alternata* on a glass slide. The spore is indicated by ‘S’ in the top left corner and appressoria are indicated with arrows. Figure adapted from Hatzipapas et al.[49].

Subsequently, the established spore will develop hyphal branching. These structures have an advantage over discrete colonies as they are able to spread over solid substrates and enable nutrient transport throughout the organism. Hyphal growth proceeds with greater frequency in areas of high nutrient concentration[40]. In young colonies, hyphae have similar sizes and extension rates and tend to branch out perpendicularly[40]. As the colony ages, branching occurs at < 90° degree angles and hyphal extension occurs mainly at the colony margin[40].

### 1.5.4 Propagation models

Prevention of fungal growth must target the impetus for adhesion and subsequent germination and proliferation. While the dark nodular fungal colonies clearly result in detrimental discolouration of COLORBOND® Steel substrates (as discussed in Sections 1.3 and 1.4), it is well reported that symbiotic relationships exist between bacteria and fungi[50]. These may result in both antagonistic and protagonistic changes in the growth rate, morphology, virulence, survival and surface adhesion of the fungal counterpart[50]. The possible involvement of a bacterial agent, in
instigating fungal attachment is, therefore, of interest.

When in contact with accommodating surfaces, bacteria propagate by forming bacterial biofilms. Biofilm formation involves changes in the behaviours and metabolism of planktonic forms of microorganisms\textsuperscript{[51]}. Through this communal growth, biofilms provide such advantages as enhanced surface attachment, encasement in an exopolymeric matrix, enhanced antimicrobial resistance, enhanced cell-to-cell communication and increased nutrient capture efficiency\textsuperscript{[51]}. A typical bacterial biofilm model is shown in Figure 1.9.

![Figure 1.9: The lifecycle of a bacterial biofilm: (i) planktonic bacteria settle and attach to a surface, (ii) once irreversibly bound, cells cluster together which is mediated by exopolysaccharide production and surface motility, e.g. twitching, (iii) microcolony formation, (iv) maturation in which the biofilm reaches maximum thickness and surface coverage and (v) dispersal of planktonic cells. Adapted from Harding et al.\textsuperscript{[51]}.](image)

Conversely, it has been shown that fungi are independently capable of colonising a range of synthetic surfaces including polymers, metals and ceramics, leading to the formation of fungal biofilms\textsuperscript{[52]}. The development of such colonies is thought to proceed in a similar manner to bacterial biofilm formation, as detailed in Figure 1.10. This process proceeds through (i) initial contact of the fungal spore or hyphal fragment to the surface and (ii) attachment to the surface via the excretion of adhesive substances followed by colony maturation (Figure 1.10 (iii) and (iv)).
During the maturation process, the monolayer of hyphae explores the surface, laying down a polymeric extracellular matrix (iii) before the colony starts to ‘layer’, through hypha-hypha adhesion (iv). This is followed by aerial growth and reproduction (v) and eventual dispersal of spores (vi).

Figure 1.10: A proposed model for the formation of a filamentous fungi biofilm, involving (i) surface adsorption, (ii) attachment, (iii) formation of monolayer microcolony, (iv) a more developed and layered microcolony with hyphal development, (v) the mature biofilm and (vi) dispersal of the planktonic phase. Adapted from Harding et al.[51].
1.6 Methods for the detection, identification and quantification of fungi and bacteria on surfaces

1.6.1 Morphological analysis

Traditionally fungal classification of microorganisms has been performed using the phenotypic approach, which is based on observable morphological criteria\[^{53}\]. In cases where the organisms cannot be visualised in their environmental state, it is necessary to culture the organism in the laboratory. However, this can introduce additional variables due to the culture media\[^{54}\], competition between co-occurring species\[^{54}\] and the time between sampling and culturing\[^{54}\]. The latter is important given that spores and hyphal fragments decline in viability over time at a rate which is species specific\[^{54}\]. In addition, it is recognised that fungal fruiting bodies are strongly influenced by environmental factors, making identification using only macroscopic features difficult\[^{55, 56}\], particularly when dependent on culturing\[^{54}\]. However, microscopic features such as hyphae, spore sacks and spores are not influenced by environmental factors\[^{56}\] and are large enough to observe using an optical microscope\[^{53}\]. Several common and distinctive spore types found in air are shown in Figure 1.11, which shows the spore types observed in a spore survey of Medallin (Colombia)\[^{57}\]. Spores can be differentiated based on their size, shape and colour.
Figure 1.11: Fungal spores present in the atmosphere. (a) Agrocybe sp., (b) Alternaria sp., (c) Arthrinium sp., (d) Beltrania sp., (e) Cladosporium sp., (f) Curvularia sp., (g) Dreschslera sp., (h) Epicoccum sp., (i) Fusarium sp., (j) Nigrospora sp., (k) Leptosphaeria sp. (l) Pithomyces sp. (m) Pleospora sp. (n) Puccinia sp. (o) Tetraploa sp. (p) Torula sp. (q) Ustilago sp.\[57]\.

If the sample to be analysed is in such a form that spores may be visually observed without sample preparation, for example, when spore settling occurs on an impermeable membrane, this method is advantageous. Because of the exposed nature of the spores on the hard, impermeable surfaces of interest in this work, optical detection is an efficient detection method. Even in cases where there are very few spores, individual spores can be detected under the optical microscope. Figure 1.12 shows two optical microscope images taken of panels that had been exposed at
Burrawang for a period of four weeks. Although infestation is minimal, several spores (circled) are clearly visible.

Figure 1.12: Optical microscope images taken from two panels which had been exposed at Burrawang for a period of four weeks. Although infestation is minimal, several spores can be distinguished on the panel’s surface.

Quantification of visible microorganisms is also possible using optical microscopy by processing the photographs using image analysis software. Such methods have previously been applied in the quantification of hyphal development\textsuperscript{[58]} and characterisation of fungal morphological growth\textsuperscript{[59]}.

1.6.2 DNA analysis

1.6.2.1 Amplification and sequencing

Recently, access to DNA sequence data has revolutionised identification of organisms. This field, known as molecular identification\textsuperscript{[55]} is now widely employed in agriculture\textsuperscript{[60, 61]}, human health\textsuperscript{[62]}, and environmental management\textsuperscript{[63]} where fungal contamination is a major concern. While DNA sequencing can be used to identify organisms, often laboratory culturing must first be performed in order to isolate or provide adequate quantities of the organisms. The time requirements of this
method are a consideration and, in addition to culturing, regions of the genome are generally amplified using polymerase chain reaction (PCR).

PCR assays use primers to specifically detect DNA sequence fragments unique to the target organism; however, this approach is dependent on the quality of the reference sequences. Most molecular identification of fungi uses the internal transcribed spacer (ITS) region\textsuperscript{55} of the fungal genome as its reference point as it is highly variable among species or even populations of the same species. This is then compared to a known DNA sequence in order to identify the organism\textsuperscript{64}. Despite the amount of work done in this area, as of 2011 less than 1 % of the estimated 1.5 million fungal species on earth have been sequenced in this way. Hence, a significant number of species are still unable to be identified in this manner\textsuperscript{55}.

1.6.2.2 Fluorescence in situ Hybridisation

Fluorescence in situ Hybridisation (FISH) is a method of targeting and detecting the presence of specific DNA or RNA sequences. In this technique, the DNA or RNA in question is hybridised with an oligonucleotide probe, which takes the form of a primer labelled with a fluorescent dye. The probe must both contain a sequence long enough to be specific towards the target organism and small enough to penetrate the cell wall\textsuperscript{65}. After cell penetration, the probe will hybridise to the complement site of the DNA or RNA. If such a site is not present, the probe will not bind and is able to be washed off. By using a primer specific to a certain target organism or organisms, FISH is capable of providing species specific information about morphology, quantity and spatial distribution of bacterial and fungal colonies without the need for time consuming culturing techniques by highlighting the specific areas in which
specific DNA or RNA sequences are localised\textsuperscript{[65]}. Hence, an advantage of this technique is its ability to be applied to untreated environmental specimens and to produce rapid imaging of organisms in their natural environments. As such, it has been used to image fungal growth on leaves\textsuperscript{[66]}, wood\textsuperscript{[67]} and polymer coated steel, as shown in Figure 1.13, in which two COLORBOND\textsuperscript{®} Steel panels have been stained with bacteria (green) and fungi (red) specific probes\textsuperscript{[68]}. 

Figure 1.13: Detection of bacteria and fungi by fluorescence in situ hybridisation (FISH). Bacteria were hybridised with EUB-Cy3 probe (green) and fungi with EUK-Cy5 probe (red). Images were collected from panels exposed at Burrawang for (a) 4 weeks and (b) 24 weeks. Scale bar represents 50 µm. Adapted from Huynh et al.\textsuperscript{[69]}

While probes for fungi have long been used, the detection of species of interest requires one to first know the DNA or RNA sequences to be targeted and to then develop and validate specific probes\textsuperscript{[70]}. Thus, the technique is not suitable for discovering new organisms and is generally limited to known organisms. Future development of this technique would ideally involve automation to remove the impact of variability of operator technique and bias, which impacts detrimentally on the reproducibility of measurements\textsuperscript{[70]}. Another vital requirement of this technique is
that the cells are fixed and treated to make the cell wall permeable so that the probe is able to traverse the cell wall and bind with the DNA. Hence, the technique involves mechanical disturbance of the cell and can therefore be viewed as a destructive form of testing\cite{70}, a limitation not associated with conventional optical microscopy.

1.6.3 **Lipid analysis**

Eukaryotic organisms can consist of hundreds to thousands of unique biomolecules\cite{71}, the type and abundance of which reflect genetic traits and phylogenetic relationships between organisms\cite{71}. Mass spectrometry (MS) is becoming an increasingly useful tool in the analysis of biological samples, enabling a wide range of characteristic biomolecules, including oligonucleotides, proteins and peptides and lipids to be detected.

Recent developments in soft ionisation methods have meant that biological molecules may be infused intact into the mass spectrometer, allowing for more detailed analysis of complicated biological molecules. Other advantages of MS are its potential for fast, high throughput analysis\cite{72} and sensitivity of detection. The fungal sterol lipid, ergosterol, for example, has also been employed as a biomarker for fungal species. It is the dominant sterol in the ascomycota and basidiomycota taxa\cite{73} and is readily detectable by MS techniques. Lipids in particular are attractive targets as biomarkers for specific organisms because they are often present in high abundance and can be readily detected using MS. Hence, they can be used to characterise different microbial species\cite{74, 75} and strains\cite{71, 76, 77}. Microbial identification using MS based profiling of glycerophospholipids (GPLs) is discussed
in greater detail in Section 1.8.
1.7 Common microbial lipids

1.7.1 Glycerophospholipids

Glycerophospholipids (GPLs) are one of the major lipid categories of eukaryotic organisms\cite{71}. The mass spectrometric ionisation and dissociation behaviour of these lipids are well documented and this broad class of lipids have the potential to act as biomarkers\cite{78}. The high selectivity and sensitivity of headgroup scans for this class of lipids (as described in detail in Section 1.8.3) makes them one of the most commonly profiled lipid classes; particularly when using shotgun lipidomics techniques such as electrospray ionisation tandem mass spectrometry (ESI-MS/MS)\cite{79}.

The lipidome of various fungal species has been widely reported to contain the GPLs, phosphatidylethanolamine (PE)\cite{75, 76, 80-84}, phosphatidylglycerol (PG)\cite{75, 76, 82, 84}, phosphatidylcholine (PC)\cite{76, 80-84}, phosphatidylserine (PS)\cite{76, 80, 82-85}, phosphatidic acid (PA)\cite{75, 76, 80, 82-84}, phosphatidylinositol (PI)\cite{76, 80, 82-84, 86} and cardiolipin (CL)\cite{76, 80, 83}, the generic structures of which are shown in Figure 1.14. These GPLs may be diacyl (containing two FAs) (Figure 1.15 (a)) or lyso (containing a single FA) (Figure 1.15 (b)).
Figure 1.14: Generic structures of common glycerophospholipids of the fungal lipidome, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI) and cardiolipin (CL) where ‘R’ represents a hydrocarbon chain.
The most abundant classes present in yeast are generally reported to be PC\textsuperscript{[71]}{[71, 84]} and PE\textsuperscript{[84]}. Kapoor \textit{et al.}, in a study of several Candida species, found the most abundant PE, PI, PS, PG and PA lipids to be those with a total of 32, 34 or 36 carbons in their acyl chains\textsuperscript{[74]}. In contrast, bacterial cells have been found to be most abundant in PE, PC and PG\textsuperscript{[87, 88]}, with some bacteria containing CL lipids\textsuperscript{[89]}.

GPLs perform a diverse range of functions within the cell. PE regulates the structure of the cell membrane\textsuperscript{[90]} and has also been implicated as a chaperone lipid for protein molecules\textsuperscript{[91]}. PG lipids are involved with electron transport facilitating photosynthesis, chloroplast development and low temperature tolerance\textsuperscript{[92]} (as reviewed by Wada \textit{et al.}). PA lipids, too, have numerous functions in eukaryotes, both in cell signalling and membrane dynamics\textsuperscript{[93]} (as reviewed by Kooijman \textit{et al.}). PS lipids function as important signalling molecules, aiding in the removal of apoptotic cells and as an enzyme cofactor\textsuperscript{[94]} (as reviewed by Vance \textit{et al.}). PI lipids act to anchor proteins to the cell membrane\textsuperscript{[95]} (as reviewed by Thomas \textit{et al.}).

1.7.2 Sterol lipids

Sterols are thought to contribute to cell membrane stability by forming intermolecular bonds with fatty acyl chains of lipids\textsuperscript{[36]}. As a fungi specific sterol
lipid, ergosterol has been utilised in the quantification of atmospheric fungal spores, where bulk air samples were taken and ergosterol specifically is extracted\textsuperscript{[96]}. The specificity of ergosterol as a biomarker for fungi has also allowed it to be used to qualitatively detect fungal contamination in agricultural produce\textsuperscript{[97]}. In addition to ergosterol, fungi contain a diversity of sterol lipids\textsuperscript{[98]}. Several of these are illustrated in Figure 1.16, below. The filamentous fungus, \textit{Epicoccum nigrum}, contains both 9(11)-dehydroergosterol and ergosterol\textsuperscript{*} as its main sterols\textsuperscript{[99]}. Similarly, ergosterol is most abundant of all sterol lipids in the yeast fungi \textit{Hortaea werneckii}, \textit{Aureobasidium pullulans} and \textit{Saccharomyces cerevisiae} and also in the filamentous fungi \textit{Alternaria alternata} and \textit{Cladosporium sphaerospermum}\textsuperscript{[98]}. The filamentous fungus, \textit{Alternaria kikuchiana} also contains mostly ergosterol with smaller amounts of lanosterol and 24-methylene-24,25-dihydrolanosterol\textsuperscript{[100]}. 24-Methylcholest-7-en-3β-ol is the second most abundant sterol in \textit{C. sphaerospermum} and \textit{H. werneckii} and 4α,24-Dimethylcholest-7-en-3β-ol, the second most abundant sterol in \textit{A. pullulans}\textsuperscript{[98]}. In contrast, another filamentous fungus, \textit{C. sphaerospermum} has as its main sterol components, 5α,8α-Epidioxy-24(R)-methyl-cholesta-6,22-diene-3-β-ol and 5α,8α-Epidioxy-24(R)-methyl-cholesta-6,9(11),22-triene-3-β-ol\textsuperscript{[101]}.

\textsuperscript{*} The systematic name for ergosterol is 24-Methylcholesta-5,7,22-trien-3β-ol.
Figure 1.16: Several sterol lipids found in fungi.
1.7.3 **Sphingolipids**

The sphingolipids (SL) of yeast differ from mammalian SL by their inositol mannosyl residues.\textsuperscript{102} The lipidome of various fungal species has been reported to contain: ceramide (Cer),\textsuperscript{76, 82, 86} inositol phosphorylceramide (IPC),\textsuperscript{76, 82, 86} mannosylinositol phosphorylceramide (MIPC)\textsuperscript{76, 82, 86, 102} and mannosylidiinositol phosphorylceramide (M(IP)\textsubscript{2}C)\textsuperscript{76, 82, 102} (Figure 1.17). Kapoor et al. identified 21 SLs, spanning the sub-classes IPC, MIPC and M(IP)\textsubscript{2}C, in eight different species of *Candida*. The most abundant IPC were those with a total of 50 or 52 carbons in the molecule and the most abundant MIPC and M(IP)\textsubscript{2}C were those with 56 or 58 and 62 or 64, respectively\textsuperscript{74}. None of the major SLs detected featured carbon-carbon double bonds in their sphingoid base or acyl chains\textsuperscript{74}. 
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Figure 1.17: Common sphingolipids of the fungal lipidome: ceramide (Cer), inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC) and mannosyldiinositol phosphorylceramide (M(IP)_2C) where ‘R’ represents a hydrocarbon chain.

1.7.4 Fatty Acyl Chains

Fatty acyl (FA) chains are generally described in the form (Y:X), where Y denotes the number of carbon atoms in the chain and X, the number of double bonds. Abbreviations such as ‘18:X’ denote FA chains of 18 carbons in length containing an unspecified number of double bonds. The FA chains that occur within larger lipids (represented as the ‘R’ chain in the figures above) are often indicative of the
organism. Both the FA chain length and its degree of unsaturation are useful biological identifiers. Organisms differ in their FA desaturases, enzymes that create a carbon-carbon double bond on a FA chain. The double bond will be installed between specific carbons in the hydrocarbon chain, depending on the specific desaturase enzyme (denoted by Δz, where z represents the position at which the double bond is formed, numbered as shown in Figure 1.18).

Figure 1.18: Fatty acyl 18:1, in which the carbon-carbon double bond is located between the C9 and C10. A double bond in this position would have been created by a Δ9 fatty acid desaturase enzyme.

Elucidation of the position of the FA double bond can provide enzymatic information about the organism. Additionally, organisms with only one FA desaturase enzyme can be broadly distinguished from those with several based on their degree of unsaturation. Hein et al. have linked FA unsaturation to yeast strain-specific genetic data, showing that the yeast, *S. cerevisiae*, contains a single fatty acid desaturase which introduces a double bond in the 9 position of the acyl chain only (Δ9). In contrast, *Yarrowia lipolytica* possesses Δ9 and Δ12 fatty acid desaturase enzymes while *C. albicans* contains Δ9, Δ12, and Δ15 fatty acid desaturase enzymes. Hence, *S. cerevisiae* can be distinguished from *Y. lipolytica* and *C. albicans* based on the incidences of double bonds in their FA chains, when grown in identical conditions (as further discussed in Section 1.7.5).
Variations in the relative abundance of certain FAs are evident in different organisms. The most abundant FAs in flowering plants, for example, are the polyunsaturated fatty acids (PUFA), 18:2, 18:3 and 16:3\textsuperscript{104}. In contrast, non-flowering plants, such as mosses, have an abundance of PUFAs with 20 carbons, in addition to the 18 carbon PUFAs found in flowering plants\textsuperscript{104}. Similarly, the FA chains 20:4, 20:5, 18:2 and 18:3 have been associated with red algae, 18:2, 18:3 and 18:4 with green and 18:2, 18:3 and 18:4 and 20:4 with brown algae\textsuperscript{104,105}.

Much of the lipid profiling of fungal species has focused on yeast. Several studies of yeast have identified GPLs with between 1 and 6 double bonds in their acyl chains\textsuperscript{71,74}. Within GPLs, the most common total acyl chain lengths, that is, the total number of carbons in both FA chains, are between 32 and 36 carbons\textsuperscript{74}. Approximately 1 % of the total GPLs, however, were comprised of molecules containing 31, 33 and 35 carbon atoms in their acyl chains\textsuperscript{71}.

Studies of the yeast \textit{S. cerevisiae} found the most commonly occurring FAs to be 16:1, 16:0, 18:1, 18:0 and 14:0\textsuperscript{71,80,86}. Minor FAs include 14:0, 14:1, 12:0 and 12:1, as well as the odd-numbered FAs 15:0, 15:1, 17:0 and 17:1. FA composition of the yeast, \textit{C. albicans}, has been reported to be dynamic and temperature dependent\textsuperscript{103}.

Lattif \textit{et al}. found that the mean number of double bonds per acyl chain (unsaturation index) of phospholipids in \textit{C. albicans} decreased with the age of the fungus, with this effect particularly strong in biofilms\textsuperscript{84}. In Verbelen \textit{et al}.’s study of brewer’s yeast FAs 16:1 and 18:1 represented 60-80 % of the total fatty acids, followed in abundance by 16:0 and 18:0, constituting 10 % and 5 % respectively\textsuperscript{106}.

Conversely, filamentous fungi tend to contain less odd numbered FA chains and are dominated by 16:X and 18:X FAs. The filamentous fungus, \textit{Cladosporium sp.}, for
example, is most abundant in 16:0, 18:1, 18:2 which constitute 20, 30 and 40 % of total FAs respectively,[107], giving rise to differing ratios of lipids. The bracket fungus *Laetiporus sulphurous* also contains predominantly the FAs 16:0, 18:1, 18:2[108] and, similarly, 88 % of the total fatty acid content of another filamentous fungus, *E. nigrum*, is comprised of FAs 16:0, 16:1, 18:0, 18:1 and 18:2[99].

1.7.5 **Additional considerations in lipid diversity**

Production of lipids is governed by a complex combination of biological processes, many of which are not yet well understood. For this reason, growth conditions are an important parameter to consider if these molecules are to be used as biomarkers[109]. A microorganism’s lipidome is influenced by its growth stage[84], available nutrients[110] and environmental factors such as temperature[99], oxygen availability[36], salinity[99], pH[99] and environmental pollutants[81].

Lipid variations are thought to arise from cellular adaptation to changing environmental conditions. Growth at low temperatures, for instance, can lead to various adaptation reactions such as increased synthesis of unsaturated fatty acids, intramolecular rearrangement of fatty acids and changes to sterol content[36]. Additionally, growth in an anaerobic environment prevents FA desaturation reactions and sterol synthesis, both of which require oxygen to proceed[36].

In a recent study of the filamentous fungus, *E. nigrum*, Ahumada-Rudolph et al., found temperature, salinity and pH all impacted upon FA and sterol lipid constitution[99]. *E. nigrum* grown at 25 °C contained a relatively high proportion of unsaturated fatty acids compared to those grown at 6 °C. High salinity at acidic pH resulted in an increase in 16:0 relative to 18:X but the same was not true in basic
conditions. In high salinity environments and also at lower temperature (6 °C) the diversity of sterol species decreased, with less ergosterol relative to 9(11)-dehydroergosterol[99].
1. 8 Mass Spectrometry as a tool for characterisation of microbial lipidomes

1.8.1 Lipidomics

The field of lipidomics is concerned with identifying, characterising and quantifying the lipids within a biological system. It also incorporates the study of extra- and intra-cellular factors on cellular lipids and overall function of the biological system\[^{85, 86, 111-113}\]. MS is a central analytical tool in the field of lipidomics due both to the ease of detection and the availability of extraction and ‘soft’ infusion techniques, which allow fragile biomolecules to be analysed both intact and fragmented in a controlled manner.

Lipids can be divided into eight different categories or classes; fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GPL), sphingolipids (SL), sterol lipids, prenol lipids, saccharolipids, and polyketides, each of which are distinguished by their common structural elements\[^{114}\]. Each class of lipids contains several lipid sub-classes which contain, in turn, many types of lipids which vary based on variables such as the length and unsaturation of their acyl chains, the number and position of hydroxyl groups and branching points. The identities of lipids in addition to their relative abundances can be used to construct a ‘lipid profile’ of a certain organism.

1.8.2 Extraction

Several studies have used MS to identify the characteristic array of fungal lipids. Most studies of this nature have used procedures to extract and purify the cellular lipids prior to mass spectrometric analysis\[^{75, 82, 83, 86, 115}\]. Commonly used procedures
are based on the Bligh and Dyer technique\textsuperscript{[116]} for phospholipid extraction and purification, reported in 1959\textsuperscript{[75, 82, 83]} or the Folch method, reported in 1957\textsuperscript{[117]}. Both methods have shown comparable lipid extraction when applied to animal tissues for samples containing < 2 % lipid, although the Folch method was twice as efficient for samples with higher lipid content\textsuperscript{[118]}. It has been demonstrated that algal lipid extraction is most efficiently achieved using 2-ethoxyethanol but is also possible using the Folch method\textsuperscript{[119]}. The latter is a more universal method, which has been more generally applied to other organisms. The majority of these studies sought to quantify the lipids detected\textsuperscript{[82, 83, 86, 115]} and used headgroup or targeted scans to increase the selectivity and sensitivity of the detection\textsuperscript{[82, 86, 115]}.

Most recently, Ejsing \textit{et al.} quantified 21 different lipid sub-classes and 250 individual molecular lipids of four strains of the yeast, \textit{S. cerevisiae}, using a two-step extraction process\textsuperscript{[120]} and targeted headgroup scans\textsuperscript{[76]}. Hein \textit{et al.} quantitatively analysed several \textit{Saccharomyces} species including \textit{S. cerevisiae} and identified over 100 unique molecules belonging to nine GPL sub-classes\textsuperscript{[71]}. A modified Bligh and Dyer extraction and a series of centrifugation and resuspension steps were used to purify and separate cellular lipids from cell residues and high performance liquid chromatography (HPLC) was used to distinguish GPL sub-classes\textsuperscript{[71]}. Kapoor \textit{et al.} conducted a similar study wherein a modified Bligh and Dyer extraction and ESI-MS/MS headgroup scans were used to identify and quantify over 200 individual GPLs in eight strains of the yeast, \textit{Candida}\textsuperscript{[74]}.

Several extraction methods, uniquely suited to analysis from a surface and for analysis of intact biomolecules are also available using MS. Liquid extraction surface analysis (LESA), for example, is a method of using a robotic system to extract
molecules of interest directly from a substrate, without the need for prior sample preparation (Figure 1.19). Using a conductive pipette tip, solvent is applied to a surface, creating a ‘liquid microjunction’ between the surface and the pipette\[121\]. In this microjunction, molecules are solvated from the surface and the liquid is taken back into the tip. The tip containing the micro-extract is then docked with a nano-ESI interface. Solvent containing sample is then infused into the mass spectrometer and ionised via electrospray ionisation (ESI).

Figure 1.19: Schematic showing the working principles of the Liquid Extraction Surface Analysis (LESA) technique showing solvent being applied to a surface to extract the analyte (1), tip rotation (2) and infusion, via the NanoESI source, into the mass spectrometer (3) (image used with permission from S. J. Blanksby).

Another method of surface extraction is desorption electrospray ionisation (DESI) which involves directing an electrospray towards a sample on a substrate\[87\] (Figure 1.20). This desorbs and ionises the molecules of interest from the surface allowing them to be directly analysed by a mass spectrometer. This approach facilitates the direct analysis of molecules from a substrate, potentially avoiding lengthy sample preparation prior to analysis. DESI can be achieved with high spatial resolution of
less than 200 µm\textsuperscript{[122-124]}. This may enable it to detect from and distinguish individual fungal colonies.

Figure 1.20: Schematic showing the working principles of desorption electrospray ionisation (DESI) mass spectral analysis (image used with permission from S. J. Blanksby).

A recent study used DESI-MS to spatially image the distribution of a polymer additive molecule from a COLORBOND\textsuperscript{®} Steel substrate\textsuperscript{[125]}. In this study, chemical modification of the paint additive was observed as a function of exposure time (Figure 1.21). This technique is ideal for studying the chemistry of inherently flat and non-absorbent surfaces like COLORBOND\textsuperscript{®} Steel.

Figure 1.21: A set of false colour images of test panels, showing the extracted ion intensities of the target ion at \textit{m/z} 737.5. Test panels have been exposed to artificial weathering for (from left to right) 0, 300, 600, 900 and 1500 hours\textsuperscript{[125]}.

Direct extraction methods are commonly teamed with solvent mixtures containing
water, methanol and/or chloroform. The selection of solvent(s) depends on the solubility of the target molecules and the properties of the sample substrate. In the example mentioned in the previous paragraph, for instance a 2 : 1 methanol/chloroform mixture was selected to most efficiently extract the large target molecules which consisted of a number of polar and non polar regions\textsuperscript{[125]}. Another consideration during solvent selection is the difficulty in generating higher charge states using less polar solvents\textsuperscript{[126]}.

1.8.3 **Lipid class profiling using targeted scans**

Microorganisms contain a complex range of molecules. As such, it is often difficult to distinguish and identify the lipid or lipid class of interest from a crude extract. Contaminants or high-abundance ions can swamp the spectrum, obscuring significant ions of lower abundance. Additionally, ambiguity can arise when multiple lipids have the same nominal mass. Depending on the individual structural characteristics of a molecule, once ionised, it can fragment in characteristic ways when subjected to collision induced dissociation (CID).

Using tandem mass spectrometry (MS/MS), the fragmentation patterns of ionised lipids can be exploited to observe molecules with common structural elements. This phenomenon can be exploited on triple quadrupole MS using an array of techniques\textsuperscript{[111]}. The use of precursor ion scans (PIS) (Figure 1.22 (c)) and neutral loss scans (NLS) (Figure 1.22 (d)) has been shown to be a powerful tool in targeting lipid molecules within crude biological extracts\textsuperscript{[127]}.
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Figure 1.22: Several scan types possible using a triple quadrupole MS, commonly used in lipidomic analysis, including (a) normal, (b) product ion, (c) precursor ion, (d) neutral loss or neutral gain, (e) multiple reaction monitoring full scan MS. Abbreviation: CID, collision-induced dissociation. Figure taken from Blanksby et al.\textsuperscript{111}.

The first level of lipidomic identification is lipid class\textsuperscript{128}. By pinpointing and detecting fragmentation patterns characteristic of a certain class of molecule, a spectrum can be generated which is unique to that class. This allows the lipids of each lipid class to be categorised together and allows for the easy comparison of a specific class between different organisms. Using this targeted approach, a significant reduction in chemical noise is also possible as the molecules detected by
the mass spectrometer are exclusively those with a very specific pattern of fragmentation.

As shown in Figure 1.23, CID of the positively charged lipid ion can result in the formation of a fragment which is characteristic to that class of lipid. Using a triple quadrupole mass spectrometer, this fragment can be specifically selected in order to detect only the molecules belonging to that class. For example, upon CID, ionised PC lipids fragment to produce a positive ion at \( m/z \) 184 (Figure 1.23 (a)) which can be detected in a positive ion mode PIS of \( m/z \) 184. In this experiment, the first quadrupole scans through the \( m/z \) range, allowing ions in the selected \( m/z \) range into the second quadrupole where they are fragmented via CID. The third quadrupole then transmits to the detector only the ion at \( m/z \) 184, correlating this to the \( m/z \) of ions that fragmented to produce it.

\[ \text{Figure 1.23: Collision induced dissociation (CID) of positively charged phosphatidylcholine (PC) and phosphatidylethanolamine (PE) ions, resulting in the fragments characteristic of their specific lipid class. (a) CID of a PC, resulting in the formation of a positively charged ion at } m/z 184, \text{ which is detected in a precursor ion scan (PIS) of } m/z 184. (b) CID of a PE which fragments to produce a neutral molecule of 141 Da. Ions which loose a molecule of this mass can be detected in a positive ion mode neutral loss scan (NLS) of 141 Da. R and R’ denote hydrocarbon chains of the formula } C_nH_{2n}. \]
Similarly, CID fragmentation of ionised PE results in a mass loss of 141 Da, corresponding to loss of the phosphoethanolamine headgroup (Figure 1.23 (b)). This can be detected in a positive ion mode NLS of 141 Da. Ions are fragmented via CID in the second quadrupole while the first and third quadrupoles scan through the m/z ratios, with a defined offset of a 141 Da loss.

In order to detect PE lipids and exclude background noise from the spectrum, the prerequisite that [M+H]$^+$ ions undergo a characteristic loss of a neutral molecule with mass 141 Da (i.e., a so-called neutral loss of 141 Da), can be set. An example of this is shown in Figure 1.24. High abundance ions can easily dominate the mass spectrum when all ions are transmitted to the detector, as in the conventional ESI-MS scan shown in (a). However, as stated, PE lipids experience a loss of a neutral molecule of 141 Da, as shown in the CID spectrum of the ion at m/z 704.6 (b). When only those molecules experiencing a neutral loss of 141 Da are detected, as in (c), the noise is dramatically reduced and the ions corresponding to PE lipids can be easily identified. Such scans can enable direct infusion of crude samples without arduous purification and separation procedures and, thus, such scan protocols are well suited towards the development of microbial identification strategies.
Figure 1.24: A comparison of spectra of a crude *Escherichia Coli* extract in *m/z* range 500-800. (a) shows a positive mode enhanced molecular ion scan (EMS) while (b) shows a CID spectrum of the ion at *m/z* 704.6, which experiences a loss of a neutral molecule of 141 Da. (c) shows a positive mode headgroup scan which specifically targets the phosphatidylethanolamine (PE) class of lipids (neutral loss of 141 Da in positive ion mode). The base peak of (c), *m/z* 704.6, can barely be distinguished from the noise in (a).
1.9 Thesis outline

In the past, approaches to the challenge of preventing fungal infestation of COLORBOND® Steel substrates have employed untargeted anti-microbial strategies. Such approaches have had little success in mitigating growth and have been inherently flawed by the lack of understanding of the nature of the crucial infesting species. Knowledge of the precise nature of the species will drive future studies in this area in a logical and convergent manner. The work detailed in this thesis, therefore, is firmly targeted towards characterisation and identification of the crucial organism or organisms which proliferate on COLORBOND® Steel roofs.

Chapter 2 describes methods specifically developed for identification, characterisation and quantification of microbial growth on COLORBOND® Steel substrates. It is hoped that these customised methods will ultimately result in a better understanding of the growth trends and will assist in assessing the efficacy of future mitigation strategies. Chapter 3 draws on the methods developed in Chapter 2 to explore the use of optical microscopy as a tool in characterising morphological and size changes over time in addition to characterising reactions of the infesting species to environmental factors. Chapter 4 investigates the lipid profiles of laboratory grown samples of fungal species which are similar to those infesting COLORBOND® Steel substrates and demonstrates that differentiation of fungal species is possible using lipid profiling. Chapter 5 further explores the scope for mass spectrometric lipid profiling methods to be used to chronicle the development and influences upon colonies infesting COLORBOND® Steel substrates. It builds upon the observations made in Chapter 3, using the techniques explored in Chapter 4, investigating temporal, geographical and seasonal factors and their influences on the
lipid profiles of the organisms studied. Chapter 6 brings the findings of the previous chapters together into a coherent overview of the problem at hand. Here, the commercial and scientific implications of the findings are discussed, in addition to recommendations for future work in this area.
Chapter 2: **Development and Evaluation of Methods to Detect, Identify and Quantify Fungal Proliferation on COLORBOND® Steel**
2.1. Introduction: Characterisation of microbial colonies on COLORBOND® Steel to date

2.1.1. Laboratory culturing and morphological identification

Studies of microorganisms on COLORBOND® Steel have been ongoing since the early 1980s and have already explored several analysis methods. Species identification of microbes on COLORBOND® Steel has been achieved through culturing on agar plates. An example, as applied to microbial infestation on COLORBOND® Steel roofing products in Singapore, is shown in Figure 2.1. Organisms have been identified to genus level using macroscopic and microscopic morphology [9, 12, 129]. Some of the species identified are listed in Table 2.1.
2.1.2. Microscopy

Visualization of fungal growth on COLORBOND® Steel surfaces has been attempted by BlueScope using scanning electron microscopy (SEM) imaging\[^{8, 9, 15}\]. This method was used primarily to distinguish between dirt particles and fungi. While this distinction is possible, as shown in Figure 2.2, it was found that, in many cases, the vacuum conditions required for this technique were incompatible with the biological
samples, resulting in the colonies rupturing\textsuperscript{[15]}.

Figure 2.2: SEM images of microbial colonies on COLORBOND\textsuperscript{®} Steel, produced at BlueScope Innovation, showing (a) a microbial colony (black dot) with hyphus-like extension and (b) collapsed cellular derived detritus formed after sample preparation and exposure in the high-vacuum environment of the SEM sample chamber. Dirt particles can be seen in both images\textsuperscript{[15]}.

More recently, colonies have been viewed by first slicing through their cross section
using a JEOL IB09010 Argon broad ion beam cross section polisher. Often used in metallurgy, this technique uses Ar ions to mill the surface, creating a very smooth, clear cross section, which can then be imaged using SEM, as shown in Figure 2.3 (a). The organism cross sectioned in this example appears on the panel as a round colony, which spreads from a central point, as exemplified by the optical microscope image shown in Figure 2.3 (b). This technique was used to assess the impact of the fungal growth on the underlying paint. These results suggest that the organisms are unlikely to be feeding on or encroaching upon the paint film itself.
Figure 2.3: (a) An SEM image of a panel having been on exposure at Burrawang for a period of 48 weeks. The yellow line shows the organism-paint top coat interface and the blue line, the paint top coat-primer interface. (b) An optical microscope image of a round colony originating from a central point, similar to that pictured in the cross section shown in (a).

2.1.3. DNA sequencing

2.1.3.1. Summary of microbial identification to date

Collaborations with the University of New South Wales (UNSW) have provided DNA sequencing data from several species present on COLORBOND® Steel panels.
These have been identified both by culturing and morphological analysis and, more recently, through DNA sequencing. Of the fungal species found, *Cladosporium sp.* and *Alternaria sp.* are most commonly identified and are therefore thought to be habitual infesters. These have been identified in samples from both Australia and South East Asia using both morphological and DNA sequencing techniques. The most commonly identified bacteria were of the *Bacillus* genus. Fungal and bacterial identifications are detailed in Table 2.1 and Table 2.2, respectively. For simplicity, the identifications shown are grouped by the genus level only and only those with a ≥ 99% match using a Genebank database search, as matched using BLAST[^130] (‘basic local alignment search tool’) are listed. BLAST is a search tool which uses an algorithm to compare regions of similarity in DNA sequences.
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

Table 2.1: Fungal genera identified by both culturing and morphology and DNA sequencing on samples collected from COLORBOND® Steel panels after outdoor exposure.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Report in which species was identified by culturing and morphology</th>
<th>Report in which species was identified by DNA sequencing (UNSW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paecilomyces</td>
<td>‘the tropics’† (1985)(^{[12]})</td>
<td></td>
</tr>
<tr>
<td>Cladosporium</td>
<td>Singapore and Darwin (1992)(^{[129]}) and ‘the tropics’ (1985)(^{[12]})</td>
<td>2 x Burrawang (2012)(^{[13]})</td>
</tr>
<tr>
<td>Alternaria</td>
<td>‘the tropics’ (1985)(^{[12]})</td>
<td>3 x Burrawang (2012)(^{[13]}) and several from Burrawang (2013)(^{[131]})</td>
</tr>
<tr>
<td>Pullularia</td>
<td>‘the tropics’ (1985)(^{[12]})</td>
<td></td>
</tr>
<tr>
<td>Penicillium</td>
<td>‘the tropics’ (1985)(^{[12]})</td>
<td>several from Burrawang (2013)(^{[131]})</td>
</tr>
<tr>
<td>Epicoccum</td>
<td></td>
<td>3 x Burrawang (2012)(^{[13]}) and Burrawang ‘lichen isolate’ (2012)(^{[132]})</td>
</tr>
<tr>
<td>Davidiella</td>
<td></td>
<td>two samples from Burrawang (2012)(^{[13]})</td>
</tr>
<tr>
<td>Curtobacterium</td>
<td></td>
<td>one sample from Burrawang (2012)(^{[13]})</td>
</tr>
<tr>
<td>Spirosoma</td>
<td></td>
<td>one sample from Burrawang (2012)(^{[13]})</td>
</tr>
<tr>
<td>Dothideomycetes</td>
<td></td>
<td>one sample from Burrawang (2012)(^{[13]}) and several samples from Burrawang (2013)(^{[131]})</td>
</tr>
<tr>
<td>Stagonospora</td>
<td></td>
<td>one sample from Burrawang (2012)(^{[13]})</td>
</tr>
<tr>
<td>Phaeosphaeria</td>
<td></td>
<td>one sample from Burrawang (2012)(^{[13]})</td>
</tr>
<tr>
<td>Entrophospora</td>
<td></td>
<td>one sample from Burrawang (2012)(^{[13]})</td>
</tr>
<tr>
<td>Sphingopyxis</td>
<td></td>
<td>one sample from Burrawang (2012)(^{[13]}) and Burrawang ‘lichen isolate’ (2012)(^{[132]})</td>
</tr>
</tbody>
</table>

† Most likely referring to South East Asia where BlueScope (then Lysaght) had operations at that time\(^{[7]}\).
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

<table>
<thead>
<tr>
<th>Fungal Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptococcus</td>
<td>one sample from Burrawang (2012)¹³¹</td>
</tr>
<tr>
<td>Cellulomonas</td>
<td>one sample from Borneo¹³³</td>
</tr>
<tr>
<td>Cladiorina</td>
<td>several samples from Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Aureobasidium</td>
<td>several samples from Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Opuntia</td>
<td>several samples from Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Sordaria</td>
<td>several samples from Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Lasiosphaeris</td>
<td>several samples from Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Cochliobolus</td>
<td>several samples from Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Phoma</td>
<td>several samples from Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Chaetothyriales</td>
<td>several samples from Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Melanops</td>
<td>Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Paraphaeosphaeria</td>
<td>Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Pleosporales</td>
<td>Burrawang (2013)¹³¹ and Burrawang ‘lichen isolate’ (2012)¹³²</td>
</tr>
<tr>
<td>Preussia</td>
<td>Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Berkleasmium</td>
<td>Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Taphrina</td>
<td>Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Arthrinium</td>
<td>Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Phaeosphaeria</td>
<td>Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Shirai</td>
<td>Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>Burrawang (2013)¹³¹</td>
</tr>
<tr>
<td>Novosphingobium</td>
<td>Burrawang ‘lichen isolate’ (2012)¹³²</td>
</tr>
</tbody>
</table>
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

Table 2.2: Bacterial genera identified on COLORBOND® Steel panels after outdoor exposure.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Report in which species was identified by DNA sequencing (UNSW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Burkholderia</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Enterobacteriaceae</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Methylobacterium</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Promicromonosporaceae</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Edaphobacter</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Sporichthyaceae</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Truepera</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Haliangium</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Acidiphilium</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Micrococcus</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Hymenobacter</em></td>
<td>Burrawang (2012)[13]</td>
</tr>
<tr>
<td><em>Sphingomonas</em></td>
<td>one sample from Burrawang (2012)[13] and Burrawang ‘lichen isolate’ (2012)[132]</td>
</tr>
<tr>
<td><em>Cellulosimicrobium</em></td>
<td>one sample from Borneo[133]</td>
</tr>
</tbody>
</table>
2.1.4. Aims

Optical microscopy (BlueScope) and lipidomics-based mass spectrometry (UoW) methods were contrasted to FISH and DNA profiling techniques performed on the same specimens by colleagues at UNSW. The efficacy of these techniques was evaluated based on their ability to detect, quantify and identify organisms. Detection capability is important in ascertaining when the infestation begins. Quantification is important as a means to monitor rate of infestation based on location and environment and is also an important parameter when comparing a sample which incorporates a mitigation strategy to a control sample. Identification is important in order to tailor mitigation strategies to specific problem species.
2.2. Materials and Methods

2.2.1. Reagents

HPLC grade methanol (MeOH) and chloroform (CHCl₃) were obtained from Crown Scientific (Sydney, NSW, Australia) and used without further purification. Ammonium acetate (NH₄OAc) was obtained from Ajax Chemicals (Sydney, NSW, Australia). Isopropyl alcohol (IPA) was obtained from Thermo Fischer Scientific (Sydney, NSW, Australia).

2.2.2. Biological specimens

Specimens were obtained from the surface of COLORBOND® Steel panels placed outdoors at BlueScope exposure sites located in Burrawang (Australia) and Kapar (Malaysia).

2.2.3. COLORBOND® Steel test panels

2.2.3.1. Preparation

Three types of test panel were used in this study. The majority of test panels were made of material taken from a coil of COLORBOND® Steel in Surfmist colour produced on the production line (offline sample). The second type of sample was prepared in the laboratory from wet paint and pre-primed steel substrate and cured in a fan forced oven. The third type was offline COLORBOND® Steel in Whitehaven colour and was used exclusively in the TSR study (refer to Section 3.2.5 for preparation details). Offline samples were obtained using the coil coating process.
detailed in Figure 1.1.

All panels were top coated with Armorpol 4400 resin system in ‘Surfmist’ colour, a white, solvent-borne, polyester-based paint system, manufactured by PPG and formulated for application on a coil coating paint-line\textsuperscript{134}. The approximate composition of the cured paints are shown in Table 2.3. ‘Resin’ was approximately 83 % polyester and 17 % melamine formaldehyde crosslinking agent and ‘matting agent’ consisted of silica particles with mean particle size 8.3 – 10.2 µm. Inorganic mixed metal oxide pigments are used in all COLORBOND\textsuperscript{®} Steel paints. ‘Pigments’ used to formulate ‘Surfmist’ colour were 98 % white (TiO\textsubscript{2}), 1.5 % black pigment and 0.5 % yellow pigment. The exact identity of these pigments is PPG proprietary information.

<table>
<thead>
<tr>
<th>Paint system</th>
<th>Resin (%)</th>
<th>Matting agent (%)</th>
<th>Pigment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Surfmist’ standard gloss</td>
<td>44</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>‘Surfmist’ high gloss</td>
<td>50</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

‘Standard gloss’ panels used in Burrawang ‘Batch 1’ and ‘Batch 2’ and Kapar sample sets came from line produced ‘Surfmist’ COLORBOND\textsuperscript{®} Steel, coil number D42794, manufactured on Coil Processing Line (CPL) #8 on the 24th of October 2012. ‘Standard gloss’ panels exposed as ‘Winter 2012’ came from line produced ‘Surfmist’ COLORBOND\textsuperscript{®} Steel, coil number D24379, manufactured at CPL#8 on the 27th of October 2011.

‘High gloss’ panels (Burrawang ‘Batch 1’) were drawn down in the laboratory using
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

a #0028 drawdown bar on Code 70A panels† (as shown in Figure 2.4). Panels were cured for 57 s in a fan forced oven set at 262 °C. After 57 s the peak metal temperature is 232 °C, the specified temperature for completion of cure.

Figure 2.4: A code 70A panel (top) and a drawdown bar (bottom), similar to that used for coating ‘high gloss’ panels. These drawdown bars are used to replicate, on a laboratory scale, the application of paint on the COLORBOND® Steel production line.

Panels for exposure at Burrawang were cut to 90 × 235 mm² and those for exposure at Kapar were cut to 110 × 130 mm² using a hydraulic guillotine (Epic Industries). These dimensions were chosen to fit the standard rack sizes at each of the exposure sites.

† Pre-primed on the paint line with Primax 1200 primer.
2.2.3.2. Outdoor exposure

2.2.3.2.1. Burrawang (Australia)

Burrawang ‘Batch 1’ and ‘Batch 2’ and ‘Winter 2012’ sample sets were placed at the Burrawang exposure site on north facing ‘normal’ racks. ‘Winter 2012’ was exposed on 28/5/2012. ‘Normal’ racks consist of a series of horizontal wooden slats to which panels are attached by placing them between two rubber washers, tightened with a screw, as shown in Figure 1.3. (a) In ‘Batch 1’, 144 ‘standard gloss’ panels and 72 ‘high gloss’ panels were exposed and in ‘Batch 2’, 72 ‘standard gloss’ panels were exposed. Exposure and collection dates of Burrawang ‘Batch 1’, ‘Batch 2’ and ‘Winter 2012 are listed in Table 2.4.
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

Table 2.4: Exposure and collection dates of panels exposed at Burrawang. Burrawang ‘Batch 1’ was denoted by a ‘B-’ prefix and ‘Batch 2 by ‘B2-’. ‘Batch 2’ was exposed 56 weeks after ‘Batch 1’ and ran for 48 weeks. Extra panels were taken from the ‘Winter 2012’ set as desired. ‘Winter 2012’ was exposed on 28/5/12 (exposure date not shown)

<table>
<thead>
<tr>
<th>Week</th>
<th>Date</th>
<th>Burrawang ‘Batch 1’</th>
<th>Burrawang ‘Batch 2’</th>
<th>‘Winter 2012’</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19/11/2012</td>
<td>exposed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>17/12/2012</td>
<td>B-4W collected</td>
<td></td>
<td>‘Winter 2012 29 weeks’ collected</td>
</tr>
<tr>
<td>8</td>
<td>14/01/2013</td>
<td>B-8W collected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11/02/2013</td>
<td>B-12W collected</td>
<td></td>
<td>‘Winter 2012 37 weeks’ collected</td>
</tr>
<tr>
<td>16</td>
<td>11/03/2013</td>
<td>B-16W collected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8/04/2013</td>
<td>B-20W collected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>6/05/2013</td>
<td>B-24W collected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>3/06/2013</td>
<td>B-28W collected</td>
<td></td>
<td>‘Winter 2012 49 weeks’ collected</td>
</tr>
<tr>
<td>32</td>
<td>1/07/2013</td>
<td>B-32W collected</td>
<td></td>
<td>‘Winter 2012 53 weeks’ collected</td>
</tr>
<tr>
<td>36</td>
<td>29/07/2013</td>
<td>B-36W collected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>26/08/2013</td>
<td>B-40W collected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>23/09/2013</td>
<td>B-44W collected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>21/10/2013</td>
<td>B-48W collected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>18/11/2013</td>
<td>B-52W collected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>16/12/2013</td>
<td>B-56W collected</td>
<td></td>
<td>exposed</td>
</tr>
<tr>
<td>60</td>
<td>13/01/2014</td>
<td>B-60W collected</td>
<td>B2-4W collected</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>10/02/2014</td>
<td>B-64W collected</td>
<td>B2-8W collected</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>10/03/2014</td>
<td>B-68W collected</td>
<td>B2-12W collected</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>7/04/2014</td>
<td>B-72W collected</td>
<td>B2-16W collected</td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>5/05/2014</td>
<td>B-76W collected</td>
<td>B2-20W collected</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>2/06/2014</td>
<td>B-80W collected</td>
<td>B2-24W collected</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>30/06/2014</td>
<td>B-84W collected</td>
<td>B2-28W collected</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>28/07/2014</td>
<td>B-88W collected</td>
<td>B2-32W collected</td>
<td></td>
</tr>
<tr>
<td>92</td>
<td>25/08/2014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>22/09/2014</td>
<td>B-96W collected</td>
<td>B2-40W collected</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>20/10/2014</td>
<td>B-100W collected</td>
<td>B2-44W collected</td>
<td></td>
</tr>
<tr>
<td>104</td>
<td>17/11/2014</td>
<td>B-104W collected</td>
<td>B2-48W collected</td>
<td></td>
</tr>
</tbody>
</table>

Nine ‘Batch 1’ samples were collected every four weeks. These were numbered as shown in Table 2.5, where the number of weeks on exposure is represented by ‘_’, so
that, for example, a panel from ‘Batch 1’, having been exposed for 32 weeks, could be named, ‘B-32W-1’. Panels 1-6 in ‘Batch 1’ were ‘standard gloss’ and panels 7-9 were ‘high gloss’. Similarly, six ‘standard gloss’ ‘Batch 2’ samples were collected every four weeks and named as described in Table 2.5.

Table 2.5: The numbering system used to name Burrawang panels from ‘Batch 1’ and ‘Batch 2’ and Kapar panels. For each sample, the number of weeks on exposure is substituted for the ‘_’. Panels (from Burrawang ‘Batch 1) named in *italics* are ‘high gloss’ panels. Unitaliced panels are ‘standard gloss’.

<table>
<thead>
<tr>
<th>Burrawang ‘Batch 1’</th>
<th>Burrawang ‘Batch 2’</th>
<th>Kapar</th>
</tr>
</thead>
<tbody>
<tr>
<td>B- _W-1</td>
<td>B2- _W-1</td>
<td>M- _W-1</td>
</tr>
<tr>
<td>B- _W-3</td>
<td>B2- _W-3</td>
<td>M- _W-3</td>
</tr>
<tr>
<td>B- _W-4</td>
<td>B2- _W-4</td>
<td>M- _W-4</td>
</tr>
<tr>
<td>B- _W-5</td>
<td>B2- _W-5</td>
<td>M- _W-5</td>
</tr>
<tr>
<td>B- _W-6</td>
<td>B2- _W-6</td>
<td>M- _W-6</td>
</tr>
<tr>
<td>B- _W-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B- _W-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B- _W-9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.3.2.2. Kapar (Malaysia)

Kapar panels were placed at the Kapar exposure site on ‘normal’ racks. In this batch, 150 ‘standard gloss’ panels were exposed. Six of these were collected every four weeks for the first 56 weeks and every 8 weeks after that. The exposure ran for 144 weeks in total. Panels were named as described in Table 2.5. Exposure and collection dates of Kapar panels are described in Table 2.6.
Table 2.6: Exposure and collection dates of panels exposed at Kapar. Panels were collected every four weeks for the first 56 weeks and every 8 weeks after that. The exposure ran for 144 weeks in total.

<table>
<thead>
<tr>
<th>Week</th>
<th>Date</th>
<th>Kapar</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8/12/2012</td>
<td>exposed</td>
</tr>
<tr>
<td>4</td>
<td>5/01/2013</td>
<td>M-4W collected</td>
</tr>
<tr>
<td>8</td>
<td>2/02/2013</td>
<td>M-8W collected</td>
</tr>
<tr>
<td>12</td>
<td>2/03/2013</td>
<td>M-12W collected</td>
</tr>
<tr>
<td>16</td>
<td>30/03/2013</td>
<td>M-16W collected</td>
</tr>
<tr>
<td>20</td>
<td>27/04/2013</td>
<td>M-20W collected</td>
</tr>
<tr>
<td>24</td>
<td>25/05/2013</td>
<td>M-24W collected</td>
</tr>
<tr>
<td>28</td>
<td>22/06/2013</td>
<td>M-28W collected</td>
</tr>
<tr>
<td>32</td>
<td>20/07/2013</td>
<td>M-32W collected</td>
</tr>
<tr>
<td>36</td>
<td>17/08/2013</td>
<td>M-36W collected</td>
</tr>
<tr>
<td>40</td>
<td>14/09/2013</td>
<td>M-40W collected</td>
</tr>
<tr>
<td>44</td>
<td>12/10/2013</td>
<td>M-44W collected</td>
</tr>
<tr>
<td>48</td>
<td>9/11/2013</td>
<td>M-48W collected</td>
</tr>
<tr>
<td>52</td>
<td>7/12/2013</td>
<td>M-52W collected</td>
</tr>
<tr>
<td>56</td>
<td>4/01/2014</td>
<td>M-56W collected</td>
</tr>
<tr>
<td>60</td>
<td>1/02/2014</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>1/03/2014</td>
<td>M-64W collected</td>
</tr>
<tr>
<td>68</td>
<td>29/03/2014</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>26/04/2014</td>
<td>M-72W collected</td>
</tr>
<tr>
<td>76</td>
<td>24/05/2014</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>21/06/2014</td>
<td>M-80W collected</td>
</tr>
<tr>
<td>84</td>
<td>19/07/2014</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>16/08/2014</td>
<td>M-88W collected</td>
</tr>
<tr>
<td>92</td>
<td>13/09/2014</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>11/10/2014</td>
<td>M-96W collected</td>
</tr>
<tr>
<td>100</td>
<td>8/11/2014</td>
<td></td>
</tr>
<tr>
<td>104</td>
<td>6/12/2014</td>
<td>M-104W collected</td>
</tr>
<tr>
<td>108</td>
<td>3/01/2015</td>
<td></td>
</tr>
<tr>
<td>112</td>
<td>31/01/2015</td>
<td>M-112W collected</td>
</tr>
<tr>
<td>116</td>
<td>28/02/2015</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>28/03/2015</td>
<td>M-120W collected</td>
</tr>
<tr>
<td>124</td>
<td>25/04/2015</td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>23/05/2015</td>
<td>M-128W collected</td>
</tr>
<tr>
<td>132</td>
<td>20/06/2015</td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>18/07/2015</td>
<td>M-136W collected</td>
</tr>
<tr>
<td>140</td>
<td>15/08/2015</td>
<td></td>
</tr>
</tbody>
</table>
2.2.4. Post exposure processing

Panels were divided into segments suitable for each analysis. Circular disks of diameter 28 mm were punched from a COLORBOND® Steel panel using an EG-3 Hydraulic box punch (Kamekura Seiki Co, Japan). Rectangular segments of approximately 5 × 3 cm for LESA MS analysis were cut using a hydraulic guillotine (Epic Industries).

Live/dead staining, fluorescence in situ hybridization (FISH) and DNA sequencing were performed at the University of New South Wales (UNSW) (Sydney, Australia), mass spectrometry at the University of Wollongong (UoW) (Wollongong, Australia) and optical microscopy at BlueScope Research (Port Kembla, Wollongong, Australia). Samples were divided as shown in Figure 2.5 (Burrawang ‘Batch 1’ and ‘Batch 2’, standard gloss), Figure 2.6 (Burrawang ‘Batch 1’, high gloss) and Figure 2.7 (Kapar panels, standard gloss).
Figure 2.5: ‘Batch 1’ and ‘Batch 2’ panels (standard gloss) collected every four weeks from Burrawang showing how they were divided for different testing methods. Disks are 28 mm in diameter (shown to scale).
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Figure 2.6: ‘Batch 1’ panels (high gloss) collected every four weeks from Burrawang showing how they are divided for different testing methods. Disks are 28 mm in diameter (shown to scale).

Figure 2.7: Panels (standard gloss) collected every four weeks from Kapar (Malaysia), showing how they are divided for different testing methods. Disks are 28 mm in diameter (shown to scale).
2.2.5. **Analysis**

2.2.5.1. **Optical Microscopy**

Optical microscopy studies were performed using an OLYMPUS BX51M optical microscope with 50 ×, 100 ×, 200 × and 500 × magnification capabilities. For both Burrawang and Kapar panel sizes, templates were prepared with 0.5 × 0.5 mm² holes distributed across the surface, as seen in Figure 2.8. Templates were printed on standard white A4 paper using a HP Color LaserJet CM6040f Multifunction Printer and the holes cut out using a scalpel blade. Once on the microscope stage, the paper template was placed over the panel and two images at 50 × magnification were taken in each of the holes in the template. Burrawang templates contained 24 holes and Kapar templates, 15, to produce a total of 48 and 30 photographs, respectively. The 24-hole template, numbered A1 to C8 is shown in Figure 2.8 (a). The template shown in Figure 2.8 (b), numbered A1 to C5, was used for the smaller Kapar panels.
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Figure 2.8: A photograph of templates used to define areas in which optical microscopy images should be taken. The Burrawang template, (a), contained 24 evenly spaced holes which were numbered A1-C8. Two photographs were taken in each of these holes, amounting to a total of 48 images. The Kapar template, (b), contained 15 evenly spaced holes which were numbered A1-C5. Two photographs were taken in each of these holes, amounting to a total of 30 images.

ImageJ software (ImageJ 1.46r, USA) was used to analyse the dark area coverage of each photograph by converting images to 8-bit images, adjusting the threshold value to 150 to create a black and white image which showed the dark particles in black and the painted COLORBOND® Steel surface in white. Particles were then analysed to calculate % area coverage of black area in terms of total surface area. Data was then exported to Microsoft Excel where the average coverage and standard deviation was calculated from the 48 (Burrawang) or 30 (Kapar) areas analysed.
The bleaching procedure, was developed in-house at BlueScope\textsuperscript{[11]} and was used in this study to assess dirt coverage on Kapar panels. Panels were photographed, sprayed with household bleach and left for two minutes before gentle rinsing with tap water. Panels were placed at an angle to dry and, once dry, were photographed again. Their resultant dark area coverage was measured as described above.

2.2.5.2. Mass Spectrometry

2.2.5.2.1. Liquid extraction surface analysis (LESA) sample preparation

Rectangular segments for LESA analysis were mounted on the LESA sampling stage. Panel segments were sampled using LESA-MS NH\textsubscript{4}OAc (7.5 mM) in both MeOH:CHCl\textsubscript{3} (2:1) and in IPA: MeOH:CHCl\textsubscript{3} (4:2:1). LESA-MS spectra were also acquired using TriVersa Nanomate\textsuperscript{®} operating in surface sampling mode.

2.2.5.2.2. Surface swabbing sample preparation

To prepare microbial extracts using the ‘swabbing’ method, 2 mL of MeOH was placed in a glass vial, into which a cotton bud (pre-rinsed with MeOH) was dipped. The cotton bud was used to swab an area of approximately 25 cm\textsuperscript{2} of the panel. This process was performed in triplicate on a single panel. This solution was centrifuged and an 80 µL aliquot of the top layer was combined with 80 µL of MeOH:CHCl\textsubscript{3} (2:1) containing NH\textsubscript{4}OAc (15 mM) in a 96 well plate. This plate was placed into the Triversa NanoMate\textsuperscript{®} autosampler for MS analysis. Swabbed areas of a Burrawang and Kapar panel are shown in Figure 2.9.
Figure 2.9: Areas swabbed on (a) B-28W-8, a high gloss panel, having been on exposure at Burrawang for 28 weeks and (b) M-24W-5, a standard gloss panel, having been on exposure at Kapar for 24 weeks. (b) is darker in colour due to tropical discolouration in South East Asia.

2.2.5.2.3. Electrospray ionisation mass spectrometry

Extracts were analysed by chip-based nano ESI-MS using a Triversa NanoMate® (Advion, Ithaca, NY, USA) operating in direct infusion mode. For both LESA and infusion experiments, mass spectra were acquired using a triple quadrupole QTRAP® 5500 mass spectrometer (AB Sciex, California, USA) with Analyst® 1.5.1 control and acquisition software (AB Sciex, California, USA). In direct infusion mode, typical experimental conditions were delivery gas pressure (0.4 psi) and spray
voltage (1.15 kV) in positive ion mode.

For each sample, full scan MS, neutral loss (NL) and precursor ion scans (PIS) in positive ion mode were obtained using parameters previously reported in literature for glycerophospholipids (Table 2.7). These scans were used to detect a set of lipids incorporating a common headgroup fragment.

### Table 2.7: Precursor ion (PIS) and neutral loss (NL) scans for glycerophospholipid headgroup analysis, undertaken in positive (+) ion mode. All scan types and collision energies are taken from values reported in the literature.

<table>
<thead>
<tr>
<th>Lipid sub-class</th>
<th>Scan range (m/z)</th>
<th>Scan Type</th>
<th>Collision Energy (eV)</th>
<th>Literature Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylethanolamine (PE)</td>
<td>200-900</td>
<td>+ NL of 141 Da</td>
<td>+28</td>
<td>[82, 135]</td>
</tr>
<tr>
<td>Phosphatidylglycerol (PG)</td>
<td>200-900</td>
<td>+ NL of 189 Da</td>
<td>+22</td>
<td>[82, 135]</td>
</tr>
<tr>
<td>Phosphatidylcholine (PC)</td>
<td>200-900</td>
<td>+ PIS of m/z 184</td>
<td>+40</td>
<td>[82, 135]</td>
</tr>
<tr>
<td>Phosphatidylserine (PS)</td>
<td>200-900</td>
<td>+ NL of 185 Da</td>
<td>+22</td>
<td>[82, 135]</td>
</tr>
<tr>
<td>Phosphatidic acid (PA)</td>
<td>200-900</td>
<td>+ NL of 115 Da</td>
<td>+25</td>
<td>[82]</td>
</tr>
<tr>
<td>Phosphatidylinositol (PI)</td>
<td>200-900</td>
<td>+ NL of 277 Da</td>
<td>+22</td>
<td>[82, 135]</td>
</tr>
</tbody>
</table>

For LESA-MS experiments, typical experimental conditions were delivery gas pressure (1.0 psi) and spray voltage (1.4 kV) in positive ion mode. A total volume of 4.0 µL of solvent was used for each experiment with 2.3 µL being dispensed forming a liquid micro-junction which was held for 1 s before 2.0 µL was reaspirated into the pipette tip. This was repeated twice using the same volume of solvent to maximise analyte concentration in the aspirated solvent prior to analysis. Dispensation height and aspiration height were both set at 0.6 mm above the surface.
2.3. Results and Discussion

2.3.1. Experimental Design

COLORBOND® Steel Surfmist panels, exposed at two BlueScope exposure sites; Burrawang (Australia) and Kapar (Malaysia) formed the basis of this project. ‘Surfmist’ refers to the COLORBOND® Steel paint colour that most closely resembles plain white, as shown in Figure 2.10. It was chosen in this experiment to provide the greatest visual contrast between the dark fungi and the background substrate. The Burrawang site was selected for this work as it has historically shown a high rate of microbial infestation and is easy to access from the Wollongong area, where the majority of the experimental work was based. Kapar was selected as antimicrobial solutions are of great interest to the South East Asian sector of BlueScope and a greater understanding of the infestation in these locations is desired. Outdoor exposure commenced in November 2012.

Figure 2.10: A colour photograph of panel B-28W-6, following 28 weeks of exposure at the Burrawang site.

In this exposure set, standard gloss COLORBOND® Steel Surfmist panels were
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exposed at Kapar and both standard and high gloss panels at Burrawang. Standard gloss panels have a slightly matt appearance and are used in most COLORBOND® Steel applications, such as roofing and fencing, where paint reflectivity is not desirable. Standard gloss paint contains more silica matting agent than high gloss. Apart from differences in optical reflectance, standard gloss panels have a water contact angle of 78-80° while high gloss has a water contact angle of 125-135°.

A clean coating used to prevent tropical discolouration on COLORBOND® Steel in South East Asia uses hydrophilic technology to inhibit dirt retention. It has been suggested that this encourages fungal proliferation. Therefore, by using both high and standard gloss test panels, hydrophobicity was introduced as an additional testing parameter at the Burrawang site. Although the effect of hydrophobicity is more relevant to the South East Asian market, more space was available on exposure racks at Burrawang and experimenters had more control over panels at the Burrawang site, therefore any ‘extra’ (i.e., high gloss) panels were exposed at Burrawang, rather than Kapar.

A total of 144 standard gloss 110 × 130 mm² panels were placed on exposure racks at Kapar with 6 panels collected every 4 weeks for the first 56 weeks and every 8 weeks following that. A total of 144 standard gloss and 72 high gloss 90 × 235 mm² panels were placed on exposure racks in Burrawang in November 2012 with 6 standard gloss and 3 high gloss panels collected every 4 weeks (Burrawang ‘Batch 1’). To account for missing data from the first exposure year and to provide comparative data with the first year, another set of panels (Burrawang ‘Batch 2’) were exposed at Burrawang in December 2013, after the primary batch had been exposed for 56 weeks. This set comprised 72 standard gloss panels which were taken
from the same production run as the initial batch, of which six panels were collected every 4 weeks. These had been stored in the laboratory between November 2012 and December 2013 and were coated with Corstrip§ during this time. This protective coating ensured that no dirt or organic matter was introduced to the surface prior to outdoor exposure. The different panel sizes used reflect the ‘standard’ panel size in each location. Panels were designed to fit onto the established exposure racks, as shown in Figure 2.11.

Figure 2.11: Surfmist panels exposed on standard exposure racks at Burrawang (Australia) (a) and Kapar (Malaysia) (b). Panel dimensions reflect the ‘standard’ panel size, designed to fit on the exposure racks.

Additional samples from the ‘Winter 2012’ batch of samples were also obtained from the Burrawang exposure site. These panels were taken from a batch of COLORBOND® Steel Surfmist panels exposed in June 2012.

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§ Corstrip is a plastic coating which is applied to COLORBOND® Steel post-painting. This layer acts as a protective coating which prevents the paint from being scratched or abraded when COLORBOND® Steel is subsequently roll formed or installed in a building application. It is generally removed as part of the installation process.
2.3.2. Optical Microscopy

2.3.2.1. Detection

Detection of microbiological matter was achieved by examining the surface of the outdoor exposed sample using a metallurgical microscope. These were compared to unexposed control samples, which are shown at different magnifications in Figure 2.12. Control samples were obtained from the same coil as the panels exposed outdoors and were covered in a protective plastic coating (Corstrip), which was removed immediately before the images were taken, ensuring that all visible features were encased in and formed part of the paint itself.

Under the microscopy conditions used, small black dots are visible on the control panels at magnifications of 100 ×, 200 × and 500 × (Figure 2.12 (b), (c) and (d), respectively). These are black pigment particles and appear to be 2 µm² in size, as measured from the image shown in Figure 2.12 (d), using ImageJ software. The ‘Surfmist’ paint used in this study contains 1 % black, 0.5 % yellow and 98.5 % white pigments. Of these, black and yellow pigment particles with an approximate diameter of 2 µm are visible, as evident in Figure 2.12 (d) and (c) and to some extent in Figure 2.12 (b). White pigment particles, at approximately 200 nm in diameter, cannot be seen at this magnification. Note that the microscope light was turned down to make the black dots more visible in the images shown in Figure 2.12. For this reason, they appear a slightly different colour than the majority of microscope images shown which are taken with the maximum possible light intensity in order to highlight organisms on the surface.
Figure 2.12: Photomicroscope images of Surfmist control substrate at (a) 50 ×, (b) 100 ×, (c) 200 × and (d) 500 × magnification, showing the topography and particles typical of the paint coating. Black and yellow pigment particles, having an approximate diameter of 2 µm are visible at (d) 500 × magnification and to a lesser extent at (c) 200 × magnification.

Detection of pigmented organisms was possible at 50 × magnification (the lowest available) after as little as four weeks of outdoor exposure at both the Burrawang and Kapar exposure sites. Figure 2.13 shows a panel from the Burrawang exposure site, at 50 × magnification, which had been exposed for four weeks. Dark colonies (as circled in red) are discernable from the light surface but little can be noted about the morphologies of individual colonies. Several colonies are barely visible, due to their small size.
Figure 2.13: A panel which had been exposed for 4 weeks at the Burrawang exposure site, photographed at 50 × magnification. At this magnification, dark colonies (as circled in red) are visible on the light surface but the morphology of the growth is difficult to discern.

Closer inspection at magnifications of 100 ×, 200 × and 500 × of these four week old colonies confirmed that they had morphology indicative of fungal spores and are readily differentiated from visible pigment particles and other features of the COLORBOND® Steel substrate. Figure 2.14 shows two photographs from a standard gloss panel after four weeks exposure at Burrawang. These were taken at 200 × and 500 × magnification and clearly show a fungal spore (circled in red in Figure 2.14 (b)) on the COLORBOND® Steel substrate.
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Figure 2.14: Optical microscopy images of microorganism(s) (as circled in red) on a COLORBOND® Steel test panel which had been exposed a Burrawang for four weeks with (a) showing the organism at 200 × magnification and (b) the blue outlined section at 500 × magnification.

Figure 2.15 shows two photographs from a standard gloss panel exposed for four weeks at Kapar. Similar to the panel from Burrawang, after four weeks, fungal spores can be seen on the surface. One such example is circled in red in Figure 2.15 (b)). In contrast to the Burrawang panel, the Kapar panel was considerably darker. Previous studies of darkening in outdoor conditions have been conducted by BlueScope Research on white COLORBOND® Steel substrates. These suggest that ΔL (or change in lightness) was reduced by approximately 0.5 units after one month, and 1 unit after four months [137]. In contrast, in Australia, ΔL was reduced approximately 0.2 units after one month, and 0.7 units after four months [137].

When in clumps on the surface, as circled in green in the bottom left of Figure 2.15 (a), the appearance of dirt is different to the fungal spores, helping to morphologically distinguish them. However, the additional dark areas and the overall darkening of the panel make the fungal spores harder to detect by visual techniques.
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Figure 2.15: Optical microscopy images of microorganism(s) (as circled in red) on a COLORBOND® Steel test panel which had been exposed at Kapar for four weeks with (a) showing the organism and dirt (circled in green) at 200 × magnification and (b) the blue outlined section at 500 × magnification.

2.3.3. Quantification

Past work performed by BlueScope Research has suggested that rates of infestation vary between Burrawang (Australia) and South East Asia\textsuperscript{15, 138}. Some studies indicate that tropical sites, such as Darwin (Australia) and Singapore show similar levels of fungal growth\textsuperscript{139}, although other studies have suggested that infestation of Clean COLORBOND® Steel was three times faster in Darwin than in Singapore\textsuperscript{11}.

At Burrawang, a rural exposure site which experiences seasonal temperature variation, infestation is thought to accelerate in the warmer months while Kapar, as a tropical location, is thought to undergo minimal seasonal variation in growth rate. One study has noted a marked acceleration in biological infestation after three years of exposure in the South East Asian environment\textsuperscript{11}.

A means of comparing the rates of growth in separate locations is required to enable future work focusing on geographic and, hence, environmental influences on
microbial infestation. In addition, quantification is required to understand the efficacy of mitigation strategies incorporated into the COLORBOND® Steel product on rates of microbial infestation.

Panels were collected every four weeks from both the Burrawang and Kapar exposure sites and sent to BlueScope Research (Port Kembla, Australia) for analysis. Due to geographical distance, the time taken in transit and the time sensitive nature of the measurements, it was not feasible that panels from Kapar be returned to the exposure site after analysis to be used for future testing. Hence, it was necessary for all quantification to be performed on separate, albeit identical, panels. For consistency, this method was also applied to the Burrawang testing regime.

A template was developed to ensure that optical microscope images were evenly distributed across the panel, in order to better represent the entire panel. For examination of the Burrawang samples a template with 24 sampling holes was developed. The templates are shown in Figure 2.9. Two images were taken in each of the labelled areas. This amounted to 48 images of the Burrawang panels and 30 images of the Kapar panels, equivalent to 0.52 % and 0.53 % of the total panel area respectively, when photographed at 50 × magnification. Table 2.8 summarises the area photographed at 50 ×, 100 × and 200 × magnification and the percentage area of the panel represented by these images.
Table 2.8: The % of panel area photographed under magnifications of 50×, 100× and 200×. Calculations assume 48 separate photographs are used for Burrawang samples and 30 for Kapar samples, as per the method described.

<table>
<thead>
<tr>
<th>Magnification</th>
<th>50×</th>
<th>100×</th>
<th>200×</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area of each photograph as measured by ImageJ (mm²)</td>
<td>2.30</td>
<td>0.575</td>
<td>0.144</td>
</tr>
<tr>
<td>Representing % of Burrawang panels†</td>
<td>0.52</td>
<td>0.13</td>
<td>0.033</td>
</tr>
<tr>
<td>Representing % of Kapar panels‡†</td>
<td>0.53</td>
<td>0.13</td>
<td>0.033</td>
</tr>
</tbody>
</table>

A magnification of 50× was ultimately chosen for several reasons. First, pigment particles are not visible at this magnification and, thus, do not interfere with the measurement. Secondly, a magnification of 50× covers four times as much area as a 100× magnification and 16 times more than a 200× magnification. 50× was the lowest magnification possible on the metallurgical microscope used.

Figure 2.16 shows the percentage of area covered by dark growth on nine individual panels exposed at Burrawang for between 28 and 48 weeks. Both standard and high gloss panels and their calculated surface area coverage, using images taken at both 50× and 100× magnification are shown.

The magnification used did not appear to negatively or positively bias the calculated area coverage. As seen in Figure 2.16, area coverage for the nine panels shown was higher when viewed at 50× magnification for four of the panels and higher when viewed at 100× magnification for five of the panels.

†† Burrawang panels have an area of 90 mm × 235 mm, totaling 21150 mm².

‡‡ Kapar panels have an area of 110 mm × 130 mm, totalling 14300 mm².
In most cases, the analyses at 50 × and 100 × magnification agreed within the standard deviation, the only exception being panel B-28W-6. Standard deviation was lower for calculations performed at 50 × than 100 × magnification. Of the nine panels at 50 × magnification, shown in Figure 2.16, only one had a standard deviation greater than half the total value. Conversely, standard deviation for the 100 × panels was always greater than half of the total value and, in one case, greater than the total value (B-32W-9). Given that the results obtained from the 100 × magnification account for a quarter of the area that the 50 × results do, it is not surprising that the latter would have a lower proportional standard deviation.

Figure 2.16: Area coverage (error bars show standard deviation) for Burrawang panels at 50 × and 100 × magnification. Selected panels had been exposed at Burrawang for between 28 and 48 weeks and included both standard (denoted by a suffix of 4 or 6) and high gloss panels (denoted by a suffix of 7 or 9).
Overall, there is an upward trend in area coverage evident in both 50 × and 100 × magnification data sets. Although the standard deviations calculated were high, it is worth noting that this reflects the actual high variation in coverage that is present on all panels. Figure 2.17 compares the photographs calculated as having highest (Figure 2.17 (a) and (c)) and lowest (Figure 2.17 (b) and (d)) area coverages on the single panel B-32W-6. Figure 2.17 (a) and (b) are taken at 50 × magnification and (c) and (d) at 100 × magnification.

Figure 2.17: A comparison of photographs used to calculate area coverages within a single panel, B-32W-6, demonstrating the variation in area coverage found across single panels and hence high standard deviation. (a) and (b) show panel B-32W-6 at 50 × magnification. (a) shows the maximum area coverage of 2.5 % while (b) shows the minimum calculated coverage of 0.4 %. (c) and (d) show panel B-32W-6 at 100 × magnification. (c) shows the maximum area coverage of 1.4 % while (b) shows the minimum coverage of 0.2 %.
Following image collection, ImageJ[140] software was used to calculate the ‘dark’ area of the photograph. Figure 2.18 shows the stages of data analysis using ImageJ where (a) shows the optical microscope image prior to processing and (b), the image after the darker microbial colonies have been converted to black pixels. The percentage area of black pixels was then calculated.

Figure 2.18: (a) Optical microscope image of an area of a test panel from the Burrawang exposure site. (b) The same image after processing in ImageJ. The areas of black and white are subsequently used to determine the percentage of the surface exhibiting fungal growth, in this case, 5.0%.

Quantification via optical microscopy also sought to measure the difference between ‘tropical discoloration’, a darkening phenomenon observed primarily in South East Asia and caused by dirt deposition, and surface darkening caused by microbial growth. The surface was exposed to household bleach, which has been found to lighten only the fungal growth and have no noticeable effect on the dirt[11].

As discussed in Section 2.3.2.1, dark areas on panels exposed at the Burrawang site are almost exclusively due to biological infestation, as seen in Figure 2.14. Dark areas on the panels from Kapar, however, show darkening due to both dirt deposition and colonisation by microorganisms, as seen in Figure 2.15. Most of the dark regions
on the surface of Burrawang panels, therefore, can be lightened to the point where they are no longer visible through the application of household bleach. The efficacy of this process when applied to Burrawang samples is shown in Figure 2.19 (a) and (b), where (a) shows heavy black infestation and (b), surface coverage after bleach application and a light water rinse. In this case, the total dark area coverage has been reduced by 98 % from 11.8 % to 0.2 %. Note that all photographs in Figure 2.19 have been taken at 100 × magnification so as to more clearly illustrate the visible changes to the substrate.

In contrast, a greater percentage of dark regions on panels that have been exposed at the Kapar exposure site are retained after bleaching. Figure 2.19 (c) and (d) show a Kapar panel pre- and post-bleaching, respectively. Hence, the first area measurement will provide an indication of the percentage of surface discoloration arising from inorganic material and yield the total percentage area coverage from both sources (Figure 2.19 (c)). The post-bleach measurement will provide an indication of the percentage of surface discoloration arising from inorganic material (Figure 2.19 (d)), allowing the microorganism percentage area coverage to be established by subtracting the influence of the dirt from the total. The percentage area coverage of panels prior to exposure, as calculated by Image J, using the method described in Section 2.2.5.1, was < 0.001 %. Example calculations for the areas shown in Figure 2.19 are detailed in Table 2.9.
Figure 2.19: Optical microscope images at 100 × magnification of panels exposed at the Burrawang exposure site (a-b) and at the Kapar exposure site (c-d) after >100 weeks of exposure. Images (a) and (c) have not been treated whilst (b) and (d) have been bleached. The Burrawang sample (a-b), in which organic growth constitutes most of the dark surface coverage, shows almost complete removal while the Kapar sample (c-d) shows less than half of the dark regions to be removed by bleaching.
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Table 2.9: Calculated total, dirt and microbial area coverages for the two areas shown in Figure 2.19.

<table>
<thead>
<tr>
<th>Area</th>
<th>Total area coverage (%)</th>
<th>Dirt area coverage (%) (after bleaching)</th>
<th>Microbial area coverage (%) (total coverage – dirt coverage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burrawang</td>
<td>11.8</td>
<td>0.2</td>
<td>11.6</td>
</tr>
<tr>
<td>Kapar</td>
<td>8.3</td>
<td>0.7</td>
<td>7.6</td>
</tr>
</tbody>
</table>

In order to assess both the dirt and biological coverage on Kapar samples, optical microscopy was used to photograph the panel before and after bleach treatment. Due to the negligible contribution of airborne dirt at the Burrawang site, Burrawang panels did not undergo the bleaching procedure. The area coverage quantification methods for both sites are summarized in the flowchart shown in Figure 2.20.
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Figure 2.20: Flowchart summarising the process undergone to calculate biological coverage on Burrawang panels (left) and both dirt and biological coverage on Kapar panels (right).
On Kapar panels, dirt particles result in a higher area coverage being detected at 100 × than at 50 × magnification. Figure 2.21 demonstrates the incompatibilities between comparing photographs at 50 × and 100 × magnifications. The image in Figure 2.21 (a) is taken at 50 × magnification and ImageJ processing resulted in calculated an area coverage of 1.1 % (Figure 2.21 (b)). This same area, photographed at 100 × magnification is shown in Figure 2.21 (c). Through the same process, area coverage is calculated to be 2.8 % (Figure 2.21 (d)). This result is not surprising, given the visibly higher percentage of darkening in the center of the area shown (i.e. the area zoomed in on in Figure 2.21 (c)). However, if the area represented in Figure 2.21 (c) is photographed at 50 × magnification, as shown in Figure 2.21 (e), the area coverage is instead calculated to be 1.7 % (Figure 2.21 (f)). If 50 × and 100 × magnification resulted in detection of the same particles, Figure 2.21 (d) and Figure 2.21 (f) should have the same percentage area coverage. However, 100 × magnification results in greater surface coverage calculation. For this reason, and to enable comparison with the Burrawang samples, 50 × magnification was used for all Kapar panels both pre- and post-bleach treatment. However, the ‘total’ dirt on Kapar panels will be underestimated given that dirt particles under approximately 2 µm² cannot be detected at 50 × magnification.
Figure 2.21: Comparisons of surface coverage calculated using optical microscope images taken at different magnifications are not suitable for comparison. Images of a Kapar panel which has been on outdoor exposure for 24 weeks. (a) shows an area at 50 × magnification. The area outlined in blue is then shown at 100 × magnification in (c). (e) shows the area from (c) taken at 50 × magnification. (b), (d) and (f) show the resulting image and calculated surface coverage after ImageJ analysis, corresponding to (a), (c) and (e) respectively. The areas shown in (c) and (e) are identical, however, due to differences in the resolution, resulting from the magnification used, the surface coverages calculated in (d) and (e) differ.
2.3.4. **Identification**

The magnification used for identification varied, depending on the target organism. In images taken at 500 × magnification (Figure 2.22), it was possible to observe the organism’s cellular structure, as seen in Figure 2.22 (a). However, at this magnification, images were often blurry, particularly when the growth extended vertically from the surface. Improving the resolution by using composite images often resulted in poor quality, grainy images, as shown in Figure 2.22 (b).

Larger organisms were therefore often photographed at 200 × magnification (Figure 2.23). This was advantageous in cases where there were possible interactions of colonies, as in Figure 2.23 (a). Figure 2.23 (b) shows the same organism shown in Figure 2.22 (a) at lower (200 ×) magnification. Cellular structure is less evident at 200 × magnification.

![Figure 2.22: Optical microscope images taken at 500 × magnification.](image)
Figure 2.23: Optical microscope images taken at 200 × magnification.

All photographs were examined for distinct microbial morphologies and organisms were morphologically categorised. Table 2.10 and Table 2.11 list the morphologies observed on panels from the Burrawang and Kapar sites, respectively. A possible identification and the panels upon which this morphology was observed are also listed.
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Table 2.10: An illustrative list of the morphologies observed on panels exposed at Burrawang for $\leq 44$ weeks. * in the second column indicates the panel from which the image shown was taken.

<table>
<thead>
<tr>
<th>Organism (optical microscope image)</th>
<th>Panels upon which observed</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td>B-4W-4* B-4W-9 B-16W-5 B-16W-7 B-32W-9 B-36W-9 B-44W-8</td>
<td>Beaked shape and size suggests spore of <em>Alternaria sp.</em></td>
</tr>
<tr>
<td><img src="image2.png" alt="Image" /></td>
<td>B-4W-6 B-4W-9*</td>
<td>Shape and growth pattern similar to <em>Aureobasidium sp.</em></td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td>B-4W-9* B-16W-5 B-32W-5</td>
<td>Textured surface, shape and size suggests <em>Epicoccum sp.</em> spore, generating germ tubes.</td>
</tr>
</tbody>
</table>
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

<table>
<thead>
<tr>
<th>Image</th>
<th>Description</th>
<th>Identification</th>
</tr>
</thead>
</table>
| ![Image](image1.png) 50 µm | Fusarium sp. | B-4W-6*  
B-32W-5  
B-44W-5 |
| ![Image](image2.png) 50 µm | Not identified. | B-12W-5*  
B-12W-6 |
| ![Image](image3.png) 100 µm | Not identified. | B-16W-7  
B-28W-9* |
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| B-20W-4 | A mature colony of *Epicoccum nigrum*. Colonies of this morphology are adherent and prolific on test panels exposed at Burrawang. The growth of individual colonies of this type are presented in Section 3.3.2.3.2. |
| B-20W-8 |
| B-24W-5 |
| B-24W-9 |
| B-28W-9* |
| B-32W-5 |
| B-32W-9 |
| B-36W-9 |
| B-40W-5 |
| B-40W-9 |
| B-44W-5 |
| B-44W-8 |

| B-28W-9* |
| B-36W-9 |
| B-40W-5 |

Unpigmented *Cladosporium sp.*
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

<table>
<thead>
<tr>
<th>Image 1</th>
<th>Image 2</th>
<th>Image 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-28W-9*</td>
<td>Not identified.</td>
</tr>
<tr>
<td>B-16W-7</td>
<td>A mature colony of <em>Aureobasidium</em> sp.</td>
</tr>
<tr>
<td>B-24W-5</td>
<td></td>
</tr>
<tr>
<td>B-28W-9*</td>
<td></td>
</tr>
<tr>
<td>B-32W-9</td>
<td></td>
</tr>
<tr>
<td>B-40W-5</td>
<td></td>
</tr>
<tr>
<td>B-40W-9</td>
<td></td>
</tr>
<tr>
<td>B-44W-5</td>
<td></td>
</tr>
<tr>
<td>B-16W-5</td>
<td>Cladosporium sp. spore.</td>
</tr>
<tr>
<td>B-20W-4*</td>
<td></td>
</tr>
<tr>
<td>B-44W-5</td>
<td></td>
</tr>
<tr>
<td>B-44W-8</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

<table>
<thead>
<tr>
<th>Image</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image 1" /></td>
<td>B-20W-4 &lt;br&gt; Spore of <em>Stemphilium</em> sp.</td>
</tr>
<tr>
<td><img src="image2.png" alt="Image 2" /></td>
<td>B-24W-5 &lt;br&gt;B-32W-5* &lt;br&gt;B-32W-9 &lt;br&gt;B-44W-5 &lt;br&gt;B-44W-8 &lt;br&gt;Not identified.</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image 3" /></td>
<td>B-36W-9* &lt;br&gt;Not identified.</td>
</tr>
<tr>
<td><img src="image4.png" alt="Image 4" /></td>
<td>B-24W-9* &lt;br&gt;B-40W-9 &lt;br&gt;Not identified.</td>
</tr>
</tbody>
</table>
Table 2.11: An illustrative list of the morphologies observed on panels exposed at Kapar for ≤ 40 weeks. * in the second column indicates the panel from which the image shown was taken.

<table>
<thead>
<tr>
<th>Organism (optical microscope image)</th>
<th>Panels upon which observed</th>
<th>Possible identity</th>
</tr>
</thead>
</table>
| ![Image 1](image1.png) | M-4W-6*  
M-8W-6  
M-12W-6  
M-16W-6  
M-20W-6  
M-32W-5 | Not identified. |
| ![Image 2](image2.png) | M-4W-6* | e |
| ![Image 3](image3.png) | M-4W-6*  
M-12W-6  
M-16W-6  
M-36W-5  
M-40W-5 | Not identified. |
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

<table>
<thead>
<tr>
<th>Sample</th>
<th>Identification</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-4W-6*</td>
<td>Not identified.</td>
<td></td>
</tr>
<tr>
<td>M-8W-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-40W-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-4W-6*</td>
<td>Not identified.</td>
<td></td>
</tr>
<tr>
<td>M-8W-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-12W-6</td>
<td>Not identified.</td>
<td></td>
</tr>
<tr>
<td>M-8W-6*</td>
<td>Not identified.</td>
<td></td>
</tr>
</tbody>
</table>

* Asterisk denotes the presence of fungal proliferation.
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

<table>
<thead>
<tr>
<th>M-8W-6</th>
<th>Not identified.</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-12W-6*</td>
<td></td>
</tr>
<tr>
<td>M-20W-6</td>
<td></td>
</tr>
<tr>
<td>M-24W-6</td>
<td></td>
</tr>
<tr>
<td>M-32W-5</td>
<td></td>
</tr>
<tr>
<td>M-40W-5</td>
<td></td>
</tr>
</tbody>
</table>

| M-24W-6         | A mature colony of *Epicoccum*    |
| M-36W-5         | *nigrum*, similar to that shown in |
| M-40W-5*        | Table 2.10.                       |
Chapter 2: Development and evaluation of methods to detect, identify and quantify fungal proliferation on COLORBOND® Steel

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-24W-6* M-40W-5</td>
<td>A hyphal network resembling <em>Aureobasidium</em> sp.</td>
</tr>
<tr>
<td>M-40W-5*</td>
<td>Not identified.</td>
</tr>
</tbody>
</table>
2.4. Mass Spectrometry

2.4.1. Lipid extraction

ESI-MS/MS of lipid extracts from COLORBOND® Steel panels was performed in positive ion mode. Based on the findings of previous research\(^{141}\), targeted scans of the GPL classes: PC, PE, PG, PS, PA and PI were undertaken.

MS/MS lipid headgroup scans were used to categorise lipids based on their specific lipid class (listed above). These scans exploit the tendency of lipids to dissociate under collision induced dissociation (CID) into class specific fragments\(^{111}\). The complex biological extracts used in this study are not chromatographically separated and, hence, it is important to separate lipid classes using MS so that comparisons can be made between samples.

Extracts were obtained using both LESA and by ‘swabbing’ the substrate with a methanol soaked cotton bud. Assessment of the ‘swabbing’ method was of interest given that, unlike LESA, it can be used to sample from surfaces without the need to physically remove and transport them to the laboratory. Such an approach is preferred when dealing with samples in the field which may be part of a customer’s COLORBOND® Steel fence or roof.

LESA, an automated sampling method, uses a capillary to apply solvent to surface, solvating molecules on the surface before taking solvent back into the capillary. Solvent, containing sample, is then infused into the mass spectrometer in the same way as direct infusion. Solvent volume and solvent dispensation height of the LESA setup was optimised.
In the ‘swabbing’ method, 25 cm\(^2\) areas of outdoor exposed COLORBOND\(^\circ\) Steel were rubbed vigorously using a MeOH rinsed cotton bud and 2 mL of MeOH. The cotton bud and the MeOH were stored in a glass jar until an aliquot was introduced into the mass spectrometer. Figure 2.24 shows images taken at 100 × magnification that highlights the extent to which this method removes material from the surface. Burrawang panels (Figure 2.24 (a – d)) show efficient removal, however some dark matter remains on the Kapar panel after ‘swabbing’ (Figure 2.24 (f)).
Figure 2.24: Panel B-36W-4, a standard gloss Burrawang panel (a) before and (b) after swabbing, panel B-36W-8, a high gloss Burrawang panel (a) before and (b) after swabbing and panel M-32W-5, a standard gloss Kapar panel (a) before and (b) after swabbing with a methanol soaked cotton bud.
2.4.2. Detection

2.4.2.1. Liquid extraction surface analysis (LESA)

Extractions from unexposed control panels were performed using both (2:1) (MeOH:CHCl₃) and (4:2:1) (IPA:MeOH:CHCl₃) solvent systems. Both solvent systems contained NH₄OAc at a final concentration of 7.5 µM. PC lipid headgroup scans (PIS of m/z 184 in positive ion mode) were performed on these extracts. The PC lipid class was chosen as it is abundant in many varieties of microorganism and is easily ionised, and thus, detected with relatively high sensitivity. It was confirmed that no material was extracted from the panel, which would interfere with lipid peaks in the m/z 700 – 800 range. The spectrum obtained from an unexposed control panel is shown in Figure 2.25 (a).

It was possible to detect PC lipids from the surface of the exposed panels after as little as four weeks of exposure. However, detection was more difficult in the less exposed samples as the incidence of microbial colonies was less frequent on the surface. Therefore, in many of the sampled areas, lipid signal could not be observed. When a characteristic peak was observed, the signal to noise ratio (S/N) was typically low.

Numerous LESA extractions from four week old samples produced no lipid signal. The scan shown in Figure 2.25 (b) represents the highest S/N obtained from a standard gloss panel exposed at Burrawang for four weeks (B-4W). The PC lipid fingerprint of microorganisms on COLORBOND® Steel has been previously investigated[142] and a peak at m/z 782.5 has been found to be the base peak with m/z 780.6 and 784.6 also present in most spectra. There are several peaks in the 580 –
680 Da range that do not correspond to known PC lipids and may represent chemical noise in these analyses. LESA is a direct analysis technique and it is, thus, expected that S/N be low compared to other techniques. After twelve weeks of outdoor exposure, the S/N was higher (Figure 2.25 (c)) and after 24 weeks the S/N is higher again (Figure 2.25 (d)) with the base peak at \( m/z \) 782.5 more intense relative to chemical noise in the \( m/z \) 400 – 700 region. Although the indicative peak at \( m/z \) 782.5 of the PC headgroup scan is detectable after four weeks of exposure, the S/N of the spectra improves significantly as exposure time increases although time exposed does not correlate to the ion abundance observed. Additionally, a ‘Winter 2012’ panel, having been exposed at Burrawang for 29 weeks was analysed at the same time as B-4W. The resultant scan, (Figure 2.25 (e)), had a higher S/N again. In this spectrum, a peak at \( m/z \) 758.6 can also be distinguished. This is also a characteristic feature of the PC lipid fingerprint of COLORBOND® Steel microorganisms\(^{[142]}\), corresponding to PC(34:2).
Figure 2.25: Precursor ion scans for m/z 184, specific to the PC lipid subclass were obtained by direct analysis of panels using the LESA technique. (a) an unexposed panel (b) a standard
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on COLORBOND® Steel

gloss panel exposed for four weeks at Burrawang, (c) a standard gloss panel exposed for
twelve weeks at Burrawang (d) a standard gloss panel exposed for 24 weeks at Burrawang
and (e) a ‘Winter 2012’ panel, exposed for 29 weeks at Burrawang and analysed on the same
day as (b). All spectra shown are those with the highest S/N obtainable from the sample.

2.4.2.2. ‘Swabbing’ extraction method

The spectrum obtained from an unexposed control panel, sampled using the
‘swabbing’ method is shown in Figure 2.26 (a). The swabbing method was
developed in May 2013 and therefore, the earliest data for Burrawang ‘Batch 1’
standard gloss and high gloss and Kapar came after 24 (Burrawang) and 20 (Kapar)
weeks. 24 weeks ‘swabbing’ (Figure 2.26 (b)), when compared to 24 weeks LESA
(Figure 2.26 (c)) has higher S/N which may be attributed to the higher panel surface
area sampled using the ‘swabbing’ method.
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2.4.3. Quantification

The suitability of both LESA and ‘swabbing’ extraction techniques combined with ESI-MS/MS to quantify changes in the amount of microbial material on the painted steel surface was assessed. Internal standards were not utilized because it is not possible to gauge the extraction efficiency of the LESA or swabbing techniques without knowing accurately the amount of material on the surface initially. The

Figure 2.26: Precursor ion scans for m/z 184, specific to the PC lipid subclass were obtained (a) an unexposed panel, extracted using the ‘swabbing’ technique (b) a standard gloss panel (‘Batch 1’) exposed for 24 weeks at Burrawang extracted using the ‘swabbing’ method and (c) the same panel extracted using the LESA technique.
ability of the LESA technique to gauge the quantity of microbial growth was assessed. In PC spectra of COLORBOND® Steel panels, the base peak is typically at $m/z$ 782.5. Hence, this peak was chosen to compare the signal intensity obtained from various panels at several locations.

2.4.3.1. LESA method

Both (2:1) (MeOH:CHCl$_3$) + 7.5 µM NH$_4$OAc and (4:2:1) (IPA:MeOH:CHCl$_3$) + 7.5 µM NH$_4$OAc solvent systems were trialled as extraction/infusion solvents for LESA and both are shown in Figure 2.27. Using both solvents, the absolute abundance of this ion was variable between samples. The average ion abundance of the $m/z$ 782.5 peak over exposure time is graphed in Figure 2.27. Spectra represented in this graph are obtained from Burrawang ‘Batch 1’ standard gloss panels. For the (MeOH:CHCl$_3$) solvent system, maximum ion abundance was approximately $2 \times 10^4$ at week four and week twenty with the majority of spectra having ion intensities below $5 \times 10^3$. Similarly, for the (IPA:MeOH:CHCl$_3$) solvent system, which was investigated from week twenty onwards, the maximum ion abundance was $1.5 \times 10^4$, however, the majority of spectra had $m/z$ 782.5 ion intensities below $5 \times 10^3$. 
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Figure 2.27: Duration of outdoor exposure compared to average $m/z$ 782.5 ion abundance for standard gloss panels exposed at Burrawang as part of ‘Batch 1’. Extracts obtained using LESA are shown. Error bars represent standard deviation for $n$ between 2 and 6.

Figure 2.28: Duration of outdoor exposure compared to average $m/z$ 782.5 ion abundance for standard gloss panels exposed at Kapar. Extracts obtained using LESA are shown. Error bars represent standard deviation for $n$ between 4 and 5.

Based on $m/z$ 782.5 ion abundance, variation between measurements is large and no trends are observed over time. This is in contrast to the whole-panel microscopy data.
(shown in Figure 2.16 and Figure A1 in the Appendix) that indicates a small increase in infestation over time. The high variation in this method may be attributed to several factors, for instance, instrumental variation and the area covered by the solvent. Area coverage varies based on the surface topography and on solvent polarity which is in turn influenced by changes in solvent composition (due to evaporation). Additionally, as discussed in Section 2.3.3, there is substantial variation in levels of microbial colonization across the panel. Using LESA, the area extracted is approximately 7 mm$^2$, approximately three times the area covered in the images taken at 50 ×, detailed in Section 2.3.3. It has been shown that, even on panels less than a year old, the area coverage at these areas can vary from almost nothing to up to 7 %. Future research could involve using internal standards to correct for these parameters but there are significant challenges in developing this for direct analysis methods.

2.4.3.2. ‘Swabbing’ extraction method

When judged by the same parameters, the ‘swabbing’ method, too, displayed highly variable ion intensities, although the area sampled was 12500 mm$^2$. This is 1800 times larger than that employed by the LESA method indicating that variability is not due to lack of surface uniformity. Indeed, ion abundance in spectra obtained via both the LESA and ‘swabbing’ methods can be more appropriately correlated to cleanliness of the instrument when the spectra was obtained. Given that internally cleaning a mass spectrometer is a complicated, delicate and time consuming process, 

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‡‡ On unweathered panels, solvent droplets typically spread to a diameter of 3 mm. hence, $\pi r^2 = \pi \times 1.5^2 = 7 \text{ mm}^2$. 

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this was performed every few months as required. Again, ion abundance is unsuitable as a quantitative technique.

Figure 2.29: Duration of outdoor exposure compared to average m/z 782.5 ion abundance for standard gloss panels exposed at Burrawang and Kapar, respectively, and for high gloss panels exposed at Burrawang. Extracts obtained using the ‘swabbing’ method from three separate 25 cm$^2$ areas on the same panel. Error bars represent standard deviation, n = 3 replicate panels.

2.4.4. Identification

Numerous studies have derived key differences in the lipidome of bacteria and fungi as well as between various species of fungi. Identification may be achieved by comparing data from organisms that have grown on outdoor COLORBOND® Steel to known lipid profiles. An advantage of LESA in this application is its ability to sample from smaller areas on the panel and, therefore, detect biological diversity across the panel.
Past studies have established lipid distributions for laboratory grown organisms such as *E. coli*[^141] (bacteria)^§§ and *C. cladosporium*[^142] (filamentous fungus)^***. Phosphatidylethanolamine (PE) headgroup scans for these organisms are shown in Figure 2.30 below. These spectra were obtained from methanolic extracts of laboratory grown organisms provided by collaborators at UNSW and analysed in 2011 and 2012, respectively. Similarities and differences in the lipid profiles can help to confirm the identity of the unknown sample. However, more work must be done to profile other varieties of known organisms for comparison.

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[^141]: Grown in Luria broth at 37 °C for 15 – 20 h.

[^142]: Grown in PYG broth at room temperature for 72 h.
2.5. Conclusions

In the case of the Kapar samples, the presence of atmospheric pollution hampers microbial detection using optical microscopy. Nonetheless, spore detection is possible after as few as four weeks exposure on all samples exposed. Similarly, quantification is hampered by the presence of atmospheric pollution in South East Asia and requires an extra ‘bleaching’ step which removes the visual contribution of biological matter, allowing the total biological and inorganic pollution contributions to be measured. Optical identification was possible through visual analysis of some organisms; however, this method is made more difficult by the very similar spore structures exhibited by many organisms.

Both LESA and swabbing extraction methods in conjunction with MS/MS utilizing targeted lipid head group scans are comparable in terms of their ability to detect lipids from COLORBOND® Steel panels. LESA may be more useful for low concentrations as it is able to target small areas and if these areas correspond to the location of a colony, substantially more lipid can be extracted relative to solvent volume. Neither extraction method is suitable for quantification of biomass due to changes in instrument performance over time and a lack of appropriate internal standards for this application.

Using MS based lipid analysis, identification of organisms from COLORBOND® Steel panels is potentially possible by comparing lipid profiles from panels to those of laboratory grown known organisms. The lipid profiles of many organisms have yet to be reported. Further work, therefore, is required to create a library of lipid profiles for known organisms in order for this potential for identification to be
explored.

Overall, optical analysis is a more efficient method of analysing the extent of biological infestation as it is quicker, less expensive and therefore, allows for a more comprehensive view of area coverage across the panel. Given that the primary objection to the growth in question is based on visual grounds, it is more appropriate that this be used to assess the extent of the problem.
Chapter 3: A TEMPORAL STUDY OF MICROBIAL COLONIES IN THEIR NATURAL ENVIRONMENT USING OPTICAL MICROSCOPY
3.1. Introduction

3.1.1. Commercial justification of study

In the absence of access to more complex microbiological techniques, microbial infestations on COLORBOND® Steel substrates have historically been chronicled using optical microscopy. Antimicrobial strategies, customer complaints and geographical variations in growth behaviour have been assessed purely upon qualitative optical observations. Currently, therefore, most of the knowledge of infestations in Australia and overseas originates from microscopic examination of the surface. To add context to the advanced analytical and microbiological techniques deployed elsewhere in this thesis, an in depth optical examination of the progression and morphology of the colonies forms an integral part of this broader study.

There are four main reasons that the work detailed in this chapter is of commercial and scientific importance. Firstly, it adds a quantitative dimension to an area of study for which there is existing qualitative and anecdotal historical evidence. Secondly, optical microscopy is the easiest, cheapest and quickest method of comparing new data to past studies and bodies of knowledge pertaining to COLORBOND® Steel. It can also be employed ‘in house’, not requiring microbial analysis to be outsourced to external organisations. Thirdly, it enables the biological diversity and symbiotic interactions to be characterised from early stages of development. Finally, practical, appearance-based parameters of commercial interest in the coatings industry, such as colour change, total solar reflectance (TSR) and visible darkening can be linked closely to the extent of microbial growth.
Chapter 3: A temporal study of microbial colonies in their natural environment using optical microscopy

3.1.2. **Fungal characterisation by optical microscopy**

The short time frame between outdoor exposure and microscopically observable colonisation enables information about the growth patterns and behaviour of fungal colonies to be derived at an early stage in their development. This allows a comprehensive chronology of colony development to be established. In this chapter, microbial morphology is discussed in terms of the characteristics described in Figure 3.1.

![Figure 3.1: Guide for morphological characterisation of microbial colonies. Adapted from Willey et al. [143].](image)

3.1.3. **Fungal quantification by optical microscopy**

The ability to quantify fungal growth is relevant to diverse areas of research with many studies quantifying fungal spores or mass in substrates such as soil [144-146], water [147] and air [148]. In samples such as these, extraction and subsequent quantification of some biomarker is preferred, as saving the sample is neither practical nor necessary. Recently, quantitative PCR methods have become widely
used to quantify fungi\textsuperscript{[149]}. Other quantification methods involve ergosterol extraction\textsuperscript{[145, 146]} and cytometry\textsuperscript{[148]}. As destructive methods, however, they do not allow for temporal mapping of discrete colonies. Optical microscopy has been used to estimate spore density in sediments\textsuperscript{[150]}. Examples of fungal quantification using optical microscopy are sparse, however, and are often limited to growth on solidified agar media\textsuperscript{[151-153]}.

3.1.4. **Fungal attachment to pre-painted steel surfaces**

On biological surfaces and on environmental samples, as discussed above, optical microscopy is not commonly used as the fungi often adhere to the substrate by penetrative mechanisms and can grow in three dimensions. The surface area coverage, however, is the most relevant quantification parameter in this work. It is surface area coverage that leads to visible surface darkening and reduction of surface properties such as aesthetic appeal and total solar reflectance.

Cross-sectional scanning electron microscopy (SEM) images previously obtained of fungi growing on COLORBOND\textsuperscript{©} Steel substrates reveal that the colonies are typically less than 10 µm thick and do not form significantly raised structures (Figure 3.2 (a)). Often colonies are a single cell thick, as shown in the close up in Figure 3.2 (b). As demonstrated in Figure 3.2 (b), there is no evidence that the colony encroaches into the polymer topcoat. For this reason, two dimensional imaging, achieved through optical microscopy imaging of the surface alone has been used to study growth rates over time and in different growth conditions. To the author’s knowledge, no such temporal quantitative area study of fungal colonies in their native environment has been attempted to date.
Chapter 3: A temporal study of microbial colonies in their natural environment using optical microscopy

Figure 3.2: Cross sectional SEM images of a typical fungal colony grown at the Burrawang exposure site following 52 weeks of panel exposure. (a) shows the entire colony atop the polymer topcoat (i), primer (ii), metal coating (iii) and steel substrate (iv). A close up of the yellow bordered area is shown in (b). SEM images courtesy of Stewart Ford of BlueScope Innovation Labs.

The work detailed in this thesis looks at adhesion, colonisation and proliferation by microorganisms in the early stages (0 – 3 years) of service lifetime. Later in the service lifetime (>10 years), as the effects of environmental surface degradation of the polymer surface occurs, adhesion mechanisms may differ from those discussed here. One such example is shown in Figure 3.3, below, which shows a COLORBOND® Steel coating system used in the early 1990s. This coating system is similar to that used in this study and has been degraded following prolonged exposure at Burrawang. In contrast to the coating seen in Figure 3.2, the resin of the topcoat has broken down to expose white TiO₂ pigments which were able to detach from the bulk topcoat. Figure 3.3 (b) shows a close up of the area indicated in Figure 3.3 (a) in which the fungal colony observed is bound directly to these pigments. Infestation of such surfaces is not investigated in this work.
Figure 3.3: Cross sectional SEM images of a fungal colony on a weathered polymer substrate. This sample was exposed for 23 years at Burrawang. (a) shows the entire colony atop the polymer topcoat (i), primer (ii) and metal coating (iii). A close up of the yellow bordered area is shown in (b) in which the fungal colony is seen to be adhering to exposed TiO$_2$ pigment particles. SEM images courtesy of Stewart Ford of BlueScope Innovation Labs.
3.2. Materials and Methods

3.2.1. Reagents

Household bleach was obtained at a local supermarket, having a hypochlorite content of 4 % w/w. Standard and high gloss paints used for side-by-side drawdowns (see Section 3.2.3.2) were obtained from PPG Industries (Clayton, Australia).

3.2.2. Biological specimens

Specimens were obtained from COLORBOND® Steel panels placed outdoors at BlueScope exposure sites located in Burrawang (Australia) and Kapar (Malaysia).

3.2.3. COLORBOND® Steel test panels

3.2.3.1. Quantification and bleaching studies

Test panels used for quantification and bleaching studies are detailed in Section 2.2.3.

3.2.3.2. Time-lapse panels

Six ‘time-lapse’ panels (as described in Table 3.1) were exposed at Burrawang on 19/11/12. Four panels were mounted on ‘normal’ and three on ‘hot’ racks (as described in Section 1.2). ‘Standard gloss’ panels came from line produced ‘Surfmist’ COLORBOND® Steel, coil number D42794, manufactured at CPL#8 on the 24th of October 2012. ‘High gloss’ and ‘standard/high gloss’ panels were prepared in the laboratory using the curing conditions detailed in Section 2.2.3.1.
Table 3.1: Gloss, rack and associated nomenclature of ‘time-lapse’ panels exposed at Burrawang.

<table>
<thead>
<tr>
<th>Gloss</th>
<th>Rack</th>
<th>Panel nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Normal</td>
<td>S.N</td>
</tr>
<tr>
<td>High</td>
<td>Normal</td>
<td>H.N</td>
</tr>
<tr>
<td>Standard/high</td>
<td>Normal</td>
<td>S/H.N</td>
</tr>
<tr>
<td>Standard</td>
<td>Hot</td>
<td>S.H</td>
</tr>
<tr>
<td>High</td>
<td>Hot</td>
<td>H.H</td>
</tr>
<tr>
<td>Standard/high</td>
<td>Hot</td>
<td>S/H.H</td>
</tr>
</tbody>
</table>

Standard/high gloss panels were prepared by side-by-side drawdown of standard and high gloss paints. Wet standard and high gloss paint was placed side-by-side on the panel, with the location indicated by the red ovals in Figure 3.4. The draw down bar is used to drag the wet paint in a perpendicular direction, as shown by the blue arrow in Figure 3.4.

Figure 3.4: An example of a side-by-side drawdown. The red ovals show where the wet paint was originally placed on the panel and the blue arrow shows the direction in which the paint was drawn down the panel. (a) shows the cured high gloss paint, (b) the cured standard gloss paint, (c) the underlying primer and (d) the metallic substrate.
A further time-lapse panel (standard gloss, normal rack) was employed specifically to study discrete individual organisms and their development during the period of November 2013 to April 2015.

3.2.4. **Analysis**

3.2.4.1. **Optical microscopy**

Optical microscopy and quantitative analysis were performed as detailed in Section 2.2.5.1.

3.2.4.2. **Image J analysis**

Time-lapse pictures were analysed in Image J\(^{[140]}\) using the method described in Section 2.2.5.1.

3.2.5. **Total solar reflectance**

COLORBOND\(^\circledR\) Steel was obtained from line produced ‘Whitehaven’ colour, coil numbers D22865S1 (coupons D22865S1-1-D22865S1-11) and Z35865 (coupons Z35865-1-Z35865-12) manufactured at CPL#8 on the 19\(^{th}\) of September 2011 and the 4\(^{th}\) of April 2011 respectively. ‘Resin’ was approximately 83 % polyester and 17 % melamine formaldehyde crosslinking agent and ‘matting agent’ consisted of silica particles with mean particle size 8.3 – 10.2 µm. Pigment used to formulate ‘Whitehaven’ colour was 100 % white (TiO\(_2\)) pigment.

The COLORBOND\(^\circledR\) Steel strip was cut into coupons measuring 100 mm × 45 mm using a hydraulic guillotine (Epic Industries). These dimensions were chosen to fit the sample holder of the spectrometer used. Coupons were exposed at Burrawang on
hot racks starting in November 2012. An additional sample was exposed every month until December 2014, inclusive, resulting in a total of 23 samples exposed. TSR was measured using a Perkin Elmer Lambda 1050 UV-Vis-NIR spectrometer fitted with an integrating sphere accessory. Spectra were recorded in the wavelength range from 2000 cm$^{-1}$ to 250 cm$^{-1}$ with a data interval of 4 nm. The spectral data were then used to calculate the TSR as per ASTM E 903-96.
3.3. Results and discussion

3.3.1. Initial colonisation of surfaces

3.3.1.1. Burrawang (Australia)

Samples were initially exposed in November 2012 and were first collected for optical analysis four weeks later. Although no darkening or fouling of the surface could be seen with the naked eye, under 50 × magnification, small dark particles were observed. When viewed at 500 × magnification, these were found to be individual spores adhering to the substrate, as seen in Figure 3.5. Figure 3.5 (a) shows a single spore, with morphology similar to Alternaria sp. The spores in Figure 3.5 (b), however, can be seen to be developing germination tubes. The presence of observable and frequent microbes after this short exposure period highlighted the ability of optical microscopy to monitor single colonies throughout their life cycle, from adherence of initial spore to development of mature colony to, sometimes, complete colony detachment. The wealth of information available through this avenue of investigation inspired the ‘time-lapse’ portion of the optical microscopy study, reported in Section 3.3.2.
3.3.1.2. Kapar (Malaysia)

At the Kapar site, and elsewhere in South East Asia, carbonaceous atmospheric fallout has been long observed by BlueScope to have a rapid effect on COLORBOND® Steel surfaces. Surfaces become rapidly darkened, with colour change noticeable to the naked eye in as little as four weeks of outdoor exposure (Figure 3.6 (ii)). This is clearly evident after eight weeks (Figure 3.6 (iii)), when compared to an unexposed sample Figure 3.6 (i). The presence of this dark discolouration hinders microscopic analysis of fungal colonies, however, the difference between the atmospheric fallout and the fungal colonies can be semi quantitatively separated by bleaching the biological matter, as discussed in Section 2.3.2.
3.3.2. ‘Time-lapse’ panels at Burrawang (Australia)

3.3.2.1. Exposure testing

‘Time-lapse’ panels of standard and high gloss were exposed at the Burrawang site on both ‘normal’ (Figure 1.3 (a)) and ‘hot’ (Figure 1.3 (b)) racks. Standard gloss paint contains silica matting agent and therefore has a lower water contact angle than high gloss paint which does not contain silica matting agent.

The ‘hot’ exposure rack (Figure 1.3 (b)) is designed to mimic the conditions on an
insulated roof, where the lack of heat loss into the structure elevates the temperature of the roofing material. ‘Surfmist®’ is an off-white colour in the COLORBOND® Steel roofing range. These panels on ‘normal’ and ‘hot’ racks typically reach maximum temperatures of 40° C and 60° C respectively[136]. This comparison aimed to observe whether higher temperatures had any effect on the fungal morphologies observed on the panels or on the growth rates of the colonies.

Panels were collected every month and were taken to BlueScope Innovation (Port Kembla, Australia) and photographed at 100 × magnification using a metallurgical microscope before being returned to the Burrawang exposure site within a few days.

3.3.2.2. Correlation with environmental conditions

To account for the effect of environmental conditions at the exposure site, daily temperature and rainfall data was obtained from the Bureau of Meteorology (BOM)[154]. Data was obtained from BOM test sites within 10 kilometers of the Burrawang exposure site. The average daily maximum temperature and the sum of the rainfall for the 28 days preceding collection date are represented in Figure 3.7. Temperature data was measured at ‘Moss Vale AWS’. Rainfall data from November 2012–March 2014 was measured at ‘Burrawang (Range St)’ and data from March 2014–April 2015 at ‘Burrawang (Spurfield)’.
Figure 3.7: Temperature and rainfall over the 28 days preceding the date of collection, measured at locations within 10 km of Burrawang, Australia\textsuperscript{[154]}. 

3.3.2.3. Growth behaviour of individual colonies

Individual colonies on time-lapse panels were observed every 4 weeks for 120 weeks. These colonies were categorised morphologically into two main types which have been observed to comprise the majority of colonies. Colonies of type I (Figure 3.8 (a)) are defined as irregular, umbonate structures with dark brown to black colouring and an undulate margin. Type II (Figure 3.8 (b)) colonies are circular and raised with an entire margin and a medium brown colour.
3.3.2.3.1. Colonies type I morphology

Sixty colonies with type I morphology were monitored over the 120 week exposure period. Targeted colonies were spread between high and normal gloss and hot and normal racks (as detailed in Table 3.2). Fifteen colonies were monitored on standard gloss, normal rack panels (S.N), fifteen on standard gloss, hot rack panels (S.H), 15 on high gloss, normal rack panels (H.N) and 15 on high gloss, hot rack panels (H.H). The colonies chosen showed nodular morphology, typical of type I colonies. Figure 3.9 shows the locations and morphologies of the 60 colonies chosen.
Chapter 3: A temporal study of microbial colonies in their natural environment using optical microscopy
Figure 3.9: Locations and morphologies of colonies chosen as examples of type I morphology. Images are annotated with panel type and [area].

Table 3.2, below, summarises all type I colonies analysed, their panel type and position in addition to the ‘exposure week’ in which they were first observed and the ‘exposure week’ when detachment was observed. Note that all panels were first exposed on 19/11/2012, hence, ‘52 weeks’ corresponds to collection on 18/11/2013 and ‘104 weeks’ to 17/11/2014. Attachment and detachment are, here, defined as ‘visible under optical microscope at 100 × magnification’ and ‘greater than 50 % reduction in size’, respectively. Colonies still attached at the end of the study (120 weeks exposure) are denoted by a ‘-‘ in the ‘week of colony detachment’ column.
Table 3.2: Summary table of type I colonies chosen for analysis. Panels are referred to by the nomenclature detailed in Table 3.1.

<table>
<thead>
<tr>
<th>Colony name</th>
<th>Panel</th>
<th>Panel Area</th>
<th>Week of colony attachment</th>
<th>Week of colony detachment</th>
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<td>B3</td>
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</tr>
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Chapter 3: A temporal study of microbial colonies in their natural environment using optical microscopy

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</tr>
</tbody>
</table>
The settlement of spores that went on to develop into type I colonies occurred typically between weeks 8 and 24 and also between weeks 52 and 68. This corresponded to the months January to May 2013 and November 2013 to March 2014. Eighteen of the 60 colonies observed in this time course study (30 % of colonies) were noted for the first time in March (weeks 16 and 68). Figure 3.10 shows the week in which each of the 60 colonies followed in this study were first observed.

Colony attachment did not correlate to rainfall but rather seemed to be seasonally and, therefore, temperature dependent. Settlement only occurred in the warmer months of the year. In both significant settlement periods, as shown in Figure 3.10 below, temperature ranged between an average maximum of 18 and 28°C (refer to Figure 3.7). As detailed in Figure 3.7, rainfall varied between a total of 40 mm (minimum) and 320 mm (maximum) per 4 week period during weeks 8 – 24 and between 45 mm and 90 mm per 4 week period during the period 52-68 weeks. These total rainfalls were not extreme or unusual events during the observation period.

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>A52</td>
<td>H.H</td>
<td>A6</td>
<td>12</td>
<td>108</td>
</tr>
<tr>
<td>A53</td>
<td>H.H</td>
<td>A6</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>A54</td>
<td>H.H</td>
<td>A6</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>A55</td>
<td>H.H</td>
<td>B5</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>A56</td>
<td>H.H</td>
<td>B5</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>A57</td>
<td>H.H</td>
<td>B5</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>A58</td>
<td>H.H</td>
<td>B5</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>A59</td>
<td>H.H</td>
<td>B5</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>A60</td>
<td>H.H</td>
<td>B5</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.10: Number of new colonies attached, by week first observed. New spore attachment occurred in summer (yellow shaded) and autumn (orange shaded).

Thirteen of the 60 colonies observed in this section of the study detached from the surface before the end of the study. The area coverage over the time course of these colonies exclusively is shown in Figure 3.11. Of the colonies which were observed to detach during the observation period, this occurred in several cases after periods of rapid growth. For example, A20, A21, A22, A23 and A24 all experienced between a 210 and 360% size increase between weeks 48 and 56. Detachment of these colonies was observed in week 60. A similar rapid growth and subsequent detachment trend is also seen in colony A12, A41 and in A46 and A52. With the exception of colonies A12 and A41, all of these colonies were found on hot rack panels. Four (A1, A4, A16 and A31) of the thirteen detached colonies detached after a period of no significant size alteration and A1 and A31, by contrast, detached gradually, decreasing in size over a period of several weeks.
Figure 3.11: Area coverage over time of colonies that experienced detachment during the course of the study.

There is insufficient evidence to suggest that rainfall is involved with the process of colony detachment. Average daily rainfall at Burrawang over the period 19/11/12 (week 0) – 9/3/15 (week 120) was 3.8 mm, thus the average total rainfall over 28 days was 106.5 mm. Total rainfall over the 28 days preceding sample collections at 16, 32, 92 and 108 weeks were significantly higher than the 28 day average (320.4, 359.4, 293.6 and 238.6 mm, respectively). Colonies A52 and A12 detached prior to the 108 week collection but there is no evidence that this was rainfall related, especially given that detachment of other colonies occurred after no evidence of heavy rain. Similarly, lower rainfall than average (less than 40 mm in total) occurred leading up to weeks 20 (24.4 mm), 36 (13 mm), 40 (17.6 mm), 48 (9 mm), 88 (6 mm) and 104 (22.2 mm) and no unusual behavior is observed at these time points. It
should be noted, however, that the volume and not the quality (drizzle, light rain or heavy rain) of rainfall was available from the BOM data used. It may be possible that a particularly heavy rain shower or particularly long dry spell influenced colony growth behavior.

As evident in Figure 3.9, colonies A16 – A25 originate from the same panel area. Although all normal gloss, hot rack colonies (A16 – A30) grew rapidly in weeks 48 – 56, the colonies A16 – A25 grew most rapidly. This observation is also true of colonies A1, A2 and A3 (Figure 3.14), all also coming from the same photograph area.

A reduction in size, from which the affected colonies subsequently recover, was observed in several colonies over the course of the study. At two time points, marked shrinking was common to several colonies. The most noticeable decrease occurred at 68 weeks and may be observed in colonies A1, A2, A3, A10, A18, A19, A25 and A29, as shown in Figure 3.12. Another shrinkage phenomenon occurred at 100 weeks and can be observed in colonies A3, A29 and A30 (Figure 3.13). Both of these occurred at times when temperature was higher than the yearly average and the rainfall below average. However, conditions like these were not extreme and were also present at week 48. No accompanying size decrease was observable in any of the colonies studied at this time, however, no or very little size increase is observed in most colonies between weeks 44 and 48. A3 and A29 experience repeated shrinking at both time points suggesting that they are particularly susceptible to this behavior. Both of these colonies first appeared in week 16 and were both present on standard gloss panels. The reasons for size reduction and subsequent recovery are not known. Causes may be environmental, such as temperature or rainfall, however there
is insufficient evidence at this stage to draw conclusions. It is clear, however that colonies can decrease in size and yet go on to increase in size. The fact that size reduction is not an inevitable precursor to colony death is relevant when assessing the health and viability of colonies.

![Graph showing colony growth and decline over weeks of exposure.](image)

**Figure 3.12:** Colonies that decreased in size at week 68. Size measurements taken at week 68 are highlighted in yellow.
Figure 3.13: Colonies that decreased in size at week 100. Size measurements taken at week 100 are highlighted in yellow.

Colony area coverage over the 120 week period of observation was graphed by panel type. Standard gloss, normal rack colonies are shown in Figure 3.14 (colonies A1 – A15), standard gloss, hot rack colonies in Figure 3.15 (colonies A16 – A30), high gloss, normal rack in Figure 3.16 (colonies A31 – A45) and high gloss hot rack in Figure 3.17 (colonies A46 – A60).

The average sizes of the colonies after one year of adhesion are shown in Table 3.3. Of the 60 colonies followed, nine detached within a year of first appearing on the surface. These colonies (A4, A12, A17, A20, A21, A22, A23, A24, A25) were excluded from the following analysis. As such, to calculate the average sizes shown in Table 3.3, 13 colonies from standard gloss, normal rack panels, 8 colonies from standard gloss, hot rack panels, and 15 each from high gloss, normal rack and high gloss, hot rack panels were used.
Table 3.3: Average and median sizes of one year old type I colonies, grouped by substrate type. Uncertainty reflects standard deviation. For standard gloss, normal rack n = 13, standard gloss, hot rack n = 8, high gloss, normal rack and high gloss, hot rack n = 15.

<table>
<thead>
<tr>
<th>Panel type</th>
<th>Average colony size (µm²)</th>
<th>Median colony size (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard gloss, normal rack</td>
<td>1500 ± 500</td>
<td>1739</td>
</tr>
<tr>
<td>Standard gloss, hot rack</td>
<td>1300 ± 600</td>
<td>1284</td>
</tr>
<tr>
<td>High gloss, normal rack</td>
<td>1000 ± 600</td>
<td>913</td>
</tr>
<tr>
<td>High gloss, hot rack</td>
<td>1000 ± 400</td>
<td>1063</td>
</tr>
</tbody>
</table>

Colonies from standard gloss panels are larger after a year’s growth than their high gloss counterparts (as determined using an independent t-test, p < 0.05). This observation is supported by the median colony size which is higher for the standard gloss colonies.

Figure 3.14: Area coverage over time of colonies A1 – A15, observed on standard gloss panels, mounted on normal racks. A • denotes week of detachment.
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Figure 3.15: Area coverage over time of colonies A16 – A30, observed on standard gloss panels, mounted on hot racks. A • denotes week of detachment.

Figure 3.16: Area coverage over time of colonies A31 – A45, observed on high gloss panels, mounted on normal racks. A • denotes week of detachment.
Figure 3.17: Area coverage over time of colonies A46 – A60, observed on high gloss panels, mounted on hot racks. A • denotes week of detachment.

3.3.2.3.2. Colonies with type II morphology

Eighteen colonies with type II morphology were monitored over the 120 week exposure period. This morphological type was less prevalent than the type I with only 18 colonies being identified across all areas photographed as part of the time-lapse study, compared to well over 60 type I colonies. Type II morphology was only observed on normal rack panels, which reach a maximum of 40°C, and not on hot rack panels, which reach a maximum of 60°C. It is possible that this morphology type is not suited to higher temperatures. Targeted type II colonies were taken from the locations detailed in Figure 3.18.
Figure 3.18: Locations and morphologies of colonies chosen as examples of type II morphology.
Table 3.4 lists all type II morphology colonies analysed, their panel type and position in addition to the month of initial attachment and detachment, defined as stated in Table 3.2. As with the type I colonies, attachment was observed in late summer and early autumn exclusively. Colonies B1 – B8 were observed in the four weeks leading up to week 16 (collected in March 2013). 13 type I colonies were also initially observed at this time point. Colonies B9-B11 and B13-B18 were first observed between 56 and 68 weeks. This corresponds to the period from December 2013 to March 2014 and was the same time period the majority of type I colonies were first observed (see Figure 3.10). Colony B12 was first observed at week 32, when it was already 2854 µm² in size.
Table 3.4: Summary table of type II colonies chosen for analysis. Panels are referred to by the nomenclature detailed in Table 3.1.

<table>
<thead>
<tr>
<th>Colony name</th>
<th>Panel</th>
<th>Panel Area</th>
<th>Month of colony attachment</th>
<th>Month of colony detachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>H.N</td>
<td>B4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>B2</td>
<td>H.N</td>
<td>B4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>B3</td>
<td>H.N</td>
<td>B4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>B4</td>
<td>H.N</td>
<td>B4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>B5</td>
<td>H.N</td>
<td>B4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>B6</td>
<td>H.N</td>
<td>B4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>B7</td>
<td>H.N</td>
<td>B4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>B8</td>
<td>H.N</td>
<td>B4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>B9</td>
<td>H.N</td>
<td>A5</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>B10</td>
<td>S/H.N (high gloss section)</td>
<td>B5</td>
<td>68</td>
<td>-</td>
</tr>
<tr>
<td>B11</td>
<td>S/H.N (high gloss section)</td>
<td>B6</td>
<td>56</td>
<td>-</td>
</tr>
<tr>
<td>B12</td>
<td>S/H.N (high gloss section)</td>
<td>B7</td>
<td>&lt;32</td>
<td>-</td>
</tr>
<tr>
<td>B13</td>
<td>S/H.N (high gloss section)</td>
<td>B7</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>B14</td>
<td>S.N</td>
<td>A3</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>B15</td>
<td>S.N</td>
<td>A7</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>B16</td>
<td>S.N</td>
<td>A7</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>B17</td>
<td>S.N</td>
<td>B3</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>B18</td>
<td>S.N</td>
<td>B3</td>
<td>60</td>
<td>-</td>
</tr>
</tbody>
</table>

Of the 18 type II colonies observed in the time-lapse images, only 5 were present on standard gloss panels, suggesting this microbial community is more suited to smoother surfaces. Figure 3.19 shows the area coverage of colonies B1 – B13 over...
the 120 week course of the study. Similar to the observation made with type I colonies (Section 3.3.2.3.1), several type II colonies experienced size decrease in weeks 48 and 68. As shown in Figure 3.19, colony shrinking of type II morphology colonies was observed at 48 weeks in colonies B1, B3 and B12. Colonies which did not decrease in size did not grow at this time. At week 68, colonies B1 and B3 are again noticed to decrease in size. In addition, there was size decrease at week 116, as noted in colonies B7, B12, B15 and B16 (Figure 3.19 and Figure 3.20).

![Figure 3.19: Area coverage over time of type II morphology colonies (as described in 3.3.2.3), B1-B13, observed on high gloss panels, mounted on normal racks at Burrawang. Weeks 48, 68 and 116, during which several colonies were observed to decrease in size, are highlighted.](image-url)
Figure 3.20: Area coverage over time of type II morphology colonies (as described in 3.3.2.3), B14-B18, observed on standard gloss panels, mounted on normal racks at Burrawang. Week 116, during which several colonies were observed to decrease in size, is highlighted.

3.3.2.4. Impact of external nutrient sources on colony growth

In one case, it has been possible to view the effect of bird droppings on growth rate of fungal colonies. This event occurred in late December 2013 or early January 2014 and was first observed at the 60 week sample collection (on 13/1/14). The panel appeared relatively clean at the next 4 week sample collection (week 64) and at week 68, no foreign material was detectable at 100 × magnification, as shown in Figure 3.21 (c).
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Figure 3.21: Position B8 on test panel S/H.N, shown at (a) 56 weeks, (b) 60 weeks and (c) 68 weeks, before, immediately following and after bird droppings appeared on the panel.

To assess the impact of this event on the fungal area coverage at the site, positions B8 (Figure 3.21) and B9, which had been covered in the bird droppings were contrasted with position B7, which experienced no such coverage. These areas were taken from the standard/high gloss, normal rack test panel and are shown in Figure 3.22.

Figure 3.22: Positions B7 (a), B8 (b) and B9 (c) on test panel S/H.N, shown at 60 weeks.

Percentage area coverage of the entire area was calculated using ImageJ software in order to incorporate all colonies within that area. Given the $100 \times$ magnification of the images obtained, the total area assessed for each area was $876 \, \mu m \times 657 \, \mu m$ or $575532 \, \mu m^2$. Figure 3.23 shows the area coverage as a percentage of the total area assessed. Each of the locations chosen were located approximately 3 cm from each other on the test panel. As noted previously, colonies which are close to each other
on the test panels show similar growth profiles. It may be expected that areas close to each other might show similar growth rates, hence, B7 is a good comparison to B8 and B9. As seen in Figure 3.23, the growth profile of percentage area coverage of area B7 over the period of 48-76 weeks exposure does not differ markedly from the areas B8 and B9. All areas experience increased coverage between weeks 48 and 64 and a slight drop in coverage between weeks 64 and 68. The more rapid growth rate observed in B8 between 48 and 64 may be attributed to the greater number of colonies in this area, compared to positions B7 and B9, as seen in Figure 3.22. Note that the area coverage may not be as accurate at week 60 for areas B8 and B9 as for the other measurements due to the presence of bird droppings obscuring the colonies.

There were no other instances observed of large amounts of organic matter being introduced onto the panel surface, whether through bird droppings or otherwise. Hence, observations in this area are limited. However, in this case there is no evidence of size increase or decrease due to the introduction of bird droppings nor was there an increase or decrease in the quantity of colonies after this event.
Figure 3.23: Area coverage of the areas B7, B8 and B9 of test panel ‘standard/high gloss, normal rack’. The time period during which the droppings were first present on the sample was sometime between 56 and weeks of exposure (highlighted).

3.3.2.5. Characterisation of species settled over time

An understanding of the species settling at different time points was attempted. Because panels are collected every four weeks, any new particles appearing on the panel can be deduced to have been airborne at some stage during the four weeks preceding collection. Hence, seasonally correlated settlement observations are possible. In this section of work, new ‘particles’ settled on the surface are defined as dark coloured objects <10 µm. These criteria are designed to target particles which could plausibly be fungal spores. As the time-lapse images are taken at 200 × magnification, it is not possible to definitively classify these particles as spores using optical methods. In the preceding work, classification has been retrospective and based on the ultimate development of the initial ‘particle’ into a mature colony. While the identification as ‘spores’ of settled particles for which no growth is
observed may, in some cases, be inaccurate, the best efforts have been made to select only those particles which are probably spores.

These observations are divided into two sections. Firstly, the appearance of the spores which developed into recognisable type I and type II colonies and secondly, those particles which settled and adhered for a short period of time before shrinking and detaching. In order to assess the extent to which spores from the atmosphere settle and propagate on these surfaces, one area from each of the panel types, S.N, S.H, H.N and H.H were assessed on the longevity of spore adhering in the four weeks preceding the 16 week (March 2013) collection. As stated previously, spore settling was highest during this period. The progress of particles settled between 12 and 16 weeks of exposure is assessed.

An area of panel S.N (B3) was found to have gained 12 new spores between weeks 12 and 16 of exposure, as circled in Figure 3.24. Of these, the red circled colonies have been analysed previously in Section 3.3.2.3.1 as type I colonies A1, A2 and A3. Colonies A1, A2 and A3 first appear as small circular particles. Spores circled in blue (Figure 3.24) had very similar initial appearance to A1, A2 and A3. These colonies adhered and grew in size to a maximum of approximately 100 µm² before disappearing after 32 weeks of adhesion, at week 48 (October 2013). Colonies circled in purple settled and adhered for several months but did not grow and eventually disappeared from the surface. Spores labelled with ‘*’ were still present on the surface at the conclusion of the observation period at week 120 (March 2015), a total attachment period of >104 weeks.
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Figure 3.24: Panel S.N, position B3 after 16 weeks of exposure (March 2013). All new particles noted on the surface are circled. Colonies A1, A2 and A3 which have been previously discussed are shown in red, colonies which grew in size before detaching are circled in blue and colonies which showed no size change before detachment are circled in purple. Numbers denote the length of time, in weeks, that the particle remained adhered to the surface, with ‘*’ denoting that the colony was still attached to the surface when the exposure period was concluded after a total of 120 weeks of exposure (February 2015).

Panel S.H, position B3 also experienced higher levels of spore settlement in the four weeks preceding week 16 (March 2013). Particles observed for the first time at the week 16 collection are circled in Figure 3.25. Numbers denote the weeks elapsed before the colony disappeared from the surface with ‘*’ denoting those attached for >104 weeks. Purple circled colonies did not increase in size whilst blue circled colonies did.
Figure 3.25: Panel S.H, position B3 after 16 weeks of exposure (March 2013). All new particles noted on the surface are circled. Colonies A1, A2 and A3 which have been previously discussed are shown in red, colonies which grew in size before detaching are circled in blue and colonies which showed no size change before detachment are circled in purple. Numbers denote the length of time, in weeks, that the particle remained adhered to the surface, with ‘*’ denoting that the colony was still attached to the surface when the exposure period was concluded after a total of 120 weeks of exposure (February 2015).

Panel H.N, position B4 at time 16 weeks is shown in Figure 3.26, below. Eight of the spores which settled here in that time period are detailed in Section 3.3.2.3.2 as type II colonies; B1-B8 (circled in red in Figure 3.26). All these colonies were present on the surface at 120 weeks when the observation period was concluded. Two colonies grew in size after settlement, also eventually forming type II colonies and again being present at 120 weeks. Six of the observed particles did not grow and
disappeared from the surface approximately 4-8 weeks after their initial settlement.

Figure 3.26: Panel H.N, position B4 after 16 weeks of exposure (March 2013). All new particles noted on the surface are circled. Colonies B1 – B8, which have been previously discussed, are shown in red, colonies which grew in size before detaching are circled in blue and colonies which showed no size change before detachment are circled in purple. Numbers denote the length of time, in weeks, that the particle remained adhered to the surface, with ‘*’ denoting that the colony was still attached to the surface when the exposure period was concluded after a total of 120 weeks of exposure (February 2015).

Panel H.H, position B4 at time 16 weeks is shown in Figure 3.27, below. Of the 34 new particles having settled in the 28 days preceding the 16 week (March 2013) collection, 11 did not increase in size, although one of these remained on the surface for 40 weeks and several others showed relatively long adhesion times of between 12 and 32 weeks. Of the 23 particles which did develop into larger colonies, 13 of these
detached after approximately 40-48 weeks of adhesion. These detachments were observed at the week 56, 60 and 64 sample collection. As discussed above, there were several time points at which mass detachment occurred and this often occurred for several colonies upon one panel area and was not necessarily noted elsewhere on the panel or on other panels. It is unclear why so many colonies from this area detached at this time point. As noted previously, colony detachment often correlates with higher than average temperature and lower than average rainfall. This is the case over exposure weeks 56-64 in which average daily maximum temperature ranged between 22 and 28 °C and rainfall was lower than usual at between 50 and 100 mm falling in total in the 28 days preceding collection. Panel H.H, position B4 may be another example of this phenomenon.
Figure 3.27: Panel H.H, position B4 after 16 weeks of exposure. All new particles noted on the surface are circled. Colonies which grew in size before detaching are circled in blue and colonies which showed no size change before detachment are circled in purple. Numbers denote the length of time, in weeks, that the particle remained adhered to the surface.

It is also important to note that there are several morphologies that, although noted to land on and adhere to the surface, do not appear to increase in size and eventually fade from the surface. The two most frequently observed organisms to experience this are shown in Figure 3.28. 32 instances of the organism shown in Figure 3.28 (a) (i)-(iv) are observed in the time-lapse areas over the 120 week exposure period and these specimens have an average adhesion duration of 24 weeks (as detailed in Table 3.5). Figure 3.28 (a) shows the organism at first adhesion (i), gradually fading at 4 (ii) and 12 (iii) weeks after adhesion and finally, complete removal 20 weeks later (iv). It can be noted in Figure 3.28 (a) (iv) that a residual outline of the organism
remains after detachment. This is typical of these morphology types and may indicate the excretion of an adhesive substance designed to anchor the organism to the surface.

Four instances of the organism shown in Figure 3.28 (b) (i)-(iv) are observed, having an average adhesion duration of 24 weeks. Figure 3.28 (b) shows the organism at first adhesion (i) and gradually fading from the surface after 8 (ii), 16 (iii) and 20 (iv) weeks of adhesion. Complete disappearance of this organism was observed after 32 weeks of adhesion (not shown).

![Image of organisms](image)

Figure 3.28: The process by which two organisms with abnormal morphologies disappear from the COLORBOND® Steel surface. These particles were typical of those which settle, do not increase in size and gradually disappear over the course of several months. (a) shows an organism on panel S/H.N (high gloss section), area B5, first observed at 64 weeks (i) and also shown at 68 (ii), 76 (iii) and 84 weeks (iv). (b) shows an alternate morphology from panel S.N, area A7, first observed at the 72 week collection (i) and imaged at 80 (ii), 88 (iii) and at 92 weeks (iv).

Abnormal morphologies observed on the time-lapse panels are shown in Table 3.5, below. It is important to note that in no cases have such morphologies been observed to increase in size. Rather, a decrease in size, characterised by a gradual fading (as demonstrated in Figure 3.28) is observed. This is in contrast to the majority of the
type I and type II colonies which, if they are removed from the surface, tend to do it suddenly and after periods of rapid growth. The morphologies shown in Table 3.5 are significant to the assessment of fungal proliferation as they may be considered to be non-problematic organisms. Although they clearly have the capacity to adhere to the substrate, their short-lived presence will not result in unsightly fungal darkening.

Due to fewer instances of the morphologies described below being observed on the panels, it was not possible to reliably contrast how the behaviour of these organisms differed on standard and high gloss panels and on standard and hot exposure racks.

The longevity of the most common morphology (pictured in Figure 3.28) (a)), for example, for which there were 31 colonies of this type observed in total was contrasted on the four panel types, S.N, S.H, H.N and H.H. adhesion duration was highly variable, with these morphology types on S.N panels averaging 30 ± 20 weeks adhesion, S.H and H.N panels both averaging 20 ± 10 and H.H 22 ± 4 weeks adhesion.

††† 95 % confidence interval.
Table 3.5: Morphologies observed on time-lapse panels during the 120 weeks of monitoring. These were morphologies which, although they adhered to the surface for several months, did not increase in size and gradually disappeared.

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Number of colonies observed</th>
<th>Average duration of adhesion (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>31</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Chapter 3: A temporal study of microbial colonies in their natural environment using optical microscopy
3.3.3. Whole Panel Area Coverage Quantification

3.3.3.1. Burrawang (Australia)

Area coverage of panels from the Burrawang exposure site was assessed every four weeks using the method discussed in Section 2.3.3. Analysis was performed between 24 and 108 weeks of outdoor exposure for Burrawang ‘batch 1’ and between 4 and 56 weeks for Burrawang ‘batch 2’. These analyses occurred between May 2013 and December 2014 and between December 2013 and December 2014, respectively. The ‘Batch 1’ sample set included both standard and high gloss panels and is shown in
As seen in Figure 3.29, in all instances where both high and standard gloss were analyzed at the same time period, area coverage for neither lies outside the standard deviation in the measurement of the other. This implies that surface contours and the differences in water contact angle associated with this, at least on the scale investigated here, do not influence the area coverage of fungal colonies.

Between 24 and 36 weeks of outdoor exposure, the area coverage for both sample types is in the range of 0.5 – 1%. Between 44-52 weeks of exposure, surface coverage lies within the range 0.5-1.5%. This rapid increase in coverage may be due to the increase in colonies or to the increase in size of established colonies, most likely a combination of both. Individual statistics for each image were not compiled in the whole panel area coverage section as the focus was placed on the variation and trends of area coverage across the whole panel. The possible reasons for changes in area coverage were explored at length in Section 3.3.2.

The increase noted may also be attributable to the weather conditions. The time period between which these samples were collected (23/9/13-18/11/13) was one in which average temperature increased and rainfall was lower than normal, as shown in the weather data in Figure 3.7.
In Figure 3.30, ‘batch 1’ and ‘batch 2’ are contrasted over the same outdoor exposure time (with exposure initiated in the month of November) but different exposure years (2012 and 2013 respectively). Five of the seven instances in which both batches were analyzed at the same time period show that the mean coverage is within the standard deviation of the measurements, with the two exceptions being at 24 and 32 weeks. This is due to several densely infested areas on the ‘batch 1’ samples at these times periods. There were no outliers in the ‘batch 2’ or ‘batch 1’ 24 week data sets. The ‘batch 1’ 32 week sample set, however, contained three outliers (2.46%, 2.38% and 2.05%) which were greater than two standard deviations (0.512) above the mean (1.02). Better correlation is noted between ‘batch 1’ high and standard gloss than between standard gloss of ‘batch 1’ and ‘batch 2’ (Figure 3.30), indicating that area

†‡‡ Batch 1 data from weeks 4–20 and from weeks 40 and 96 is not available.
coverage is possibly more dependent on environmental conditions year to year than to the surface topography.

Figure 3.30: Area coverage on Burrawang ‘Batch 1’ and ‘Batch 2’ standard gloss panels, weeks 4-108.¶¶¶

3.3.3.2. Kapar (Malaysia)

Area coverage of panels from the Kapar exposure site was assessed every four weeks from 24 weeks until 52 weeks and the every eight weeks from week 60 until week 120, when the analysis concluded, using the method discussed in Section 2.3.3. Panels were photographed at 50 × magnification both pre- and post-bleach treatment, as described.

¶¶¶ Batch 1 data from weeks 4 – 20 is not available, nor is data from weeks 40 and 96.
Area coverage was more variable than Burrawang. When treated with bleach, which removed the black colour of organic fungal colonies, the area coverage was reduced to a consistent value of $< 1\%$, implying that dirt fouling, despite its impact on product in South East Asia, comprises a lesser percentage of the total dark surface coverage. Bleach treatment was discontinued after week 96.

### 3.3.4. Geographically dependant morphological observations

Growth morphology differs between Burrawang and Kapar. Burrawang colonies are discrete round or nodular shaped colonies (see Figure 3.8) whilst at the Kapar exposure site, colonies are both smaller in size and show more hyphal growth than their Burrawang counterparts. A comparison of the two growth types is important for developing mitigation strategies as in many cases, morphology based on optical

**** Data is not available prior to 24 weeks.
microscopy is the major informer of the success or failure of a given mitigation strategy. The Kapar study has been less in depth than the Burrawang study for logistical reasons. The following comparison is based upon general observations at each of the sites.

3.3.4.1. Typical morphologies observed at Burrawang

Burrawang, as previously discussed, shows a prevalence of type I colonies (Figure 3.8 (a)) and less frequent, but common on normal rack panels, type II colonies (Figure 3.8 (b)). Although these are the most prevalent, it is important to note that hyphal growth does occur, although it should not be considered the norm. Figure 3.32 shows the growth behaviour of an organism at Burrawang on a ‘batch 2’ panel (standard gloss panel, normal rack) which was observed every four weeks which developed extensive hyphal growth. It has been observed that this growth behaviour is accelerated in warmer months, while in colder months, detachment of the hyphae is observed. Figure 3.32 (a) shows the organism after the panel had been exposed for 12 weeks (March 2014). 24 weeks later, new hyphal growth is seen (b) with more extensive networks developing 48 weeks after first observation (c). Image (c) is taken in December 2014, when temperatures are higher than average and the most rapid fungal growth is observed. After a period of rapid growth, (d) shows these hyphal extensions becoming thinner and in several areas disappearing. This image was taken in June 2015, 68 weeks after the organism was first observed in a period of lower temperatures. It is therefore hypothesized that the low temperatures and frosts experienced at Burrawang during the winter months are not conducive to sustained hyphal growth of the type pictured in Figure 3.32. A comprehensive temporal
analysis of the organism shown in Figure 3.32 may be found in Appendix B, Figure B1.

Figure 3.32: A colony which developed an extensive hyphal network shown at (a) 4 weeks growth, (b) 28 weeks growth, (c) 48 weeks growth and (d) 68 weeks growth.†††† (a) and (b) show the organisms at 500 × magnification and (c) and (d) at 100 × magnification.

3.3.4.2. Typical morphologies observed at Kapar

There are two main morphologies observed on the Kapar panels. Figure 3.33 shows two areas from the M-72W-5 panel (collected after 72 weeks of exposure at Kapar) which show growth patterns typical of the Kapar location. Heavy growth can be seen in Figure 3.33 (a) which was close to the panel drip edge (i.e., the edge of the panel

†††† Images (a) and (b) taken by P. Barker, formerly of BlueScope.
angled down where rain water runs off). Heavier than normal growth is expected close to the drip edge but at the Kapar site, it has been noted that this influence extends further from the edge than is the case in other locations such as Burrawang. The area shown in (a) is approximately 2 cm from the drip edge while higher than normal growth would be typical on the first 1 cm of edge at Burrawang. This may be due to higher rainfall and/or humidity in Kapar. The colonies seen in Figure 3.33 (a) are similar in appearance to the nodular type I colonies observed at Burrawang. Figure 3.33 (b) shows an area approximately 10 cm from the drip edge upon which less fungal coverage is evident. Morphological phenomena typical in Kapar are seen in the top and bottom left of the picture (circled). At the top left, a nodular colony, similar to type I, of lighter brown colour than are typically present at Burrawang can be observed. In the bottom left of (b), a colony can be seen extending hyphae. As discussed above, this is infrequently seen at Burrawang. Hyphal growth at Kapar is discussed further below.

Figure 3.33: Two areas of panel M-72W-5, exposed for 72 weeks at Kapar. (a) shows an area of high fungal growth and colonies resembling type I. (b) shows alternate morphologies typical of the Kapar exposure site, namely, lighter coloured colonies resembling type I and hyphal growth.
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Up to approximately 100 weeks of exposure at Kapar, the panels show similar morphologies to Burrawang. After this, however, prevalent morphologies diverge. Figure 3.34 shows an area of a panel exposed for 96 weeks (a) and one exposed for 104 weeks (b). In Figure 3.34 (a), morphologies are similar to those shown in Figure 3.33, however, some hyphal growth is also seen extending from the middle of the image to the right edge. Notably, this hypha appears to be originating from the central type I colony. In another panel, collected after 104 weeks of exposure (b), extensive hyphal networks are seen in several locations on the panel. After 120 weeks of exposure, between 30 and 50% of the panel shows the kind of extensive hyphal networking shown in Figure 3.34 (b), with the majority of this concentrated in the centre of the panel. This supports earlier observations made by BlueScope scientists\textsuperscript{[139]} that fungal coverage increases dramatically in South East Asia after three years of exposure.

![Images from Kapar panels (a) M-96W-5 and (b) M-104W-5 at 50 × magnification showing hyphal development.](image)

Particles similar to those ‘abnormal morphologies’ noted at Burrawang have also been observed on panels collected from Kapar. The morphology seen in Figure 3.28
(i) is also observed at Kapar, as shown in Figure 3.35 (a) (circled in red). These were frequently seen on Kapar panels throughout the 120 week period in which they were observed. Like Burrawang, these particular abnormal morphologies have not been observed to expand in size. As the Kapar colonies are not monitored over time, it is not known if they shrink and fade from the surface as they do at Burrawang. Also evident in Figure 3.35 (a) are the light coloured colonies (circled in blue), similar to type I, and hyphal networks described above. Additionally, algal growth can be seen on the Kapar samples. This is located typically no more than 3 cm from the drip edge. Algal growth is seen, in Figure 3.35 (b), to localise around and perhaps adhere to the fungal hyphae.

![Abnormal morphologies observed at samples harvested from Kapar after 128 weeks of exposure. (a) shows lighter coloured type I resembling colonies and hyphal branching in addition to abnormal morphology (i) (circled), as observed on Burrawang samples. (b) shows green algal growth near the drip edge of the panel.](image)

3.3.5. **Total solar reflectance**

TSR of panels exposed on the Burrawang hot rack was measured every four weeks with the first TSR sample exposed four weeks after the first ‘batch 1’ sample,
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December 2012. Test panels in ‘Whitehaven’ colour were mounted on hot racks with one panel exposed every 4 weeks beginning on 17/12/2012 and concluding 26/08/2014. TSR measurements were continued every four weeks until 07/04/2015. Figure 3.36 compares the TSRs of four individual samples over the first year of their exposure at Burrawang. Two samples are exposed in December, prior to the warmer months and two in May prior to the colder months. All samples start with a TSR value of 76.7 ± 0.3 % and after a year of exposure, all have TSR values of 73.3 ± 0.3 %. However, the samples exposed in December show a more rapid drop in TSR in the first 20 weeks of exposure (a gradient of −0.16, as seen in Figure 3.36), in contrast to the May exposed samples which decrease more gradually over the first 20 week of exposure (a gradient of −0.05, as seen in Figure 3.36). Between weeks 24 and 52, the May exposed samples show a steady decrease (a gradient of -0.06) whilst the December exposed samples show a much slower rate of TSR decrease (a gradient of -0.02). Gradients shown in Figure 3.36 were calculated by graphing the average of both data points from common exposure months (e.g. May 2013 and May 2014 or December 2012 and December 2013). At weeks where only one data point existed, this was used instead of the average. TSR changes of all samples are shown in Appendix B, Figure B 2 and Figure B 3.

‡‡‡‡ TSR measurements prior to May 2014 were conducted by V. Chapman of BlueScope.
Figure 3.36: Change in TSR over the first 52 weeks of outdoor exposure. Samples exposed in December (prior to warmer months) are contrasted with samples exposed in May (prior to cooler months).

No targeted correlation experiments were conducted on the relationship between TSR and area coverage. Panels for TSR analysis were mounted on hot racks as the panel sizes required by the UV-Vis Spectrophotometer were not conducive to mounting on the normal racks. Additionally, Whitehaven colour was chosen for this portion of the work as it was thought to produce the most significant TSR changes.
3.4. Conclusions

Microscopic observations of growth trends on COLORBOND® Steel substrates are a simple, fast and cost effective method of comparing the severity of microbial infestation on panels installed outdoors at different geographical locations. However, in order to create a benchmark against which laboratory infestation models and the effect of mitigation strategies can be compared, a study of the typical behaviour of fungal colonies in outdoor conditions must be established.

Time-lapse observations have revealed that spore settlement and initial adhesion at Burrawang occur exclusively during summer and autumn. Future studies seeking to assess adhesion to substrates and growth at this location should expose surfaces prior to summer, preferably in October. This timing would take full advantage of both the peak spore settlement and peak growth periods.

More rapid growth is experienced in warmer months of the year and there is no evidence to suggest that attachment is linked to rainfall. Detachment of colonies may be linked to rapid growth and hence to warmer environmental conditions, given that detachment occurred exclusively in the summer months and is more prevalent on ‘hot rack’ samples.

The majority of colonies at the Burrawang site are nodular shaped colonies of a dark brown colour. Circular shaped colonies are also frequently observed although hyphal branching is rare. Conversely, the Kapar site shows lighter coloured nodular shaped colonies for the first two years of exposure and extensive hyphal branching networks become common after this time.

Comparisons of the fungal area coverage of both high and standard gloss panels at
Burrawang and standard gloss panels at Kapar reveal that standard and high gloss panels do not experience significantly different levels of fungal infestation nor do panels exposed at the same time of year, one year apart. Bleaching studies of Kapar samples showed that, after the first few weeks, microbial growth took over from dirt adhesion as the main contributor to surface darkening. This highlights the importance of the development of an antimicrobial strategy in the South East Asian market.
Chapter 4: **LIPID PROFILING OF LABORATORY CULTURED FUNGAL SPECIES – A PRELIMINARY ANALYSIS**
4.1. Introduction

Lipid profiling is capable of differentiating between species and even strains of organisms, based on their unique combination and relative abundances of lipids (see Sections 1.7 and 1.8). In general, phospholipids and sterols are the major lipid components of fungi[109] and depending on species, growth stage and environmental conditions, generally comprise about 17 % dry weight of the organism[109].

One classification of lipid that is particularly useful in this regard and also lends itself to mass spectrometric analysis is the glycerophospholipids (GPLs). The most abundant GPLs in filamentous fungi are phosphatidylcholine (PC) and phosphatidylethanolamine (PE)[109]. In some species, phosphatidylserine (PS) and phosphatidylinositol (PI) comprise a significant amount (typically between 5 – 10 % of total GPL). Comprehensive GPL profiles of many fungal species have been obtained (see Section 1.7.1). It is important, however, that comparisons between species are conducted within the same environmental parameters.

As concluded in Section 2.5, further work is required to facilitate identification of organisms by lipid profiling. Hence, this chapter details preliminary work seeking to compare the GPL profiles of three ubiquitous fungi using the analytical techniques laid out in Section 1.8.3. Organisms were grown under identical conditions, which are potentially responsible for the fouling of COLORBOND® Steel substrates. Profiles were statistically contrasted in order to understand how usable this tool is to differentiate fungi of the Ascomycota phylum based on their lipid profiles.

Several organisms potentially responsible for the microbial proliferation on COLORBOND® Steel substrates were identified (See Table 2.1). Based on these identifications, and the availability of high quality samples, C. cladosporides, A.
*alternata* and *E. nigrum* were chosen for lipid profiling.
4.2. Materials and Methods

4.2.1. Reagents

HPLC grade methanol (MeOH) and chloroform (CHCl₃) were obtained from Crown Scientific (Sydney, NSW, Australia) and used without further purification. Ammonium acetate (NH₄OAc) was obtained from Ajax Chemicals (Sydney, NSW, Australia).

4.2.2. Biological specimens of laboratory grown organisms

MeOH extracts from *Alternaria alternata* (A. alternata), *Cladosporium cladosporides* (C. cladosporides) and *Epicoccum nigrum* (E. nigrum) cultures were each provided in triplicate, courtesy of Dr Tran Huyhn (UNSW) and were prepared as described below. Fungi were streaked out on malt extract agar plates from frozen stock. They were inoculated in Peptone Yeast Glucose (PYG) broth and the liquid cultures of fungi were incubated at room temperature for a period of three to four days, until growth was seen. Both were shaken at 200 rpm and washed three times with sterile Milli Q water and suspended in ~5 mL of HPLC grade MeOH.

4.2.3. Mass spectrometry

Methanolic extracts of biological specimens were centrifuged (1500 g for 10 minutes) and a 80 µL aliquot of the top layer was combined with 80 µL of MeOH:CHCl₃ (2:1) containing NH₄OAc (15 mM) in a 96 well plate. This plate was placed into the Triversa NanoMate® autosampler for MS analysis. Spectra were
obtained using Analyst Software (AB Sciex) using methods described in Section 2.2.5.2.3.

4.2.4. Data analysis

Raw spectra were deisotoped and the sum molecular composition or ‘brutto lipids’ were identified using Lipidview™ Software (AB Sciex). Deisotoping removes complications due to the presence of isotopes by collecting the peaks arising from different isotopes of the molecule and attributing them to that molecule. The ‘brutto lipid’ does not take into account the length of, or presence of double bonds in individual acyl chains and reflects only the sum of atoms in the acyl chains present in the molecule.

Glycerophospholipid classes, PE, PC, PS, PA and PI were processed in Lipidview with mass tolerance: 0.3, minimum S/N: 5 and minimum percent intensity: 1 in the \( m/z \) range 600 to 900. Relative abundance of lipids was calculated based on peak area and data was normalised for each sample to give a percent composition of lipid class for each brutto lipid identified. A table of brutto lipids and their percentage of the total lipid class was exported to Microsoft Excel 2010 where it was normalised to percentage of total identified lipids in each lipid class. Brutto lipids identified in the PE, PC and PS lipid classes were used to create a Principle Components Analysis (PCA) plot (Pearson (n)) using Microsoft Excel 2010 with XLSTAT\textsuperscript{155} statistical add on software. Normalised data was imported in the statistical software program, SPSS statistics software\textsuperscript{156} where statistical significance was investigated using one way ANOVA and Tukey post hoc tests.
4.3. Results and discussion

4.3.1. Data processing

The glycerophospholipid classes PE, PC and PS were the most easily detectable in the samples and produced scans with consistently high S/N. The glycerophospholipid classes PG, PI and PA produced spectra of varying quality, and are thus excluded from the following comparison.

To evaluate the headgroup scan data, raw spectra were deisotoped, and identified at the brutto level using LipidView™ software. LipidView™ is a processing program enabling molecular characterisation and quantification of lipid species from mass spectral data. Using LipidView™, it is possible to search through all peaks appearing in PC headgroup scans which correspond to ionised PC lipids. The program deisotopes and tabulates these assignments for all samples along with their corrected peak intensities. Exporting this table into Microsoft Excel allows the corrected intensities of the peaks to be normalised to the sum of all intensities, effectively expressing each lipid ion as a percentage of the total PC lipids in the spectra. Average normalised ion intensities were calculated from three replicates, as were standard deviation and standard error.

4.3.2. Principle component analysis

Principle component analysis (PCA) is a statistical method of comparing complex populations with multiple variables. PCA determines linear combinations of the original variables to explain the variance in the data. The multiple variables are then reduced to the two most significant variables, or principle components, (F1 and F2)
and can be displayed on a two dimensional score plot known as a PCA plot. F1 is the principle component that explains the most sample variation and F2, the second most.

Figure 4.1 shows the three replicates of the three organisms presented in PCA plots based on the lipid classes, PC (a), PE (c) and PS (e). The first two principle components represented 77.21 % (PC lipid class), 74.76 % (PE) and 75.79 % (PS) of the variability between the nine samples, indicating that most of the sample variation is accounted for by a single plot. These plots show that in the case of the PC (Figure 4.1 (a)) and PE (Figure 4.1 (c)) lipid classes, the samples clustered into 3 distinct groups by species. In both plots, C. cladosporides clustered most tightly. Better separation of groups was noted in the PC plot (Figure 4.1 (a)). In the PS lipid class plot, no grouping of species was evident.

Figure 4.1 (b), (d) and (e) show the PCA loadings plots for PC, PE and PS lipid classes respectively. These graphically represent the contribution of individual lipid species to the two principle components shown and can therefore be used to identify the lipids that vary the most between fungal species. For example, in Figure 4.1 (a), A. alternata is right-shifted, representing a high score for F1. In the loading plot shown in Figure 4.1 (b), the lipids species contributing most to a positive F1 score are PC(36:5) and PC(36:6).
Figure 4.1: Principle component analysis (PCA) plots constructed using (a) phosphatidylcholine (PC), (c) phosphatidylethanolamine (PE) and (e) phosphatidylserine (PS) data in XLSTAT. The influence of the individual lipids within each lipid class are
shown in (b) (PC), (d) (PE) and (f) (PS). ‘F1’ denotes the first principle component, or the component representing the most variation between samples. ‘F2’ is the second principle component and represents the second most variation.

Given that the PC and PE PCA plots grouped samples into fungal species, PE and PC lipids were pooled together to construct the PCA plot shown in Figure 4.2. A PCA plot incorporating all identified PC, PE and PS lipid species was also constructed and showed a similar grouping of samples of each fungal species to that shown in Figure 4.2 (a) (see Appendix C). When PE and PC lipids are used together, 49.00 % of variance is explained by F1 (x-axis) and 22.42 % by F2 (y-axis), amounting to a total 71.42 % of variance.

Figure 4.2: Principle component analysis (PCA) plot constructed using identified PE and PC lipids in XLSTAT. The influences of the individual lipids on the two most significant principle components (F1 and F2) are shown in (b).

It is evident in the loading plot shown above (Figure 4.2 (b)) that PC and PE lipids having the same fatty acyl composition frequently cluster together, implying that fatty acyl composition is similar in both PC and PE lipid classes for each organism. For example, *A. alternata* is right-shifted (Figure 4.2 (a)), having a positive F1 value.
The lipids which most strongly contribute to a positive F1 value are PE(36:5), PE(36:6), PC(36:5) and PC(36:6), as shown in the far right of Figure 4.2 (b). Similarly, PE(34:1), PE(34:2), PE(34:3), PC(34:1), PC(34:2) and PC(34:3) group to the far left of Figure 4.2 (b).

4.3.3. Statistical analysis

Deisotoped, normalised data was imported into statistical software, SPSS. There were no outliers in the PE, PC or PS data sets, as assessed by inspection of a boxplot for values greater than 1.5 box lengths from the edge of the box. The percent relative abundance of each lipid within the three lipid classes was normally distributed for all three fungal species, with the exception of A. alternata PE(34:1) (p = 0.02), as assessed by the Shapiro-Wilk’s test (p = 0.05). ANOVA analysis followed by Tukey post hoc testing at 99 % confidence level was used to assess differences between the normalised lipid abundances in the three organisms. Lipids identified in the three organisms tested were displayed graphically using Excel, as shown in Figure 4.3, where error bars represent standard error.
Chapter 4: Lipid profiling of laboratory cultured fungal species – a preliminary analysis

Figure 4.3: A comparison of the phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidyserine (PS) lipid classes of the fungi, *A. alternata*, *C. cladosporides* and *E. nigrum*. Error bars show standard error and an * indicates statistical differences at the 99% confidence level, as identified using SPSS.

The PC and PE lipid classes showed similarities. The most abundant PC lipid in all three organisms was PC(36:4) and PE(36:4) was the most abundant PE lipid. The distribution of PC and PE lipids in all organisms was similar with the next highest abundance lipid being PC(34:2) and PE(34:2). In contrast, the most abundant PS lipid in all organisms was PS(34:2), followed by PS(36:4). There were also no differences noted between the PS lipids.
Chapter 4: Lipid profiling of laboratory cultured fungal species – a preliminary analysis

The results indicate difference between *A. alternata*, and the other two organisms in lipids PC(34:1), PC(36:3), PC(36:5) and PE(36:3). As noted earlier, the right-shifting of *A. alternata* in the PC PCA plot (Figure 4.1 (a)) can be explained by the higher relative abundance of PC(36:5) and PC(36:6). As shown in Figure 4.3, these lipid species comprise a greater proportion of PC lipids in *A. alternata*, than the other fungal species.
4.4. Conclusions

Glycerophospholipid classes PC, PE and PS were detected in the three filamentous fungi tested. Differences exist between organisms in several lipids of the PC and PE classes. PS lipids, although detectable in all three organisms analysed here, did not show statistically significant differences between organisms. PCA plots based on combined PE and PC lipid data in addition to PCA plots based on PE and PC lipid data, respectively, grouped replicates of organisms.

The PC and PE lipid classes were instrumental in differentiating between these organisms, while the PS lipid class showed little variation between the species analysed. These results suggest that lipid profiling is able to identify differences in lipid profile, even between similar organisms.
Chapter 5: **LIPID PROFILING OF ORGANISMS INFESTING COLORBOND® STEEL SUBSTRATES USING MASS SPECTROMETRY**
5.1. Introduction

As discussed in Chapter 3, optical observations of outdoor exposed COLORBOND® Steel test panels show a diversity of fungal morphologies. There have been variations in the colonies observed, both in their growth rates and morphological appearance. This diversity is observed between different geographical locations, in different seasonal environments, upon different surface roughnesses and at different durations of outdoor exposure.

The work detailed in this chapter attempts to marry the optical observations detailed in Chapter 3 with the lipid profiling technique discussed in Chapter 4. Chapter 4 demonstrated that differentiation of fungal species, theorised to be the main infesters of COLORBOND® Steel substrates, is possible through mass spectrometry based lipid profiling. Although the same cache of lipids are observed, statistically significant differences are observed between relative abundances of PC and PE lipids. In this chapter, the lipid profiles extracted from panels exposed outdoors are contrasted in complement to the optical observations detailed in Chapter 3.
5.2. Materials and Methods

5.2.1. Reagents

Reagents were obtained as detailed in Section 4.2.1.

5.2.2. Biological specimens obtained from outdoor exposed COLORBOND® Steel test panels

Organisms were obtained directly from the surface of COLORBOND® Steel test panels following outdoor exposure at BlueScope exposure sites located in Burrawang (Australia) and Kapar (Malaysia), as detailed in Section 2.2.3. Replicates were obtained from sections of the same exposure panel, as depicted in Figure 2.9.

5.2.3. Mass spectrometry

Organisms were removed from COLORBOND® Steel panels using the ‘swabbing’ technique detailed in Section 2.2.5.2.2. Methanolic microbial extracts were further prepared using the method described in Section 4.2.3.

5.2.4. Data analysis

5.2.4.1. Data processing

Data were processed using a combination of LipidView, Microsoft Excel 2010 with XLSTAT statistical add on software and SPSS statistical software using the method described in Section 4.2.4.

As in Chapter 4, the glycerophospholipid classes PE and PC were detectable in the
samples and produced high S/N. PC was more easily detectable and thus, more data is available from this lipid class. Targeted head group scans for the glycerophospholipid classes PS, PG, PI and PA were performed however S/N was consistently low.

Raw spectra of headgroup scan data were deisotoped and identified at the brutto level, as mentioned in Section 4.3.1. Average normalised ion intensities, standard deviation and standard error were calculated from a minimum of three replicates.

5.2.4.2. Statistical processing

Deisotoped, normalised data was imported into SPSS statistical software. Outliers were detected by inspection of boxplots generated by SPSS. Samples with multiple extreme outliers were excluded from analysis§§§§. Less extreme outliers (> 1.5 and < 3 box lengths from box) were not excluded*****. The percent relative abundance of each lipid within the PC lipid class was normally distributed for most sample sets, as assessed by the Shapiro-Wilk’s test (p > 0.05). Due to the limited number of replicates available, in cases where the data was not normally distributed, statistical analysis was performed regardless. Brutto lipids identified in the environmental samples analysed were the same as those identified in the laboratory grown fungi detailed in Chapter 4. The normalised abundances of these lipids were displayed graphically using Excel, where error bars represent standard error.

§§§§ One sample: M-24W-5-C.

***** Two samples: B-24W-4-B and B-28W-4-C.
5.3. Results and discussion

5.3.1. Comparison of environmental samples

5.3.1.1. Temporal effects on lipid profile

Lipid profiles of Burrawang ‘batch 1’ samples (exposed 19/11/12) were compared at time points 24, 28, 32 and 100-104 weeks. Two B-100W samples and one B-104W sample were pooled to provide a sample set where n = 3. The PE lipid profile of sample B-28W is not shown due to fewer than three high quality samples being obtained.

The majority of significant differences exist between the samples which differ in exposure time by more than a year and this is most evident in the PE lipid class. A PCA of both the PC and PE data was performed as described in Section 4.3.2 and the resulting PCA plots are shown in Figure 5.1. Three replicates from each time period were plotted based on the PC (a) and PE (b) lipid classes. The first two principle components explained 65.96 % of variance (PC) and 79.27 % (PE), indicating that more of the sample variation was accounted for by the PE plot. In both PE (a) and PC (c) plots, there is no clear clustering of the 24, 28 and 32 week samples. In both plots, the 100 – 104 week samples are right shifted compared to the other samples (with the exception of B-28W in the PC plot). In the PE plot, this is negatively correlated to the amount of PE(36:4), which is significantly lower in the 100 – 104 week sample relative to the others, as discussed above. In this set of samples, the variation in relative abundance of PE lipids is significant enough to separate them into two groups of approximately six months and approximately two years of exposure.
outdoor exposure.

Figure 5.1: Principle component analysis (PCA) plots constructed using (a) phosphatidylcholine (PC) and (c) phosphatidylethanolamine (PE) data in XLSTAT. The influences of the individual lipids within each lipid class are shown in (b) (PC) and (d) (PE). ‘F1’ denotes the first principle component, or the component representing the most variation between samples. ‘F2’ is the second principle component and represents the second most variation.

Figure 5.2 shows PC and PE lipid profiles. Statistically significant (p < 0.01) differences are seen in the abundance of PE(36:4) between both the 24 and 32 week samples and the 100-104 week sample. Small variations between samples are also
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Evident in the low abundance (comprising < 10% of total lipid class) lipids, PC(36:5), PC(36:6), PE(34:1) and PE(36:6).

![Figure 5.2: Phosphatidylcholine (PC) (a) and phosphatidylethanolamine (PE) (b) lipid profiles of samples from the Burrawang ‘batch 1’ set, collected at various time points. PE lipid profile of ‘28 weeks exposure’ is excluded due to insufficient quality PE spectra from this time point. Error bars show standard error. * denotes statistical significance (p < 0.01).](image)

The variation in ‘Winter 2012’ samples exposed at Burrawang on 28/5/12 was also compared, as shown in Figure 5.3. PE and PC lipid profiles of samples harvested in triplicate were compared after 29 and 53 weeks of outdoor exposure. In this case, no statistical variation was observed between any of the PC lipids identified, however statistical differences were observed in PE(36:4) and the low abundance lipid, PE(36:4). Like the lipid profiles shown in Figure 5.2, the most abundant PC and PE lipids were PC(36:4) and PE(36:4) respectively and the major source of sample
variation was in the relative abundance of the lipid PE(36:4).

In contrast to the samples examined in Figure 5.2, the older sample exhibited a relative increase in the proportion of PE(36:4). In addition to the duration of exposure, the samples were also exposed and harvested in different seasonal conditions. The samples shown in Figure 5.3 were harvested in summer (29 weeks) and winter (53 weeks) and the panels exposed between 24 and 32 weeks duration were collected in the winter months, whilst the 100 – 104 week panels were collected in summer. The influence of seasonal variation is addressed in Section 5.3.1.3.

Figure 5.3: Phosphatidylcholine (PC) (a) and phosphatidylethanolamine (PE) (b) lipid profiles of samples from the Burrawang ‘Winter 2012’ set, collected after 29 and 53 weeks of exposure. Error bars show standard error. * denotes statistical variation at the 99 % confidence level.

A comparison of PC and PE lipid profiles was performed on Kapar samples after 20
and 134 weeks of outdoor exposure, as shown in Figure 5.4. Statistically significant differences in percent lipid abundances were seen between the 20 and 134 week samples in lipids PC(34:1), PC(36:1), PC(36:3), PC(36:4), PC(36:6), PE(34:2), PE(36:3) and PE(36:5). In both PC and PE classes, the older sample showed a relative decrease in PC and PE (36:3) and an increase in PC(36:4) and PE(34:2).

![Graph showing lipid profiles](image)

Figure 5.4: Phosphatidylcholine (PC) (a) and phosphatidylethanolamine (PE) (b) lipid profiles of samples from the Kapar sample set, collected at 20 and 134 weeks of outdoor exposure. Error bars show standard error. * denotes statistical variation at the 99% confidence level.

Unlike the Burrawang samples (both ‘batch 1’ and Winter 2012), Kapar samples differed in the most abundant PC lipid, PC(36:4), over time. While a variation was seen in Burrawang samples in the most abundant PE lipid, PE(36:4), this lipid’s relative abundance did not change over time in the Kapar samples. Despite these
differences, the most abundant PE and PC lipids (PC(36:4) and PE(36:4)) are the same as the Burrawang samples.

5.3.1.2. Geographical impact on lipidome

Samples obtained from Burrawang ‘batch 1’, after 24 or 28 weeks of outdoor exposure were pooled together††††, as were samples obtained from Kapar after a period of 20 and 24 weeks of exposure‡‡‡‡. These two data sets were used to investigate the influence of geographical location on lipid profile over a 20 – 28 week duration of outdoor exposure and are graphed in Figure 5.5. Differences in relative lipid abundance were observed between 4 of the 9 PC lipids identified (PC(36:2), PC(36:3), PC(36:4) and PC(36:5)) and one PE lipid (PE(36:5)). Both PE and PC profiles of the Burrawang samples are highest in the (36:4) lipid, with (36:3) being the next most abundant in both lipid classes. Notably, however, Burrawang samples contain more (36:4) relative to (36:3) compared to the Kapar samples.

†††† Pooled samples: B-24W-4 ×3 (PC and PE) and B-28W-5 ×3 (PC), B-24W-4 ×3 (PE).
‡‡‡‡ Pooled samples: M-20W-5 ×3 (PC and PE) and M-24W-5 ×3 (PC), M-20W-5 ×3 (PE).
Chapter 5: Lipid profiling of organisms infesting COLORBOND® Steel substrates using mass spectrometry

Figure 5.5: Phosphatidylcholine (PC) (a) and phosphatidylethanolamine (PE) (b) lipid profiles of samples from Burrawang ‘batch 1’ and Kapar, both exposed in late 2012 and collected at between 20 and 28 weeks of exposure. For PC, n = 6 and PE n = 3. Error bars show standard error. * denotes statistical variation at the 99% confidence level.

After a period of two years at Burrawang and Kapar respectively, significant variation exists between 4 of the 16 lipids analysed, PC(34:2), PE(34:2), PE(36:2) and PE(36:3), as shown in Figure 5.6. In contrast to Figure 5.5, in which most variation was present in the PC lipid profile, after an exposure period of > 2 years, most variation exists between PE lipids. The Kapar samples, were higher in the lipids PC(34:2), PE(36:2) and PE(36:3) and lower in PE(34:2) than the Burrawang samples. After two years, the Burrawang and Kapar samples showed similar proportions of most abundant lipids of each class, PC(36:4) and PE(36:4), both comprising approximately 70 and 40% of total lipid class respectively.
Figure 5.6: Phosphatidylcholine (PC) (a) and phosphatidylethanolamine (PE) (b) lipid profiles of samples from Burrawang ‘batch 1’ and Kapar, both exposed in late 2012 and collected after a period of > 2 years. Error bars show standard error. * denotes statistical variation at the 99 % confidence level.

5.3.1.3. Seasonal variation at Burrawang exposure site

Panels exposed in November 2012 (batch 1) and May 2012 (Winter 2012) at Burrawang (see Section 2.2.3.2.1) were compared after similar exposure times to assess the influence of season on the lipid profiles of species present. These time periods were chosen to minimize overlap of time periods. Hence, any species profiled would have settled in different months (with the exception of species settled between 19/11/12 and 17/12/12 in which both the ‘batch 1’ panels and Winter 2012 panels were simultaneously exposed).

A comparison of Burrawang ‘batch 1’ 24 weeks (collected 6/5/13) and ‘Winter 2012’
29 weeks (collected 17/12/12) are shown in Figure 5.7. Of the PC lipids identified, the percentage relative abundance of one, PC(36:5), was found to differ between the sample sets. PC(36:5), however, is a relatively low abundance lipid at between 5 and 10% of total PC lipids identified. This low variation suggests that seasonal environmental variation does not influence the PC and PE lipid profiles of these organisms. It may also suggest that there is not a significant difference in the organisms settling and propagating on the surface during the winter-spring and summer-autumn seasons.

Figure 5.7: Phosphatidylcholine (PC) (a) and phosphatidylethanolamine (PE) (b) lipid profiles of samples exposed at Burrawang in May 2012 (‘Winter 2012’) and in November 2012 (‘batch 1’), highlighting seasonal variation in infesting species. Error bars show standard error. * denotes statistical variation at the 99% confidence level.
5.3.1.4. Lipidomic changes at Burrawang based on surface hydrophilicity

Standard and high gloss samples exposed as part of Burrawang ‘batch 1’ from between 28 and 32 weeks were contrasted (Figure 5.8). Both PC and PE lipid profiles showed no statistically significant differences between the sample sets, supporting the conclusions drawn based on optical observations of high and standard gloss substrates (Section 3.3.2) that standard and high gloss substrates do not result in variation between infesting species.

Figure 5.8: Phosphatidylcholine (PC) (a) and phosphatidylethanolamine (PE) (b) lipid profiles of samples from Burrawang ‘batch 1’, highlighting variation in infesting species based on different surface roughnesses. Error bars show standard error. * denotes statistical variation at the 99 % confidence level.
5.4. Conclusions

PE and PC lipid profiles were used to compare microbial growth taken from COLORBOND® Steel test panels exposed outdoors. The test panels varied in their duration of outdoor exposure, geography of exposure sites and in the environmental conditions at the time of exposure and collection.

The most abundant PE and PC lipids in all samples were PE(36:4) and PC(36:4) respectively. PE(36:3) and PC(36:3) were also evident in all samples, in addition to (34:2) and (36:2) species. The commonalities between the lipids identified and their relative abundances between all samples suggest similarities between the species infesting this particular substrate in both the Australian and South East Asian environments, supporting the findings of Chapter 3.

Comparisons of lipid profiles from Burrawang panels with different exposure durations indicated greater variation in PE lipids over PC lipids. Samples from Kapar with different exposure durations differed in the abundance of several lipids from both the PC and PE lipid classes. No clear links could be drawn between exposure duration and trends in the lipid profile. The lipid profile differences observed at < 2 years and > 2 years of exposure reflect the observation made in Chapter 3 that distinct morphological changes occur after two years of exposure at Kapar.

It is possible that the temporal variations observed were environmentally influenced. However, the seasonal comparison of Burrawang samples conducted in this chapter suggests a minor influence. There is insufficient data available from this study to investigate this phenomenon in depth.

Comparisons of the lipid profiles at both exposure sites, Burrawang and Kapar, did
not uncover consistent geographically influenced changes in lipid profiles. While some differences between Burrawang and Kapar were observed, these were not consistent at the two time periods investigated, suggesting an influence from other factors. Finally, growth on high and normal gloss panels exposed at Burrawang did not vary, supporting the optical observations of growth on these surfaces, detailed in Chapter 3.

The variables investigated in this chapter are not comprehensively indicative of those present in the environment, nor can they be tested independently. While an effort has been made to differentiate samples based on easily quantifiable variables, it is essential to note that any changes observed may be due to a wide range of uncontrollable environmental variables. The results presented in this chapter have relevance as a preliminary baseline for future understanding of lipid profiling of these organisms, however, laboratory controlled experiments are required before this is possible.
Chapter 6: **CONCLUSIONS, FUTURE DIRECTIONS AND COMMERCIAL IMPLICATIONS**
6.1 Conclusions

For over 30 years, microbial growth has been identified as a problem affecting COLORBOND® Steel substrates. Microbial proliferation on these surfaces has both aesthetic and energy efficiency implications, hence, there is a strong commercial incentive to limit its growth. In the past, however, there has been a limited understanding of the organisms involved, their behaviour and their impact on the surface.

The overarching aim of this body of work was to develop and deploy methods of detecting, characterising and identifying the microorganisms infesting these unique substrates and uncovering as much as possible about their life cycles. The study detailed in this thesis forms the initial step in comprehending the nature of the microbial proliferation and informing the manner in which future inquiries should proceed.

6.1.1 Methods

In order to investigate the extent and effects of microbial growth on COLORBOND® Steel substrates, sets of test panels were deployed at two outdoor exposure sites at Burrawang (Australia) and Kapar (Malaysia), as detailed in Chapter 2. Complementary characterisation methods were deployed upon these samples after various periods of exposure. Of these, optical microscopy was most useful for detection and quantification and, to some extent, for identification. The COLORBOND® Steel substrate lent itself to optical microscopy, being essentially a flat, non-reactive white polymer film which did not break down or change in composition significantly over the duration of the study. Historically, optical
microscopy has been used commercially in assessing microbial growth and other factors in paint performance. This, in addition to its ease of use, speed, low cost and ability to be non-destructive to the surface and microorganisms makes it the preferred test method for assessment of microbial proliferation on COLORBOND® Steel substrates.

Lipid profiling of the infesting organisms using mass spectrometry was also performed. While detection of fungal lipid profiles was possible, quantification and identification presented several obstacles. The efficiency of extraction of fungal colonies from COLORBOND® Steel substrates for lipidomic analysis is poorly understood, as are the interactions between lipids and the surface, presenting problems when utilising internal standards. In order to develop this method as a quantitative technique, a substantial body of work would be required.

A preliminary study of the differential capacity of mass spectrometry based lipid profiling was conducted on three model fungal species, grown under identical conditions in the laboratory (Chapter 4). The results suggested that differentiation of the fungal species based on the PE and PC lipid classes was possible. However, the identification and differentiation of environmental samples using this method faces several limitations, notably the lack of lipid profile reference databases for a wide range of organisms and the insufficiently reported effect of environmental variables on lipid composition (Chapter 5). Future work on this topic is discussed in Section 6.2.

6.1.2 Learnings

Colonies observed on Burrawang-exposed test panels consist of two main colony
types, circular and nodular, both of a dark brown colour. Over time these expand in area and sparse hyphal growth is occasionally observable. Nodular light brown colonies are present on Kapar-exposed panels during the first two years of exposure. The findings of this work also support the previous observations that extensive hyphal growth occurs in South East Asian environments after an exposure period of approximately two years, a phenomenon which is not well understood.

The time-lapse microscopy performed on panels exposed at Burrawang and presented in Chapter 3 has not been previously attempted on COLORBOND® Steel substrates. The findings represent several advances in the understanding of the life cycle of the infesting organisms. Through analysis of these images, the deposition and subsequent growth of spores has been found to occur exclusively in the summer and autumn months. Furthermore, while several spore morphologies are observed to adhere to the surface, a limited number are able to proliferate and prosper. These successful organisms are able to continuously grow by increasing their area coverage of the surface and may double in size over a period of less than a year, an effect which is accelerated during the warmer months of the year. In general, the results suggest that colony growth is more rapid in warmer conditions, more accelerated growth being observable during the summer months and on the ‘hot’ rack samples. Commercial implications of these findings are discussed in Section 6.3.

Based on the findings of Chapters 3 and 5, it is likely that environmental conditions influence the growth behaviour of organism grown outdoors. The full extent of this effect both on colony morphology and lipid profile is unclear. These environmental influences mean that future outdoor exposures must be stringently controlled in order for outdoor test samples to be comparable to one another. The commercial
implications of this observation for future outdoor testing of COLORBOND® Steel are discussed further in Section 6.3.
6. 2 Future directions

The study detailed in this thesis spans several diverse research areas and incorporates both commercial and scientific concerns. As such, many interesting findings were unable to be adequately pursued. This section addresses research topics which would directly complement the findings detailed in this thesis.

Although commercially routine, the testing of outdoor exposure panels introduces many uncontrollable experimental variables. Stochastic environmental variables are not a major concern for BlueScope’s long term (> 10 years) outdoor paint durability studies but are particularly relevant to microbial growth assessment, given the short exposure time needed for meaningful measurements and observations of microbial growth. Future studies would investigate, in a controlled environment and using species of interest, the impact of pertinent environmental variables on these organisms. This may enable correlations to be drawn between external conditions and morphological and lipidomic trends. Variables to address may include: rainfall, frequency and duration, time between rain and sample collection, nutrient availability and type, aerosol pollution, ambient temperature, panel temperature, ambient humidity and solar radiation. This may help to justify differences observed between exposure sites and at different times of year. It may also have implications for cell robustness and surface adhesion, factors of particular relevance to this work.

Although not reported in this thesis, a preliminary lipid profile study was performed on the organisms profiled in Chapter 4, following a period of starvation and desiccation. Preliminary results showed a notable difference in lipid profile between the lipid profiles of the organisms before and after treatment.

Furthermore, an understanding of the adhesion mechanisms of the problematic
fungal species, specifically the wild type of these organisms, is required. Mass spectrometry based glycomics is a promising way to do this and a study of methods has been recently outlined in the literature\textsuperscript{[157, 158]}. In this way it would be possible to understand from a chemical perspective the nature of the chemical interactions which must be inhibited.

Despite the work in this thesis focusing partially on samples from Kapar (Malaysia), geographical and logistical issues have prevented this region being studied as comprehensively as Burrawang. The findings of this work suggest that, while similarities exist, the colonies in Kapar are distinct, in both morphology and lipid profile, from those in Burrawang. Hence, further study of the temporal progression and identity of colonies in the South East Asian region would be beneficial. In order for a comprehensive antimicrobial strategy to be developed, which is effective in both locations, this relationship should be better understood.

There are limitations to what mass spectrometry based lipid profiling can currently achieve in terms of identification and differentiation of fungal species due to the lack of comprehensive fungal lipid databases under standardised and fluctuating conditions. Whilst some studies have developed lipid profiles for a select suite of organisms and conditions, further development of this field would require lipid profile libraries to be made available for comparison.

This work has begun to describe the steady state infestation of COLORBOND\textsuperscript{®} Steel surfaces and the associated consequences. This work does not, however, address the phenomenon of spectacular isolated microbial outbreaks which are often the subject of customer complaints. The localised extreme infestations are suspected to be due to particular types of local vegetation (e.g. overhanging trees) which harbour either
specific microbial species capable of heightened infestation or provide a higher density of suitable infesters than other locations. Because this form of infestation is more noticeable and aesthetically problematic, future studies would benefit from focusing on locations in which this occurs with a focus on understanding the surrounding flora and the time resolved genesis of infesting species.
6.3 Implications for BlueScope

6.3.1 Assessment of microbial growth

Outdoor exposure testing plays a vital role in BlueScope’s assessment of the durability and performance properties of COLORBOND® Steel products. Various methods already exist for measuring paint durability in field testing experiments. Until now, no such methods were developed for assessment of microbial growth. Using similar methods to those developed and described in this thesis, BlueScope can characterise and quantify infestations. This may have applications to a range of COLORBOND® Steel products, from roofing and other building products to cool room panels, upon which there is concern about a range of microorganisms.

BlueScope’s assessment of COLORBOND® Steel products is also reliant on accelerated testing for assessing the performance of products in short time frames. In this work, the deposition and propagation behaviour of spores settling on the substrate in the outdoor environment has been established. While not a comprehensive study of this behaviour, the increased understanding of factors such as spore density on the surface, duration of adhesion and typical germination behaviour could be used to develop accelerated testing regimes which mimic outdoor conditions.

Such testing protocols could be carried out under standardised conditions in the laboratory and used to rapidly assess antimicrobial surface treatments in tandem with the more time consuming outdoor exposure experiments. Such testing protocols have already begun to be developed at the University of Wollongong as part of the ARC Research Hub for Australian Steel Manufacturing, of which the University of
Wollongong and BlueScope are major contributors. These methods are based on the infesting species already identified and the spore density at which they typically settle outdoors. Preliminary experiments suggest that the initial growth behaviour is very similar morphologically to that observed outdoors at Burrawang.

### 6.3.2 Outdoor exposure testing protocols

Currently, these outdoor testing protocols are tailored towards paint durability studies. This study reiterates the importance of highly controlled outdoor exposure testing of COLORBOND® Steel and suggests that testing protocols employed for assessment of antifouling roofing materials must be distinct from other testing methods such as paint durability testing. In addition to the diverse factors that exacerbate both issues, microbial growth is also more rapid in its progression than, for instance, degradation of the paint film.

Such a protocol has recently been instigated based on the findings of this work and it is hoped that the results of this will further inform the development of antifouling testing, increasing the efficiency and reliability with which potential commercial products can be assessed. For example, given that successful spore deposition occurs in summer and autumn at the Burrawang site, efficient testing regimes in this location would expose test panels in October and assess after a maximum of six and again after a minimum of twelve months. It is important, when considering time frames, to maximise understanding of microbial succession whilst being conscious of costs and commercial advantages of such testing.

This work has highlighted the effect of environmental variables on microbial growth. Hence, care must be taken when making comparisons of test samples exposed in
different locations or at different time periods. Such comparisons may not provide useful information regarding the type or growth stage of the organisms. Additionally, during any comparison of COLORBOND® Steel products, test panels should be exposed in the same location at the same time to make a valid comparison of the surface’s antimicrobial properties.
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Figure A 1: A scatter graph of the 50 × magnification data shown in Figure 2.16 and discussed in Section 2.3.3 (samples exposed at Burrawang in November 2012 as part of ‘batch 1’). Data has been fitted with a linear regression line. A Pearson correlation analysis (p < 0.01) indicates a weak positive correlation between weeks of outdoor exposure and % area coverage.
Supporting information to Chapter 3:

Table B 1: Abnormal morphologies settled on time-lapse panels.

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## Appendix B

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<td>A6</td>
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### Appendix B

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<th>A2</th>
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Figure B 1: The developmental history of a colony, ‘Charlie’, observed on a standard gloss panel which was mounted on a normal rack at Burrawang in November 2012. Images were
Appendix B

gained at 500 × magnification after the panel had been exposed outdoors for a period of (a) 16 weeks, (b) 20 weeks, (c) 24 weeks, (d) 28 weeks, (e) 36 weeks, (f) 40 weeks, (g) 48 weeks, (h) 52 weeks, (i) 56 weeks, (j) 60 weeks, (k) 68 weeks, (l) 72 weeks, (m) 80 weeks, (n) 104 weeks. (o) and (p) shown images at 500 × magnification at 16 and 104 weeks respectively. Settlement and germination of the initial spore occurred prior to 16 weeks (March 2013) (a). Hyphal extension is then observed in addition to the 90° branching of one hypha (b), (c), (d). During the Winter months (June and July 2013), growth slows and the cell wall thickens (e) and (f) and the youngest hypha, at the bottom left, shrivels (g). In the space of 4 weeks between (g) and (h), the organism has put out several new hyphae. This image was taken in November 2013 when the weather at Burrawang was becoming warmer. Also visible in (h) is the presence of a spore in the bottom left corner. This spore goes on to develop into a Type I colony, as seen in (i) – (m). The outline of the now detached Type 1 colony can be seen at the bottom left of the image (n). Little hyphal growth is seen between (i) and (l), during the colder months of the year. Some of the newly developed hyphae narrow. By 80 weeks (m), some hyphae have detached and 24 weeks later (104 weeks), the original colony had broken into fragments which appear to be generating hyphae (n). Images (o) and (p) show the overall growth of the colony followed from 16 (o) to 104 (p) weeks. Images (a) – (f) provided by Dr Philip Barker.
Figure B.2: Change in TSR over time. Panels are exposed four weeks apart starting on 17/12/2012 and TSR is measured every four weeks.
Appendix B

Figure B 3: TSR of samples over a 120 week period. One sample was exposed every month. Samples are colour coded by the season in which they were first collected for analysis (after 4 weeks of outdoor exposure). Red denotes summer, orange: autumn, blue: winter and green: spring.
Figure C 1: (a) A principle component analysis (PCA) plot constructed using all identified lipids of the phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) lipid classes using XLSTAT. The influence of each individual lipid is shown in (b).
Appendix D

APPENDIX D

A movie of time-lapse images:

A time-lapse movie containing all ‘time-lapse’ images referenced in this thesis may be viewed at:

https://youtu.be/0QaoKd0bFlk