Investigation of the microbial communities colonizing pre-painted steel used for roofing and walling

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Summary

Microbial colonization of pre-painted steel, commonly used in roofing applications, impacts their aesthetics, durability and functionality. Understanding the relevant organisms and the mechanisms by which colonization occurs would provide valuable information that can be subsequently used to design fouling prevention strategies. Here, next-generation sequencing and microbial community finger printing (T-RFLP) were used to study the community composition of microbes colonizing pre-painted steel roofing materials at Burrawang, Australia and Kapar, Malaysia over a 52 week period. Community diversity was low and was dominated by *Bacillus spp.*, *Cyanobacteria*, *Actinobacteria*, *Cladosporium sp*., *Epicoccum nigrum*, and *Teratosphaeriaceae sp*. Cultivation based methods isolated approximately 20 different fungi and bacteria, some of which, such as *E. nigrum* and *Cladosporium sp*., were represented in the community sequence data. Fluorescence in situ hybridization imaging showed that fungi were the most dominant organisms present. Analysis of the sequence and T-RFLP data indicated that the microbial communities differed significantly between locations and changed significantly over time. The study demonstrates the utility of molecular ecology tools to identify and characterize microbial communities associated with the fouling of painted steel surfaces and ultimately can enable the targeted development of control strategies based on the dominant species responsible for fouling.

Introduction

Microorganisms in the environment have the ability to colonize almost all surfaces, including manmade surfaces (Rosado, *et al*., 2014). Colonization by these microbial communities can cause degradation of various materials leading to aesthetic and structural damage. Such effects range from pigment based discoloration to staining and even deterioration of structural integrity through microbially induced corrosion of metal and concrete based materials (Ciferri, 1999). Microorganisms are capable of colonizing a wide variety of natural surfaces, and are therefore found on a broad spectrum of manmade products, including paintings, wood, paper, metals and concrete structures (Stephen and MacNaughton, 1999; Blanchette, *et al*., 2004; Michaelsen, *et al*., 2006). The colonization of the walls and air-conditioning ducts of homes and offices is associated with 'sick building syndrome' where the colonizing organisms contribute to the ill health of humans (Skov and Valbjorn, 1987; Wargocki, *et al*., 2000; Burge, 2004). The colonization of interior and exterior surfaces is unsurprisingly associated with a wide range of microbes and in most cases can be habitat specific (Ortega-Calvo, *et al*., 1991; Dubosc, *et al*., 2001; Gaylarde, *et al*., 2007).

The microbial community composition depends on several environmental factors, including temperature and humidity (Gaylarde and Gaylarde, 2005), and the physical properties of the surface. For example, fungi were found more than bacteria on painted surfaces (Gaylarde and Gaylarde, 2005) although cyanobacteria, heterotrophic bacteria and algae were also found on painted surfaces. Algae were more prevalent than cyanobacteria on roofing materials in South Eastern and North Western areas of the United States where it is humid (Gaylarde and Gaylarde, 1999). Fungi and cyanobacteria are able to survive under conditions of high UV exposure and repetitive cycles of desiccation and rehydration (Yancey, *et al*., 1982; Potts, 1994) and their survival under adverse environmental conditions is linked to their ability to form biofilms (Gorbushina, *et al*., 2004). Biofilm formation has been shown to increase the

tolerance of communities to stress (Burmolle, *et al*., 2006; Lee, *et al*., 2014) and this includes protection from desiccation (Flemming and Wingender, 2010) where the hydrated biofilm matrix retains water necessary for the viability of the community.

Once microorganisms have colonized a surface, they can alter the properties of the materials through a number of different processes. The substratum may, for example, be oxidized by the production of organic acids (Gu, *et al*., 1998) as well as nitric and sulphuric acids (Sand and Bock, 1991; Sand, 1997) and the production of such acids along with other metabolic by products may also cause corrosion (Hamilton, 1985; Boopathy and Daniels, 1991). The rate of corrosion has been shown to be considerably higher with mixed microbial cultures than for pure cultures and most fouling in environmental settings involves microbial communities rather than isolated populations (Beech and Sunner, 2004). In addition to surface deterioration, the accumulation of biomass can result in the appearance of dark staining (Berdahl, *et al*., 2008). This has significant impacts on cultural and historical items such as paintings, sculptures and monuments. Damage or discoloration caused by colonization on materials may result in failure of the materials, deterioration of function and customer dissatisfaction, all of which have significant economic impacts as well as social impacts, in the case of culturally important items. While largely aesthetic, discoloration of building materials can also alter the engineered properties of such materials. For example, some roofing materials are designed to reflect solar infrared radiation to reduce heat transfer to the interior of the building (Berdahl, *et al*., 2008). Colonization and discoloration inhibit solar reflection and thus retard the beneficial properties of the materials.

Colonization of roofing materials represents a significant challenge for the invading community. Microbial colonization occurs under all weather conditions, including extremes of temperature, desiccation, intermittent nutrient loading and periods of intense UV exposure (Berdahl, *et al*., 2008). Such habitats may also be relatively limited in nutrients as they are

105 designed for water runoff and to not accumulate environmental materials on their surfaces.
106 Despite representing a relatively extreme habitat, bacteria and fungi can colonize the roofing
107 materials, resulting in the consequences described above. Some microorganisms adapt to
108 such adverse habitats by forming resistant structures such as spores, which can rapidly
109 resume vegetative growth when conditions become favorable again (Willetts, 1971;
110 Nicholson, *et al*., 2000). Additionally, some organisms produce pigments that are protective
111 from the UV and such pigment production also contributes to the aesthetic issues associated
112 with fouling (Quesada and Vincent, 1997; Rossi, *et al*., 2012). While the problem of
113 microbial infestation of building roofs is well known, there is little information on the
114 communities involved as well as their impacts on such materials.

115 To characterize the microbial communities on roofing materials and how such communities
116 change over time, molecular techniques including community sequencing, T-RFLP and
117 fluorescence in situ hybridization (FISH) techniques were used. Panels of pre-painted steel
118 sheeting were deployed at two locations, Burrawang (Australia) and Kapar (Malaysia), on
119 exposure racks and were analyzed at monthly intervals over a period of 12 months. This was
120 performed as two separate time series at the Burrawang site, the first for 12 months and a
121 second for 6 months, deployed one year after the first series was exposed. The correlation
122 between environmental factors and the microbial communities that formed over time was also
123 investigated. The results indicated that these materials were colonized by communities
124 characterized by 216 fungal and 562 bacterial species, but that were primarily dominated by
125 less than 10 species of each domain. When the two sites were compared, the composition of
126 fungi and bacteria were significantly different and they also changed over time. While there
127 were clear patterns in community change over time, environmental factors, such as rainfall
128 and temperature, seemed have no clear effect on the community structure.

129

130 # Results

131 ## Microbial biomass on panels fluctuated over time

132 DNA yields from the Burrawang panels over 12 months also suggested an increase in
133 biomass up to 36 weeks followed by a decrease on older panels (Fig. 1A). FISH analysis
134 illustrated that there were more fungi than bacteria except at week 48 at Burrawang (Fig. 1B
135 and Figs. S1A and S1B). Both bacteria and fungi increased in total surface coverage up to
136 week 20, at which point, there was sudden decrease in total biomass. Subsequently, the
137 biomass increased to approximately 1% surface coverage from week 32 to the end of the
138 experiment. Eight fungi, including *Epicoccum* spp., *Cladosporium* spp. and *Alternaria* spp.
139 and four bacteria, including *Micrococcus* spp. and *Sphingopixis* spp. were isolated from the
140 panels over 12 months (Tables S1 and S2). While the panels were clearly impacted
141 aesthetically at the end of the 12 month trial, no significant deterioration of the material
142 occurs over this time scale.

143 ## The community composition changed over time

144 To compare the variability in the bacterial and fungal community composition between the
145 Kapar and Burrawang sites, T-RFLP analysis was performed on biological triplicates of
146 exposure panels from the Burrawang and Kapar sites. The communities changed over time as
147 shown by analysis using the Bray-Curtis similarity algorithm followed by non-metric
148 multidimensional scaling MDS plots (Clarke, 1993) and PERMANOVA to identify factors
149 responsible for the most diversity between samples. The centroid (the mean lowest
150 dissimilarity among triplicates) distances were used to calculate the average position of the
151 triplicate samples.

152 The MDS plot showed that fungal communities on the Burrawang and Malaysia panels
153 formed two separate groups during the 52 week experiment based on both T-RFLP data (Fig.

2A) and sequencing data (Fig. 2B). The bacterial compositions at both sites shared more similarity than the fungal communities and this observation was again supported by both the T-RFLP (Fig. 3A) and sequencing data (Fig. 3B). At 40 weeks, the bacterial composition was the most similar between the two geographically distinct sites. PERMANOVA analysis of T-RFLP data showed that time and geographical site accounted for the greatest differences in community composition (p=0.001) for the fungi (Table 1). The bacterial communities from the Burrawang and Kapar panels were also affected by the exposure time (p=0.001) but were not affected by location (p=0.493) (Table 2).

The fungal communities were dominated by a limited number of operational taxomic units

There were a total of 287 Operational Taxonomic Units (OTUs) identified for Burrawang time series 1 panels (26 in total) and 216 OTUs identified for the Kapar samples (26 in total). In comparison to the number of OTUs observed for the early (4 weeks) to late (52 weeks) samples, the number of OTUs was constant at 79 for Burrawang time series 1 samples but decreased for Kapar samples from weeks 52 to 55. For the second year of sampling at Burrawang (time series 2), a total of 190 OTUs were identified from seven samples over a period of 36 weeks. The fungal community composition changed over time on all panels and was dominated by four OTUs that represented up to 90% of the total community at both sites. BLAST analysis using Genebank suggested that these four OTUs corresponded to *Teratosphaeriaceae sp.* (97% identity), *Cladosporium sp.* (100% identity), *Epicoccum nigrum* (100% identity) and *Aureobasidium pullulans* (99% identity). For the Burrawang site, *E. nigrum* was present as a relatively high proportion of the community, 30-36%, in the first three months (Fig. 4A). *Cladsporium sp.* was initially present on the Burrawang panels at a high frequency, but subsequently declined in abundance from months one (44%) to four (3.9%). In contrast, the *Teratosphaeriaceae sp.* increased from week 20 onwards (Fig. 4A).

This OTU was also found to be highly abundant in the second exposure series, suggesting that this organism is reproducibly an early colonizer of the pre-painted metal panels at the Burrawang site (Fig. 4C). Approximately 45-80% of the microbial communities from the first three months at the Burrawang site were attributed to *Cladosporium sp.* and *E. nigrum*. The *Teratosphaeriaceae sp.* detected on the Burrawang samples, was also the most abundant species detected in the Kapar samples from the first month, ranging from 40-90% of the fungal community (Fig. 4B).

The abundance of *Cladosporium sp.* on the Burrawang panels correlated with temperature (correlation coefficient r = 0.76) over the 52 weeks incubation period, with a higher abundance in summer compared to winter. There were no other clear relationships between community composition and any of the environmental parameters quantified (Fig. 5A and B).

The bacterial community has low diversity

For the bacterial communities, 562 OTUs were identified for the 52 samples of both sites. For the first set of samples, among these OTUs, 37% were *Bacillus spp.*, 15% were cyanobacteria and 43% were actinobacteria, accounting for 97% of the total community. The most abundant OTU observed on the panels collected in the initial stages of the experiment for both Burrawang (time series 1) (Fig. 6A) and Kapar (Fig. 6B) was assigned to the genus *Cellulosimicrobium*. For the later time points, cyanobacteria were also present in high abundance (Fig. 6A). The abundant bacteria found on the second exposure series (time series 2) at Burrawang were *Streptomyces sp.* and *Veillonella sp.*, suggesting that, in contrast to the fungi, the bacterial colonizers were not consistent between years (Fig. 6C).

Discussion

A number of studies have reported that microorganisms contribute to the damage of man-made structures and materials. However, relatively few of these studies have focused on identifying the species responsible and their patterns of colonization over time. This limitation could be due to the observation that many environmental microorganisms are not readily cultivable in the laboratory. To augment such studies, we have combined culture based studies with molecular techniques to investigate the community composition as it forms on painted steel surfaces over time at two geographically separate sites. Based on Illumina MiSeq sequencing of rRNA gene amplicons, the microbial diversity on panels exposed at either Burrawang or Kapar over one year were studied and was repeated at Burrawang the following year to determine if the pattern of colonization was reproducible. Over 900 OTUs ($\geq$ 97% identity) were identified based on sequencing, while only approximately different 20 bacteria and fungi species were isolated by cultivation, consistent with the observation that that only 0.1 to 1% of environmental bacteria are cultivable on standard media under aerobic conditions (Ferguson, *et al*., 1984). *Cladosporium sp.*, *E. nigrum* and *Aureobasidium pullulans* were the most frequently isolated fungi and this generally agrees with the community sequencing data. Based on the fungal sequencing data, *Cladosporium sp.*, *E. nigrum* and *Aureobasidium sp.* appear to be the initial colonizers of the panels. A comparison of the sequencing data and cultured isolates indicated that these were the only three organisms common to both datasets and thus, the panels may also be heavily colonized by fungal organisms that were not culturable under the conditions used here. After 30 weeks of exposure, the Burrawang panels were dominated by an OTU that most closely matched *Teratosphaeriaceae sp*. This group of fungi belong to the class of Dothideomycetes that includes species that inhabit inhospitable niches such as the surfaces of bare rocks (Quaedvlieg, *et al*., 2014). Members of this group have been isolated from Eucalypt trees in NSW, Australia (Quaedvlieg, *et al*., 2014) suggesting they are present in the natural

environment and this source of the fungus could seed colonization of the panels by transport through the air. Based on the community composition data, future work could seek to recover the some of the more fastidious organisms such as *Tetratospaeriaceae* through the use of more oligotrophic media as opposed to the more general isolation media used here that have typically be used for environmental organisms (Reasoner and Geldreich, 1985; Machuca and Ferraz, 2001; Magnusson, *et al*., 2003)

Bacteria and fungi commonly co-inhabit building materials such as limestone (Mitchell and Gu, 2000) and concrete (Giannantonio, *et al*., 2009). Finding from frescoes and masonry in a Russian cathedral showed that the autotrophic, nitrifying bacteria were found to be the first colonizer on frescos. Heterotrophic microorganisms including bacteria and fungi grew and utilized the cellular components of the first colonizers, this hypothesis was supported by the finding that most of the heterotrophs were able to hydrolyze bacterial and yeast cell walls (Bock, *et al*., 1988; Karpovich-Tate and Rebrikova, 1991). Although there was a high diversity of bacteria, the majority of sequence reads belonged to *Bacillus* and *Cellulosimicrobium spp*. Thus, the community was dominated by a relatively limited set of bacteria. The community sequencing data shows that four bacterial groups dominated both sites, which was supported by the microscopy images indicating that the communities are not morphologically diverse. FISH based analysis suggested that the fungi were the more dominant group based on overall biomass. Hence, the bacteria may not be the primary colonizers of the roof surfaces.

It was observed here that the microbial communities found on the Burrawang panels fluctuated seasonally, with a maximum 28 d mean of 27˚C in mid-summer and a minimum mean of 11˚C in mid-winter. This was supported by one study showing that indoor and outdoor fungi varied according to season, with a maximum count in summer when *Cladosporium sp.* was the most abundant, compared to *Penicillium sp.* and *Aspergillus sp.*

252 that were the dominant organisms in winter (Medrela-Kuder, 2003). In contrast, the samples
253 from Malaysia did not show any apparent differences throughout the year possibly owing to
254 the relatively constant temperatures in Kapar throughout year (27-30˚C). While water is
255 required for growth, our data suggested that there was no correlation between community
256 composition and rainfall and this may suggest that water was not a limiting factor given the
257 relatively high rainfall throughout the year.

258 The roof panels represent a harsh environment, where microorganisms have to contend with
259 high levels of solar radiation, high temperature, intermittent water availability, desiccation
260 and relatively low nutrient inputs. However, despite these challenges, the panels were
261 relatively heavily fouled by the end of the experiment, and the biomass of the fouling
262 community showed a steady increase over time, suggesting that the communities were viable.
263 This survival may be a consequence of adaptation to these habitats, where such communities
264 would experience extended periods of dry weather (Burrawang in particular), suggesting they
265 must be capable of surviving desiccation stress, possibly through production of osmotic
266 protectants such as osmolytes (Gaylarde and Gaylarde, 2005) as well as biofilm matrix
267 components (Flemming and Wingender, 2010). Biofilms consist a consortium of
268 microorganisms such as bacteria and fungi settling in a matrix of extracellular polymeric
269 substances (O'Toole, *et al*., 2000). The individual cells in the biofilms are protected by their
270 special biofilm structures from environmental stresses such as UV, desiccation (Costerton, *et*
271 *al*., 1995; Morris and Monier, 2003).

272 *Bacillus* species often dominate in soil and painting environments, and their survival could be
273 explained by their ability to form spores (Gorbushina, *et al*., 2004). It is therefore not
274 surprising that we also observed a relatively high proportion of *Bacillus* species in the
275 community sequence data, although it remains possible that they are inactive on these
276 surfaces. Finally, it is not possible to rule out that some of the OTUs observed derived from

277 dead cells, where the residual DNA was extracted. Thus, the low abundance organisms could
278 represent those that have been transported, e.g. by wind or rain, to the panels, but were
279 incapable of growth. Further work will be required to determine what proportion of the low
280 abundant organisms are viable and capable of growth.

281 This study provides valuable information on the microbial composition and changes from the
282 roof panels and demonstrated that fungi and bacteria are found in all samples but that the
283 overall community diversity is low, where such communities were typically dominated by six
284 organisms representing 99% of the entire community. The fungal communities were initially
285 colonized and dominated by *Cladosporium sp.*, *E. nigrum* and *Aureobasidium sp.* followed
286 by a change in community composition, what was dominated by *Teratosphaeriaceae sp.*
287 during the later stages at Burrawang. A replicate exposure study one year later showed
288 similar fungal communities colonized the painted steel coupons, while in contrast, there were
289 differences in the bacterial communities between the two replicate studies. These results
290 suggest that a few fungi grew on the panels as the main active organisms and therefore the
291 strategies to fouling control on these roofing materials would be related to limiting the food
292 source and control these active organisms.

293

294 ## Experimental Procedures

295 ### Study site and sampling

296 The samples were collected monthly over 52 weeks in triplicate at Burrawang, NSW,
297 Australia (34.6000°S, 150.5167°E) and Kapar, Malaysia (3.1397°N, 101.3678°E). The
298 Burrawang panels were 9.5 cm x 23.5 cm and the Kapar panels were 11 cm x 13 cm (Fig.
299 S2). Both types of panels were mounted on exposure racks consisting of a series of horizontal
300 wooden slats to which panels are attached by placing them between two rubber washers,

301 tightened with a screw. Coupons with diameter 2.8 cm were punched out from the panels for
302 microscopic observation. The exposure study was repeated in the first half of the subsequent
303 year at Burrawang, NSW.

304 **Temperature and rainfall data collection**

305 To determine the impact of rainfall and temperature, the daily maximum temperature and
306 rainfall data were collected and averaged for the 28 d before the panels were collected. Moss
307 Vale Australian Weather Station (AWS) was used for temperature data as it is closest to
308 Burrawang. Rainfall data was collected from Range Street, Burrawang. All data were
309 extracted from the Bureau of Meteorology website (http://www.bom.gov.au/climate/data/).
310 Temperature and rainfall in Kapar, Malaysia were collected from the Selangor State from
311 Jabatan Meteorologi Malaysia (www.met.gov.my).

312 **Cultivation and isolation**

313 Fungi and bacteria were isolated from samples by swabbing the sample surfaces and
314 incubating on malt extract agar (fungi) and R2A agar (bacteria) as general cultivation media.
315 The agar plates were incubated at room temperature for 3-5 d until growth was observed.
316 Single colonies were then collected on a fresh agar plate for isolation.

317 **Fluorescence *in situ* hybridization (FISH)**

318 After the samples were transferred to the laboratory, the coupons were fixed overnight with
319 4% paraformaldehyde, and kept at 4°C until use. Fixed coupons were then dehydrated in a
320 series of ethanol at room temperature: 50%, 80% and 96% for 3 min each and air dried.
321 Hybridization was performed in a hybridization chamber at 46°C in hybridization buffer [0.9
322 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate (SDS) and 30%
323 formamide] for 3 h. Fluorescent probes were used at a concentration of 5 ng μl^{-1} labeled
324 probe hybridization buffer [0.9 M NaCl, 20 mMTris-HCl (pH 7.4), 0.01% SDS and 30%
325 formamide]. The oligonucleotide probes (IDT DNA, Iowa) used were EUB338

(GCTGCCTCCCGTAGGAGT) which binds to bacterial ribosomal RNA (Stahl, *et al*., 1989; Amann, *et al*., 1995) and EUK516 (ACCAGACTTGCCCTCC) which binds to the fungal ribosomal RNA (Amann, *et al*., 1995). Probes EUB338 and EUK516 were labeled with the fluorochromes, indocarbocyanine (Cy3) or indodicarbocyanine (Cy5), respectively. The coupons were incubated in 100 µl of hybridization mixture of oligonucleotides, then carefully rinsed and further incubated at 48°C in wash buffer [2 mMTris-HCl, 5 mM EDTA, 0.01% SDS and a variable amount NaCl to optimize probe specificity] for 20 min. The wash buffer was then carefully rinsed off with Milli-Q®water, the slides were air-dried in the dark and stained with 1 µg ml^{-1} of 4'6-diamidino-2-phenylindole (DAPI) solution for microscopic observation. Microscopy analysis was performed using an inverted Confocal Laser Scanning Microscope (Nikon, C1si) with excitation wavelengths of 408 nm, 561 nm and 637 nm for DAPI, Cy3 and Cy5 labeled probes, respectively. ImageJ software version 1.36b (http://rsb.info.nih.gov/ij/download.html) was used to estimate the proportion of bacteria (stained with Cy3), fungi (stained with Cy5) against total microorganisms (stained with DAPI). The analysis was performed on fives images from different locations of the coupons in triplicate.

DNA extraction from the panels and microbial cultures

Total genomic DNA was extracted using the modified CTAB/phenol method (Rogers and Bendich, 1994; Cubero, *et al*., 1999). An area of 100 cm^2 of the exposed panels was swabbed, using a sterile cotton bud. For bacterial and fungal cultures, overnight cultures grown in LB (bacteria, 37˚C) or peptone yeast extract glucose (fungi, room temperature) were pelleted by centrifugation. The incubated cells or swabbed materials were incubated with 20 µl of 200 U ml^{-1} lyticase for 24 h at 37 ˚C. The samples were incubated with 0.4 ml of 5% CTAB and 0.4 ml of phenol:chloroform:isoamyl alcohol (25:24:1) mixture and placed in the FastPrep bead beater (MP Bio) for 2 min (speed setting 5.5) to lyse cells. Samples were centrifuged at full

speed (18,620 x *g*) for 5 min. The top aqueous phase was transferred to a new tube and mixed with an equal volume of chloroform:isoamyl alcohol, followed by centrifugation at (18,620 x *g*) for 5 min at 4˚C. The top aqueous layer was collected and mixed with two volumes of 30% (w/v) PEG (Polyethylene glycol average molecular weight 1,450 g $mole^{-1}$) to precipitate the DNA. This mixture was incubated at 4˚C overnight and centrifuged (18,620 x *g*) for 10 min at 4˚C. The pellet was washed with 200 µl of 70% ice cold ethanol, air dried for 1 h and re-suspended in 50 µl of sterile Milli-Q® water. Extracted DNA was quantified using a Nanodrop™ND-1000 spectrophotometer (Thermo Fisher) and visualized on 1% agarose gel.

PCR amplification

For T-RFLP and colony sequencing, PCR amplification of the V3 region of the 16S rDNA gene (for bacteria - forward primer V3F 5'AC GTCCAGACTCCTACGGG 3', reverse primer V3R 5'TTACCGCGGCTGCTGGCAC 3') or the internal transcribed spacer region (ITS1) of the 18S rDNA gene (for fungi - forward primer ITS1F 5'CTTGGTCATTTAGAGGAAGTAA 3', reverse primer ITS4 5'TCCTCCGCTTATTGATATGC 3') was performed. PCR reactions contained 12.5 µl molecular grade water, 12.5 µl of EconoTaq™Plus Green 2X Master Mix (Lucigen), 0.75 µl of each forward and reverse primer (0.3 µM concentration), and 1.0 µl DNA template in 25 µl total volume. Reaction conditions were: initial denaturation at 95˚C for 4 min, followed by 30 cycles of 30 s at 95 ˚C, 30 s at 55 ˚C, 60 s at 72˚C and a final extension for 10 min at 72 ˚C. PCR products were visualized on a 1% agarose gel. For T-RFLP, the PCR reaction was performed in triplicate and pooled prior to restriction digestion and subsequent analysis (described below).

Illumina sequencing and data analysis

374 Illumina sequencing was performed by the Research and Testing Laboratory (Lubbock, TX)
375 using an Illumina MiSeq instrument. PCR amplification and sequencing of the 16S and 18S
376 rRNA genes used the primers 28F (5' GAGTTTGATCNTGGCTCAG 3') – 519R (5'
377 GTNTTACNGCGGCKGCTG 3') that target conserved regions flanking the V1-V3 region of
378 the 16S rRNA gene in bacteria and primers (5' TGGAGGGCAAGTCTGGTG 3', 5'
379 TCGGCATAGTTTATGGTTAAG 3') for the 18S rRNA gene of fungi (Hume, *et al*., 2012).
380 Data were quality filtered, and processed using the Quantitative Insights Into Microbial
381 Ecology QIIME v 1.5.0-dev pipeline(Caporaso, *et al*., 2010). Bacterial and fungal sequences
382 were assigned into groups based on their barcodes. The sequences were clustered into
383 operational taxonomic units (OUTs) by *de novo* clustering using UCLUST reference-based
384 algorithm (Edgar, 2010). Clustering was conducted at the 97% identity level using the Silva
385 database version 104 (Pruesse, *et al*., 2007). The most abundant sequence from each OTU
386 was selected as a representative sequence for that OTU and taxonomy of that OTU was
387 assigned via the Silva database version 104 and GenBank
388 (http://www.ncbi.nlm.nih.gov/genbank/).

389 **Microbial community fingerprinting analysis by T-RFLP**

390 For genomic DNA extracted from the panel surfaces as above, the V3 region of the bacterial
391 16S ribosomal subunit and the ITS region of the fungal 18S ribosomal subunits were
392 amplified. PCR amplification was performed in triplicate with the primers ITS1F and ITS4
393 (for fungi) (White, *et al*., 1990) and V3R and V3F (for bacteria), PCR was performed as
394 described above. The forward primers of V3 and ITS1-4 region were fluorescently labeled
395 with 6-FAM. PCR products were purified using DNA Clean & Concentrator Kit (Zymo
396 Research Corporation) and 200 ng of purified PCR products were digested using 5 U of the
397 *Hae*III restriction endonuclease (NEB Biolab) for 4 h at 37˚C, followed by denaturation of
398 the restriction enzyme at 65˚C for 20 min. Subsequently, 20 ng of purified digested products

were submitted to the Ramaciotti Centre for Genomics, UNSW using an Applied Biosystems 3730 DNA Analyser.

Analysis of T-RFLP profiles

The T-RFLP profiles of the FAM-labeled terminal fragments were analyzed using PeakScanner V1.0 software (Applied Biosystems, Forster City, CA, USA). GSLIZ 500 fragment ladder was used for comparison of the T-RFLPs. The standard range was set from 30-550 bp, and fragments less than 30 bp or bigger than 550 bp were eliminated from the analysis. All of the fragments that had a fluorescence unit of 1 or higher were extracted from Peak Scanner and analyzed with the online T-REX software (available at trex.biohpc.org) (Culman, *et al*., 2009) to distinguish the true peaks from background fluorescence. The data was subjected to noise filtering and T-RFLP Alignment. Bray-Curtis similarities were calculated, Centroid distances were used to average the triplicate samples and multi-dimensional scaling (MDS) techniques were used to examine community structure among the panels using PRIMER 6 software.

Statistical analyses

PERMANOVA was used to test for the significant differences among the microbial structures among the panels from different incubation sites and time of exposure.

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561 **Table 1**. Comparison of fungal diversity in Burrawang and Kapar samples based on T-RFLP
562 analysis

Source	df	Pseudo-F	P (perm)
Time	12	2.0841	0.001
Site	1	3.4384	0.008
Time and Site	9	2.3523	0.001

563

564 **Table 2**. Comparison of bacterial diversity in Burrawang and Malaysian samples based on T-
565 RFLP data.

Source	df	Pseudo-F	P (perm)
Time	12	3.3969	0.001
Site	1	0.93533	0.493
Time and Site	9	2.6498	0.001

566

567

568 Figure Legends

569 Figure 1. Total DNA extracted from painted steel coupons exposed at Burrawang, NSW
570 Australia, over a 52 week period (A), error bars represent standard error, n=3. Surface
571 coverage of bacteria and fungi based on FISH images (B), error bars represent standard error,
572 n=15.
573 Figure 2. Comparison of fungal diversity on painted steel coupons in two geographically
574 distinct locations, over a 52 week period. The MDS plot is based on centroids of triplicate
575 samples from Burrawang (green symbols) and Kapar (blue symbols) based on (A) T-RFLP
576 profiling or (B) the community sequencing of the ITS region. The week of sample collection
577 is indicated by the number associated with the symbols and comparisons of triplicate coupons
578 were based on the fourth root transformed data using the Bray Curtis Similarity matrix.

579 Figure 3. Comparison of bacterial diversity on painted steel coupons in two geographically
580 distinct locations, over a 52 week period. The MDS plot is based on centroids of triplicate
581 samples from Burrawang (green symbols) and Kapar (blue symbols) based on (A) T-RFLP
582 profiling or (B) the community sequencing of the 16S rRNA gene. The week of sample
583 collection is indicated by the number associated with the symbols and comparisons of
584 triplicate coupons were based on the fourth root transformed data using the Bray Curtis
585 Similarity matrix.

586 Figure 4. Fungal OTUs that were present at $\geq$ 1% abundance of the total community for
587 painted steel coupons from (A) Burrawang (time series 1), (B) Kapar and (C) Burrawang
588 (time series 2), as sampled for weeks 4-52. Data represent the average of duplicate samples.
589 The legends for bars shown in A are the same for B and C.

590 Figure 5. The six most abundant fungi species on (A) Burrawang and (B) Kapar painted steel
591 coupons collected from exposure racks, compared with the maximum temperatures and

592 rainfall. Maximum temperature and rainfall were calculated from the mean of maximum
593 temperature and mean of rainfall of 28 d preceding collection. OTUs were determined based
594 on community sequencing of the fungal ITS region.

595 Figure 6. The relative abundance of dominant bacterial species (>1% of the total community
596 abundance) found on exposed, painted steel coupons from (A) Burrawang (time series 1), (B)
597 Kapar and (C) Burrawang (time series 2) based on community 16S rRNA gene sequence
598 data.

599