Fungus-bacteria interactions in decomposing wood: unravelling community effects

A thesis submitted to Cardiff University for the degree of Doctor of Philosophy

By

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Blessed be the name of God forever and ever, to whom belong wisdom and might.
He changes times and seasons; […]
He gives wisdom to the wise and knowledge to those who have understanding;
He reveals deep and hidden things; He knows what is in the darkness, and the light dwells with Him.
To You, O God of my fathers, I give thanks and praise,
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Chapter 3: Dr. Anna Evans contributed an R script to query the NCBI database via Primer-BLAST.

Chapter 4: Dr. Jen Hiscox and Dr. Mel Savoury collaborated on the field and lab work as part of a complementary study. Casey Morris and Sindy Kaur Reyat assisted with lab work.

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Summary

Bacteria have long been known to inhabit decomposing wood, but much of their ecology remains unexplored. There are indications that bacterial communities are linked to interactions with fungi, which dominate the dead-wood environment. Manipulative field experiments were combined with meta-taxonomic DNA sequencing to examine fungal effects on bacterial communities, in wood precolonised by *Vuilleminia comedens*, *Trametes versicolor* or *Hypholoma fasciculare*. After one year in the field, *H. fasciculare* retained its territory in the wood and showed a significantly different bacterial community to the other treatments, where precolonisers did not retain territory. Bacterial communities were significantly correlated with developing fungal communities. Samples where cord-forming basidiomycetes were dominant showed a distinctive bacterial community. Bacterial community structure and richness was significantly associated with wood pH, but not with woodland site location. Over a shorter period of 12 weeks, fungal precolonisers significantly delayed bacterial colonisation. *V. comedens* and *H. fasciculare* were associated with distinct bacterial communities, but *T. versicolor* was not. In fungal-uncolonised wood, seasonal differences were apparent at 84 but not at 14 days. Bacterial communities were dominated by *Proteobacteria*, with *Burkholderiaceae* enriched in precolonised samples. *Acidobacteria* were a major component of the 1-yr samples, but not the earlier-stage samples. In contrast to previous studies, *Actinobacteria* were never abundant. Overall, there was strong evidence for fungal control of bacterial communities in decaying wood.

Interactions between wood-decay fungi and three strains of fungal-migratory *Paraburkholderia* were examined in agar microcosms. Mycelial extension rates of eight fungi were unaffected by bacterial migration, but two strains of *Phanerochaete* were significantly inhibited. Bacteria were also introduced into pairwise competitive interactions between fungi. One *Phanerochaete* strain showed a significant reduction in competitive performance when inoculated with *Paraburkholderia*. The presence of bacteria made the outcomes of inter-fungal interactions more unpredictable, indicating bacteria can reciprocally influence fungal communities in decaying wood.

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List of Publications

Publications directly associated with thesis:

Johnston, S.R., Boddy, L. & Weightman, A.J. 2016. Bacteria in decomposing wood and their interactions with wood-decay fungi. *FEMS Microbiology Ecology* 92: fiw179. (DOI: 10.1093/femsec/fiw179)

Hiscox, J., Savoury, M., **Johnston, S.R.**, Parfitt, D., Müller, C.T., Rogers, H.J. & Boddy, L. 2016. Location, location, location: priority effects in wood decay communities may vary between sites. *Environmental Microbiology* 18: 1954–1969 (DOI: 10.1111/1462-2920.13141)

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Boddy, L., Hiscox, J., Gilmartin, E.C., **Johnston, S.R.** & Heilmann-Clausen, J. 2016. Wood decay communities in angiosperm wood. In: *The Fungal Community* (eds. Dighton, J. & J.White) Taylor & Francis Group, 2017. (ISBN-10: 1498706657)

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Chapter 1. General Introduction

1.1 The decomposing wood environment

Decomposing wood is an essential part of forest ecosystems; the ecological lifetime of a tree far exceeds its biological lifespan. As wood decays, it provides habitat for many organisms, including invertebrates, bryophytes and birds (Stokland *et al.* 2012). This habitat is dominated and regulated by microbial communities, particularly wood-decay fungi. The complex lignocellulose structure of wood is recalcitrant to decay, and can be substantially decomposed only by a restricted set of basidiomycete and ascomycete fungi (de Boer *et al.* 2005). Fungal activity releases nutrients and modifies the physical wood environment, making it amenable to colonisation by macro-organisms. The properties of the wood environment thus reflect the activities of these fungi: nutrient levels in the wood are altered by mycelial translocation, and the intensely competitive and territorial ecology of wood-decay fungi induces complex spatial structure within the resource (Fig 1.1) (Boddy 2000; Watkinson *et al.* 2006). At a smaller scale, bacteria modify the structure of individual wood cells, both encountering and interacting with the fungal community (Greaves 1971).

Wood decay is not a single ecological process, but a dynamic process with multiple stages (Boddy 2001). In large trees, the heartwood is decomposed by specialist heart-rot fungi whilst the tree is still alive. Sapwood decay often also begins in the standing tree: dead branches are colonised by canopy fungi whilst still attached to the truck. Once wood falls to the forest floor, it becomes available to a new set of decomposers; this forest floor period is the best-characterised phase of wood decomposition.

Wood is a challenging environment to study: bulky, opaque and physically difficult to probe. For most purposes, destructive sampling is necessary, meaning that the same resource cannot be followed through time. However, wood also has advantages as a study system: it is robust, and relatively easy to resolve patterns in three dimensions. The versatile metabolism of wood-decay fungi means that they are exceptionally amenable to cultivation: decades of studies (*e.g.* Boddy *et al.* 1987) have shown that the dominant decay fungus can reliably be isolated from a visible territory. This allows a degree of experimental manipulation that is impossible in many microbial systems including the bacterial community of wood, much of which cannot be cultured (Folman *et al.* 2008).

1.2 Wood-decay fungi

Wood decay fungi possess a fascinating and unusual biology: multicellular microorganisms, existing as distinct genetic individuals, which reach large macroscopic sizes.

Figure 1.1 Complex spatial structure within a decaying beech (*Fagus sylvatica*) trunk. Each area delimited by black pseudosclerotial plates represents the territory of a fungal individual. Regions stained dark brown are indicative of areas of high bacterial activity, known as bacterial wetwood.

These individuals may fragment and reconnect innumerable times, discriminating self from non-self by somatic incompatibility loci (Boddy & Hiscox 2016). Quantifying them in terms of number of individuals (as for animals) or number of cells (as for many bacteria) is therefore invalid, and given the lack of a reliable way to estimate fungal biomass (Baldrian *et al.* 2013), the area of territory occupied is often the most meaningful proxy for fungal abundance. Fungi that rely on spores for dispersal are termed resource-unit-restricted, as the mycelium is bounded by its resource; in contrast, the mycelium of non-resource-unit-restricted fungi can spread beyond its current resource and forage for new ones (Boddy & Hiscox 2016). This ability means that the latter fungi are (theoretically) bounded in neither time nor space, but rather are able to survive and grow indefinitely.

Beyond taxonomic classification, wood decay fungi are often most meaningfully categorised by their ecological strategies – especially when considering them in terms of functional activity and interactions with other organisms. The classic CSR (competitive, stress-tolerant, ruderal) framework has been usefully adapted to describe fungal communities (Boddy & Hiscox 2016). In particular, competition drives and shapes fungal community development through a series of direct, confrontational competitive interactions

(Boddy 2000). Among the most competitive fungi are the cord-formers (Boddy 1993). Mycelial cords are organised aggregations of hyphae protected by a rind, and they are an organ that enables a foraging life strategy. Cord-forming fungi are able to produce networks many metres wide across the forest floor, foraging for resources, gaining rapid access to newly-fallen wood, and redistributing cytoplasm and nutrients across their territory.

1.3 The role of bacteria in the dead-wood environment

In contrast to wood-decay fungi, the bacterial community in decomposing wood has barely been described (Valásková *et al.* 2009; Sun *et al.* 2014; Kielak *et al.* 2016b; Rinta-Kanto *et al.* 2016), much less explored in functional terms (Hoppe *et al.* 2014). Chapter 2 summarises the current state of knowledge on bacteria in wood. Bacterial wood decomposition has been observed both structurally and chemically (Greaves 1969; Brown & Chang 2014), but direct bacterial contributions to wood decay are considered negligible in oxic environments (Greaves 1971; Clausen 1996; de Boer *et al.* 2005). Historically, the limitations of culture-based techniques have frustrated attempts to understand bacterial communities in wood (Murray & Woodward 2003). More recently, microcosm work indicates that bacteria are selected both by the wood environment and wood-decay fungi (Folman *et al.* 2008; Hervé *et al.* 2014). Bacteria potentially compete with fungi for nutrients in this oligotrophic environment, but conversely the two groups of organisms may interact synergistically, *e.g.* bacteria providing nitrogen by fixation, and fungi releasing carbon from the resource by enzymatic activity. Many studies point to *Proteobacteria* being the dominant phylum in wood, with *Acidobacteria* consistently also a major component (*e.g.* Folman *et al.* 2008; Valásková *et al.* 2009; Sun *et al.* 2014; Hoppe *et al.* 2015; Kielak *et al.* 2016; Rinta-Kanto *et al.* 2016).

The indications of non-random association between bacteria and fungi in wood suggest that bacteria should be considered with reference to fungi in the resource (Hoppe *et al.* 2014; Kielak *et al.* 2016b). A growing weight of evidence indicates the profound mutual implications of fungus-bacteria interactions across a range of environments (Partida-Martinez & Hertweck 2005; Nazir *et al.* 2014; Partida-Martínez 2017). This reinvigorates the theory that bacteria influence the dead-wood environment via their interactions with wood-decay fungi (Greaves 1971). At the same time there is a growing realisation that many eukaryotes exist in interdependent association with their microbiomes, and that the fungal microbiome is no exception (Partida-Martínez 2017; Schulz-Bohm *et al.* 2017).

1.4 Thesis aims

Against this background, the study described in this thesis set out to explore interactions between fungal and bacterial communities in wood. Such a large topic cannot be exhausted in one thesis, so by necessity this work selected a few aspects on which to focus. Three overarching hypotheses have shaped the direction of the work: firstly, that bacterial community composition in decaying wood is dependent on the identity of the dominant fungus; secondly, that development of these communities is visible over time; and thirdly, that wood-decay fungi form intimate associations with specific bacteria, both beneficial and detrimental.

Chapter 2 reviews the literature on bacteria in wood, focussing specifically on what information is available about their interactions with wood-decay fungi. The diversity, provenance and metabolic capabilities of wood-dwelling bacteria are considered first, before reviewing the types of potential interaction between bacteria and wood-decay fungi. A version of this chapter has been published in *FEMS Microbiology Ecology* (Johnston *et al.* 2016).

Chapter 3 explores a methodological issue uncovered during the course of the work: unwanted fungal co-amplification during bacterial qPCR on mixed DNA samples. *In silico* and *in vitro* analyses were used to elucidate the nature and extent of the issue.

Chapter 4 examines direct fungal influence over bacterial communities in decomposing wood, by manipulating the fungal community in a field situation. It also tested for correlation between bacterial communities and the incoming fungal communities developing through succession. Fungal and bacterial communities were determined by marker gene sequencing. The experiment was conducted over multiple sites spread across the region, encompassing geographical variation. Co-varying factors such as wood pH and local soil pH were explored for their influence on the community relationship.

Chapter 5 follows directly on by exploring fungal influences in the very earliest stages of bacterial wood colonisation, including the effects of an early-stage, primary colonising fungus. It also tracked inter- and intra-seasonal bacterial community development.

Chapter 6 focusses in on a specific interaction: bacterial migration along the hyphae of wood-decay fungi. This study employed laboratory manipulation to assess the host range of three migratory strains of *Paraburkholderia* isolated from wood-decay fungi, and to quantify effects on the fungal hosts. Two specific host abilities were considered: mycelial extension rate (growth), and performance in inter-fungal competitive interactions.

Chapter 7 draws together the findings of the previous chapters, and considers some emerging themes. It points to future avenues to be explored with respect to fungus-bacteria interactions in decomposing wood. Finally, it summarises the overarching conclusions from the work as a whole.

Chapter 2. Bacteria in decomposing wood and their interactions with wood-decay fungi

A version of this chapter has been published as:
Johnston, S.R., Boddy, L. & Weightman, A.J. 2016. Bacteria in decomposing wood and their interactions with wood-decay fungi. *FEMS Microbiology Ecology* 92: fiw179. (DOI: 10.1093/femsec/fiw179)

2.1 Abstract

The fungal community within dead wood has received considerable study, but far less attention has been paid to bacteria in the same habitat. Bacteria have long been known to inhabit decomposing wood, but much remains underexplored about their identity and ecology. Bacteria within the dead-wood environment must interact with wood-decay fungi, but again, very little is known about the form this takes; there are indications of both antagonistic and beneficial interactions within this fungal microbiome. Fungi are hypothesised to play an important role in shaping bacterial communities in wood, and conversely, bacteria may affect wood-decay fungi in a variety of ways. This literature review considers what is currently known about bacteria in wood and their interactions with fungi, and proposes possible associations based on examples from other habitats. It aims to identify key knowledge gaps and pressing questions for future research.

2.2. Introduction

Globally, fallen wood stores more than 73 billion tonnes of carbon (Pan *et al.* 2011) and provides habitat for a wide range of saproxylic (*i.e.* dead-wood-inhabiting) organisms (Stokland *et al.* 2012). Understanding the rate, mechanisms and control of wood decomposition is of major ecological and economic importance, and the key to doing so lies in understanding the microbial communities that effect and regulate decomposition. Fungi are the dominant agents of wood decomposition, but it has long been known that bacteria also inhabit dead wood (Greaves 1971). There are indications of great bacterial diversity within wood (Zhang *et al.* 2008; Věýtrovsky *et al.* 2011; Sun *et al.* 2014; Hoppe *et al.* 2015), but bacteria are very poorly understood compared with fungi in the same environment. Wherever bacteria and fungi co-occur they must interact with and influence each other (Fig 2.1), yet although wood-decay fungi are well-known for being highly competitive (Boddy 2000) relatively little attention has been paid to the fungus-bacteria relationship (de Boer *et al.* 2005). Fungus-bacteria interactions have already been studied in other contexts for their importance in medicine, agriculture, and food and drink (Frey-Klett *et al.* 2011), but have been explored far less with respect to decomposition. The suite of bacteria that surround and interact with a fungus effectively constitute its microbiome, and as such, they must be considered together. The aim of this literature review is to synthesise the current state of knowledge about bacteria in wood and how they interact with wood-decay fungi, so as to identify key areas for future exploration.

2.3. Diversity of bacterial communities in wood

Information on bacterial communities in decomposing wood is surprisingly scare, given how well saproxylic fungal communities have been studied. This disparity is doubtless partially due to the greater propensity of fungi to enter culture – there is a long history of successful isolation of fungal decay communities from wood (*e.g.* Boddy *et al.* 1987). In contrast, whilst there are studies that have looked at the culturable fraction of saproxylic bacteria (*e.g.* Murray & Woodward 2003; van der Wal *et al.* 2007), a large and variable proportion are unculturable (Folman *et al.* 2008). Culture-based studies can thus, at best, only indicate part of the bacterial community. Because the culturable *proportion* of total bacteria varies, plate counts can never be used for quantitative comparison in this context. For example, a microcosm experiment recorded that in the absence of wood-decay fungi, 61% of bacteria colonising wood blocks could be cultured; when a white-rot fungus was introduced, the culturable proportion dropped to 1% (Folman *et al.* 2008). Unfortunately,

the limitations of culture-based surveys mean that much older literature in this field is of restricted usefulness. Whilst culture-based approaches can no longer be used for whole-community characterisation, they remain highly useful for exploring specific relationships (*e.g.* Nazir *et al.* 2014). Culture-based studies have also succeeded in isolating new genera from dead wood, including members of the difficult-to-culture phylum *Acidobacteria* (*e.g.* Folman *et al.*, 2008). The accessibility and high throughput of next-generation DNA sequencing and associated metagenomics opens the door to more comprehensive study of saproxylic bacterial communities. This review will therefore pay special attention to studies that have used molecular methods to assess bacterial diversity in wood (Table 2.1), drawing on culture-based studies as well where applicable.

Bacterial diversity is far lower in wood than in soil (Hervé *et al.* 2014; Sun *et al.* 2014), and is highly influenced by the underlying soil type; nonetheless, there is a high level of intra-site heterogeneity (Sun *et al.* 2014). The bacterial community varies dependent on the wood's state of decay, with bacterial richness increasing as the wood decomposes (Hoppe *et al.* 2015). Heartwood and sapwood contain markedly different bacterial communities, but communities in heartwood are apparently more diverse (Zhang *et al.* 2008); nonetheless, bacteria may be more abundant in sapwood (Jeremic *et al.* 2004). There are indications that bacterial communities differ between tree species (Folman *et al.* 2008; Hoppe *et al.* 2014, 2015; Prewitt *et al.* 2014). The water content, pH, and C:N ratio of the wood affect the bacterial community, as does the forest management regime (Hoppe *et al.* 2015). Bacterial abundance and richness is highest at advanced stages of wood decay, but does not show a clear pattern for phylum-level community composition (Kielak *et al.* 2016b; Rinta-Kanto *et al.* 2016). These studies offer a tantalising insight into saproxylic bacterial communities, but the field is still young and the conclusions are tentative.

There are parallels between the microbial decomposer communities in wood and leaf litter. In leaf litter, bacterial and fungal communities show linked dynamics and both are also influenced by the same abiotic drivers of C:N ratio, nutrient availability, water content and pH (Purahong *et al.*, 2016). In another study on deciduous leaf litter, fungi showed less dependence than bacteria on environmental variables, such as water availability and ambient temperature (Liu *et al.* 2016). However, it is very difficult to extrapolate results from one habitat or taxonomic group to another. For example, dominant tree identity is a major fungal community driver in both soil and litter, but less important for bacteria in litter and unimportant for bacteria in soil (Urbanová *et al.* 2015).

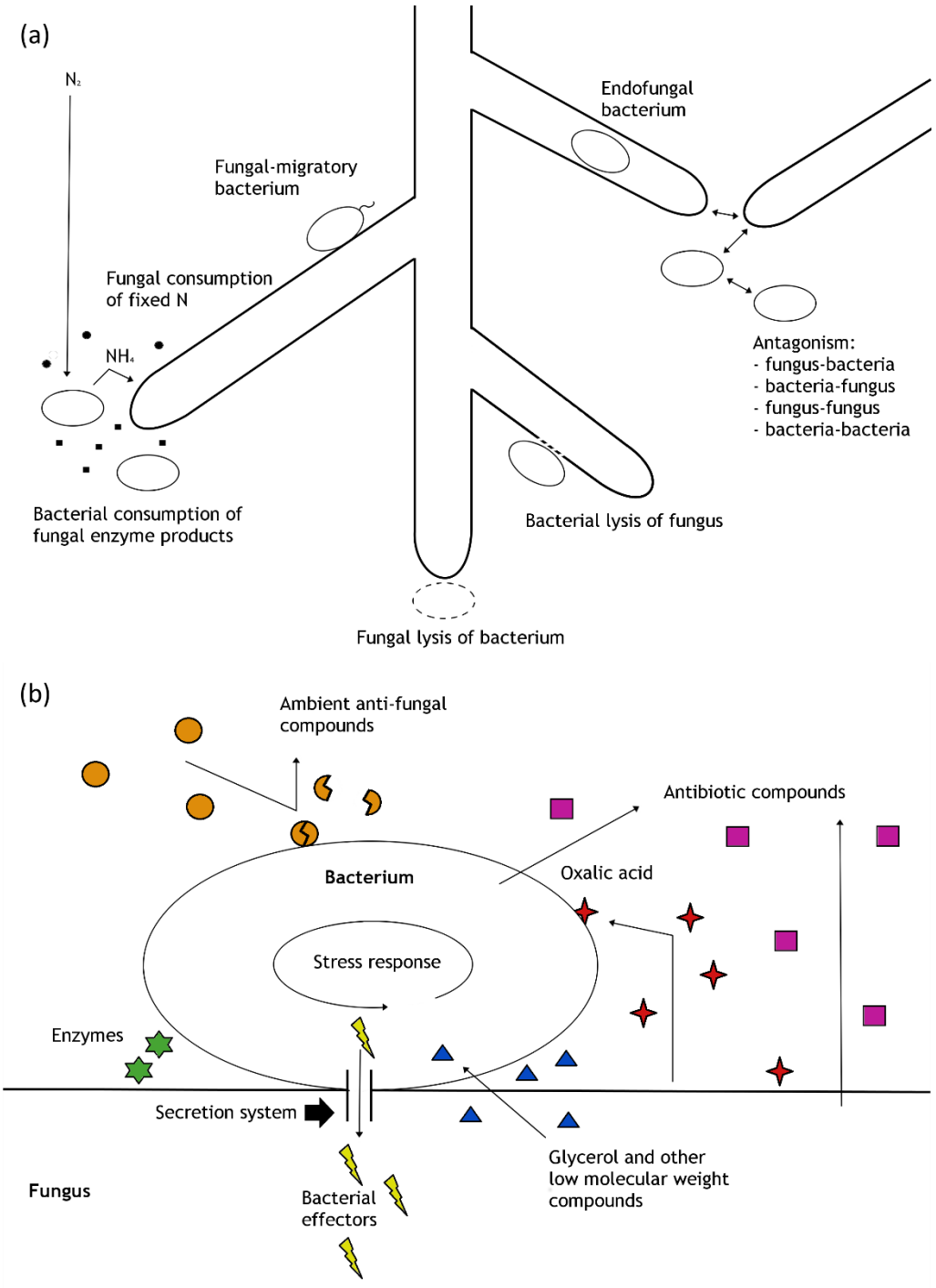

Figure 2.1 Potential fungus-bacteria interactions occurring in wood; not all have so far been observed. The interactions may be obligate (*e.g.* some endosymbioses) or facultative (*e.g.* predation). In many cases it is not clear whether a given interaction is beneficial to one, both or neither of the partners. The outcomes indicated may occur via more than one route: for example, fungi could receive fixed N by mutualism with diazotrophs, by lysing the bacteria, or by predating on nematodes *etc*. that had in turn predated on bacteria. (a) Whole-organism level interactions. (b) Molecular mechanisms of interaction; see main text for details of each.

Table 2.1 Marker gene-based studies of bacteria in wood, and the major phyla reported in each.

Study	Wood species	State of decay	Location	Marker gene	Method	Major bacterial phyla
Folman *et al.*, 2008	*Fagus sylvatica*	7-10 months' colonisation	Lab	16S rRNA	DGGE and sequencing	*Beta-proteobacteria; Gamma-proteobacteria; Acidobacteria; Bacilli*
Hervé *et al.*, 2014	*Fagus sylvatica*	Sawdust, 3-5 months' colonisation	Lab	16S rRNA	NGS amplicons	*Alpha-, Beta-* and *Gamma-proteobacteria*
Hoppe *et al.*, 2014	*Fagus sylvatica, Picea abies*	Not reported	Temperate woodland (Germany)	*nifH*	NGS amplicons	*Alpha-proteobacteria*
Hoppe *et al.*, 2015	*Fagus sylvatica, Picea abies*	Kahl (2012) decay class 1-4 (3-27 yrs)	Temperate woodland (Germany)	16S rRNA	NGS amplicons	*Alpha-proteobacteria; Actinobacteria; Acidobacteria*
Kielak *et al.*, 2016a	*Pinus sylvestris*	Classified as early, middle or late decay based on density	Temperate woodland (The Netherlands)	16S rRNA	NGS amplicons	*Alpha-proteobacteria; Acidobacteria*
Rinta-Kanto *et al*., 2016	*Picea abies*	Range from '"recently dead" to "almost decomposed" (Mäkinen *et al.*, 2006)	Boreal forest (Finland)	16S rRNA	NGS amplicons	*Alpha-proteobacteria; Acidobacteria*
Sun *et al.*, 2014	*Picea abies*	2-4 months' colonisation	Boreal forest (Finland)	16S rRNA	NGS amplicons	*Proteobacteria; Bacteroidetes; Acidobacteria; Actinobacteria*
Valásková *et al.*, 2009	*Betula sp., Fagus sylvatica, Quercus robur, Pinus sylvestris*	Reported only as "high"	Temperate woodland (The Netherlands)	16S rRNA	Clone library	*Alpha-, Beta-* and *Gamma-proteobacteria; Acidobacteria; Firmicutes*
Zhang *et al.*, 2008	*Keteleeria evelyniana*	Not reported	Not reported; sub-tropical?	16S rRNA	Clone library	*Alpha-, Beta-, Gamma-* and *Delta-proteobacteria; Actinobacteria; Acidobacteria*

In functional terms, (Greaves 1971) classified saproxylic bacteria into four groups based on their role in decomposition: bacteria that make wood more water-permeable without affecting its structural integrity; bacteria with (albeit limited) decomposition ability; bacteria that stimulate fungal decomposition; and bacteria that inhibit fungal decomposition. These classes maintain their relevance today, but present a challenge: how best to relate broad-scale, whole sample taxonomic information from sequencing to fine-scale functional abilities. Even the bacteria amenable to culture can show vastly different properties depending on medium, *etc*. (Murray & Woodward 2003). Truly making sense of the bacterial communities in wood will depend upon linking their identity and function, even for species that can be cultivated.

Any consideration of bacteria in wood should take into account the presence and identity of wood-decay fungi, as current evidence strongly indicates that they greatly influence the bacterial community (Folman *et al.* 2008; Sun *et al.* 2013; Hoppe *et al.* 2014). In the soil environment, areas under close fungal influence have distinctive bacterial communities (Warmink & van Elsas 2008). In *Picea abies* logs, fungal diversity correlated negatively with bacterial abundance, and there are indications that certain bacterial taxa co-occur preferentially with particular fungi (Rinta-Kanto *et al.* 2016). Discerning ecologically realistic patterns is challenging, due to the huge number of potentially significant variables; for example, the effects of fungal inoculation on bacteria alter over time (Sun *et al.* 2013). It is also virtually impossible to establish fungus-free bacterial controls when inoculating wood from soil (de Boer *et al.* 2010).

Fungi profoundly influence the wood physical environment by lowering the pH, excreting metabolites such as oxalic acid and translocating nitrogen and phosphorus into the resource (Watkinson *et al.* 2006; de Boer *et al.* 2010; Rudnick *et al.* 2015). It should be noted that whilst translocation increases the bulk N and P content of the wood, they are contained within hyphae and only available to bacteria that can access the hyphal contents. Experimental evidence shows that dead mycelium also provides a rich and largely labile nutrient source which supports a distinct bacterial community (Brabcová *et al.* 2016). However, in the environment fungi recycle cytoplasm from senescent hyphae to other parts of the mycelium, so not all of these nutrients are available to other decomposers (Watkinson *et al.* 2006).

Wood-decay fungi have repeatedly been associated with *Burkholderiaceae* (Seigle-Murandi *et al.* 1996; Lim *et al.* 2003; Yara *et al.* 2006; Folman *et al.* 2008; Valásková *et al.* 2009; Sato *et al.* 2010; Hervé *et al.* 2014; Prewitt *et al.* 2014; Sun *et al.* 2014). A widespread

and versatile family of bacteria, it crops up alongside fungi with remarkable regularity, and not only in wood (de Boer *et al.* 2005; Frey-Klett *et al.* 2011). Moreover, there are indications of close, specialized associations between fungi and the genus *Burkholderia* (including the recently-defined *Paraburkholderia*) involving collaborative pathogenicity (Partida-Martinez & Hertweck 2005); intimate mycelial associations (Lim *et al.* 2003); endosymbiosis (Sato *et al.* 2010); co-migration and detoxification of antimicrobials (Nazir *et al.* 2014); and successional persistence (Hervé *et al.* 2014). An analysis of global soil microbiota found significant co-occurrence between *Burkholderia*/*Paraburkholderia* and fungi (Stopnisek *et al.* 2015). The same study also analysed the proteome of *Paraburkholderia glathei* when grown alone or with fungi, and found that when fungi were present the bacteria expressed fewer proteins associated with starvation, but upregulated stress responses, suggesting that the bacteria gained nutrients from the fungus but experienced antibiosis and/or unfavourable chemical conditions (Stopnisek *et al.* 2015).

It has been suggested (Greaves 1971; Frey-Klett *et al.* 2011) that bacterial activity in the earliest stages of decay renders the wood more accessible to fungi. Whilst bacteria may detoxify certain compounds inhibitory to fungi, notably in treated wood (Greaves 1971; Clausen 1996) experiments using fresh, sterile wood show that fungi are competent wood decayers in the absence of bacterial conditioning (*e.g.* Hiscox *et al.* 2010). Similarly, although it is sometimes suggested that bacteria are the earliest colonists of dead wood, there is little evidence on whether or not that is the case (van der Wal *et al.* 2007). Given that wood-decay fungal propagules are latently present in functional wood (Parfitt *et al.* 2010), for this to hold true bacteria would likewise have to be latently present, and/or colonise wood very rapidly once conditions were favourable.

2.4. Bacterial colonisation of wood

The provenance of saproxylic bacteria communities and their means of colonisation are largely unknown. Bacteria have limited motility and are unable to cross air voids, meaning that colonisation is likely to be slow without some means of carriage into the wood. Essentially, bacteria in a woody resource have four possible points of origin: the soil; the air; the wood itself; and fungi or other organisms colonising the wood. The relative importance of these sources is likely to vary under different conditions; the bacterial community in attached dead branches is probably very different from that in wood on the forest floor.

2.4.1. Edaphic and atmospheric sources of bacteria

Soil represents a rich source of potential colonists for wood in ground contact, and the underlying assumption of many studies is that it is the main point of origin for bacteria in wood (*e.g.* van der Wal *et al.* 2007; Folman *et al.* 2008; Hervé *et al.* 2014). These show that a subset of soil bacteria are competent to colonise wood, but do not indicate to what extent this occurs under natural conditions. Underlying soil type was a good predictor of bacterial assemblage in experimental wood blocks (Sun *et al.* 2014), which suggests either an edaphic origin of saproxylic bacteria, or an indirect influence of soil: for example, via an altered fungal community.

Movement via airborne spores and other propagules is a major means of bacterial dispersal, which has led to the widespread view that all bacteria are, or have the potential to be, ubiquitous: a view that has since been challenged (Green & Bohannan 2006). Nevertheless, the air could represent another means for bacteria to arrive at decomposing wood. In woodlands, rainfall creates bio-aerosols of bacteria and fungal spores, presumably contributing to their dispersal (Huffman *et al.* 2013). An experiment on bacteria in woodland pools indicated that the community composition was not dispersal limited, suggesting that airborne dispersal is effective at least across local scales (Bell 2010).

2.4.2 Bacterial endophytes

As mentioned above, wood-decay fungi exist in living trees as latent propagules which spread as mycelia when the branch or trunk is no longer functional in water conduction (Parfitt *et al.* 2010). Scanning electron microscopy indicates that at least some, if not all, living trees also host abundant bacterial endophytes in their wood (Jeremic *et al.* 2004). However, there is very little literature on bacterial endophytes in wood, and the identity of these endophytes is as yet uncertain.

2.4.3 Bacteria co-colonisation with other organisms

Paraburkholderia terrae BS001 has been shown to migrate across soil with wood-decay fungi, including the aggregated mycelial cords of *Phanerochaete velutina* (Nazir *et al.* 2014). This demonstrates active bacterial movement, as the apical growth of fungal hyphae rules out the possibility of passive carriage. *P. velutina* had a lower bacterial 'carrying capacity' than several other fungi, suggesting that cords may be less conducive to migration than are fine hyphae. The presence of *P. terrae* BS001 can also facilitate the movement of other bacteria which would otherwise not be competent to migrate along hyphae (Warmink *et al.* 2011). Independent hyphal migration has also been observed for several other members of the *Burkholderiaceae* and strains of *Dyella japonica* (Warmink & van Elsas 2009;

Nazir *et al.* 2012). It has been suggested that other fungus-associated bacteria such as *Collimonas* may share this migratory ability (Leveau *et al.* 2010). Such behaviour raises the possibility that when fungi colonise a resource, they bring a suite of bacterial travelling companions. In this manner, saproxylic bacteria could use foraging fungal mycelium as a conduit to new resources.

Other saproxylic organisms, particularly invertebrates, may transfer bacteria from one woody resource to another. Bark beetles can carry bacteria phoretically (Mercado *et al.* 2014), and introduce them into trees during the construction of galleries. This has also been suggested as a source of nitrogen-fixing bacteria in wood (Griffiths *et al.* 1993).

2.5. Wetwood

The presence of bacteria in living trees is most obvious in bacterial wetwood. Wetwood, also known as wet-heartwood or watermark, is a condition where the heartwood of a living tree becomes saturated and discoloured. This change may be accompanied by blocked vessels, gas build up, and the presence of a fetid liquid. The term refers to a suite of phenomena, probably with multiple causal agents but broadly similar manifestations, making it hard to disentangle the exact role bacteria play. Whilst in some tree species (*e.g. Salix sachalinensis*) wetwood is a serious disease, spreading to sapwood and ultimately killing the tree (Sakamoto & Sano 2000), in others it seems to be an almost-ubiquitous part of maturation (*e.g. Ulmus americana*) (Murdoch & Campana 1983). Wetwood is often attributed to bacterial activity, but there is no clear evidence whether this is true for this latter form, where there are no apparent ill-effects to the tree: it could equally be caused by physical processes, and bacteria secondarily colonise and modify the habitat.

Wetwood is frequently associated with the presence of anaerobic, methanogenic, pectinolytic prokaryotes, which could account for many of the observed symptoms (Schink *et al.* 1981a). Although wetwood can form around the site of fungal infections, within the wetwood itself fungi are likely to be excluded by low O_2 concentrations, large amounts of organic acids and inhibitory metabolites (Worrall & Parmeter 1983). If fungi are indeed absent from wetwood, it represents an almost unique wood habitat in this respect.

2.6. Bacterial metabolism in wood

2.6.1 Bacterial nitrogen fixation in wood

It has long been recognised that dead wood plays host to nitrogen-fixing (diazotrophic) bacteria, which provide an independent source of nitrogen to the system

(Cornaby & Waide 1973; Sharp & Millbank 1973). Many studies have focussed on coniferous forests of the Pacific Northwest, and used acetylene reduction as a measure of nitrogenase activity (reviewed by Son 2001). Interpretation and comparison of these results requires caution, as the exact methodology used varies; acetylene reduction has been criticised for its sensitivity to experimental parameters (Giller 1987), although the effects may not be as serious as suggested (Son 2001). Additionally, the conversion factor used to calculate N-fixation from acetylene reduction is not consistent (Son 2001; Brunner & Kimmins 2003), and there is evidence to suggest that the true conversion rate may vary between sample types (Hicks *et al.* 2003b). In light of these difficulties, acetylene reduction should perhaps be regarded as semi-quantitative, suitable for comparison within but not between studies.

The picture that emerges of N-fixation in dead wood suggests a highly dynamic process, influenced by many factors (Hicks *et al.* 2003a). Wood water content is consistently positively correlated with N-fixation (Larsen *et al.* 1978; Jurgensen *et al.* 1984; Brunner & Kimmins 2003; Hicks *et al.* 2003a), possibly because it creates better microhabitats for the anaerobic/microaerophilic diazotrophs (Spano *et al.* 1982; Hicks *et al.* 2003b). The optimum temperature for fixation is 30°C (Hicks *et al.* 2003b), which may explain higher N-fixing activity in summer than in winter (Jurgensen *et al.* 1984; Sollins *et al.* 1987). The requirements for high temperature and high moisture suggest an interplay of factors that determine seasonal fixation patterns (Hicks *et al.* 2003b). The effect of tree species on fixation is unclear, with multiple authors reporting significant differences between species (Jurgensen *et al.* 1989; Griffiths *et al.* 1993; Hoppe *et al.* 2014), and others reporting none (Sollins *et al.* 1987; Hicks *et al.* 2003a). Nitrogen fixation in forest ecosystems is likely to be limited by the availability of molybdenum, which is necessary for nitrogenase synthesis, and possibly also by other micronutrients (Silvester 1989).

Nitrogen fixation increases as decay proceeds (Larsen *et al.* 1978; Spano *et al.* 1982; Jurgensen *et al.* 1984), although two studies found that fixation peaked before dropping off in the most advanced stage of decay, perhaps because the latter studies included more decayed wood than the former (Hicks *et al.* 2003a). An experiment using a finer resolution time-series over 6 years revealed considerable variation within the overall increase in N fixation (Griffiths *et al.* 1993). In the very early stages of decay, N fixation will be limited by the rate at which diazotrophs can colonise the resource. If nitrogen-fixing bacteria rely on carbon from fungal activity (see Section 2.7.1), they may also experience a growth lag whilst fungi colonise and start to decompose the wood. Diazotrophic activity is higher in sapwood than in heartwood, and higher again in bark; low fixation has been recorded in heartwood,

possibly because it is the fraction most refractory to decomposition (Griffiths *et al.* 1993; Brunner & Kimmins 2003; Hicks *et al.* 2003b) and often contains inhibitory extractives.

The identity of the saproxylic diazotrophs is underexplored, but *Clostridium* and *Klebsiella* have been cultured (Spano *et al.* 1982). A survey of *nifH* nitrogenase genes in decaying wood indicated *Rhizobiales* was the predominant identifiable order, with *Rhodocyclales, Pseudomonadales, Rhodospirillales, Sphingomonadales* and *Burkholderiales* also present; however, most of the saproxylic *nifH* variants could not be matched to known bacteria (Hoppe *et al.* 2014). These bacterial orders have also been identified previously in 16S rRNA gene surveys of decaying wood (Folman *et al.* 2008; Valásková *et al.* 2009).

2.6.2 Bacterial wood decomposition

Bacteria are well known to be capable of cellulose decomposition, although their contribution to overall wood decay is restricted by small size and limited movement (Greaves 1971; Clausen 1996) unlike fungi with mycelial growth (de Boer *et al.* 2005). Various bacteria from woodland soil possess enzymes involved in the breakdown of cellulose/ cellulose products, including members of the *Acidobacteria*, a common phylum in dead wood (Lladó *et al.* 2015; Table 2.1). Some cellulolytic bacteria apparently use new, uncharacterised means of metabolising cellulose without expressing the usual enzymes (López-Mondéjar *et al.* 2016). Certain bacteria in wood break down pectin (Schink *et al.* 1981b; Clausen 1996), although in some cases this may be a strategy to access cellulose (Lynd *et al.* 2002). Evidence has emerged of bacteria with lignin-decomposing abilities, albeit to a lesser extent than fungi (Bugg *et al.* 2011; Brown & Chang 2014). An actinobacterium, *Amycolatopsis* sp. 75iv2, can use lignin as a sole carbon source (Brown & Chang 2014). Previously-unknown ligninolytic bacterial enzyme systems have been found, unlike those deployed by fungi, and environmental metagenomics may reveal more (Brown & Chang 2014). Lignin-model compounds are frequently used to screen for activity, and whilst they may not be fully representative, there is also evidence of bacterial depolymerisation of natural lignin (Salvachúa *et al.* 2015).

Many bacteria are thought to favour easily accessible, low molecular weight compounds present during early decay, or released by fungal activity (de Boer & van der Wal 2008). Under such a scenario, it would be expected that bacteria would be most numerous at the start of decay, and would be displaced by fungi as the latter become established and the most labile components are used up (Clausen 1996). Conversely, the absolute number of bacteria may be maintained or even increase, but shift towards bacteria adapted to fungal co-existence, living on the products of fungal decomposition. Fungi may affect bacterial

decomposition in other ways, too: for example, *in vitro* a forest soil bacterium, *Clostridium phytofermentans*, lyses fungal hyphae to increase its own cellulose decomposition, presumably due to acquisition of fungal nutrients (Tolonen *et al.* 2015).

Again, work on forest soils and leaf litter can offer clues as to the roles of fungi and bacteria in complex polymer decomposition. *Acidobacteria* from a forest soil showed a range of enzymatic abilities, including the capacity to break down chitin (a fungal cell wall component) and cellobiose (a cellulose breakdown product) (Lladó *et al.* 2015). Importantly, the dominant taxa in terms of DNA abundance do not necessarily match the most active taxa based on RNA transcripts (Žifčáková *et al.* 2016). Fungal and bacterial biomass in soil does not vary greatly between seasons, but their patterns of transcription activity do show strong seasonal effects (Žifčáková *et al.* 2016).There is evidence for some degree of functional redundancy in litter-decomposing communities (Purahong *et al.* 2015).

Mixed communities of bacteria show greater decomposition ability in wood than individual species (Schmidt & Liese 1994), which implies that bacterial contributions to wood decomposition may have been underestimated. Nonetheless, total bacterial decomposition is likely to remain negligible compared to fungi, due to the latter's size and superior access to material: factors which would also allow fungi to decompose wood at a faster rate. One situation in which bacteria do play a major role in wood decomposition is in wet/waterlogged wood such as cooling towers and archaeological structures; the low oxygen concentrations under these conditions are inhibitory to most fungi, leaving bacteria as major agents of decomposition (Kim & Singh 2000). A fluid-filled environment is also far more conducive to bacterial movement (facilitating colonisation) than a dry material. Bacterial wood decomposition is usually slow and incomplete, and thus wooden artefacts can be preserved for centuries under these conditions (Björdal 2012).

Bacterial wood decomposition is often described based on the physical patterns produced in the wood ultrastructure, and is can be grouped into four main types (reviewed Greaves 1971; Clausen 1996; Kim & Singh 2000). These categories are based on the morphology of wood substratum following decay, rather than the taxonomic affiliations of the bacteria involved. 'Tunnelling' bacteria decay a convoluted path inside the cell walls (Kim & Singh 2000), which they may enter via pit chambers (Greaves 1969). They can act on all components of the cell wall and may be able to degrade/modify lignin, at least to some extent (Kim & Singh 2000). 'Erosion' bacteria create depressions in the wall from inside the lumen, which follow the path of cellulose microfibrils; they rarely affect the middle lamella, and probably lack the ability to degrade lignin (Greaves 1969; Kim & Singh 2000). 'Pitting'

bacteria produce small, shallow indentations (Greaves 1971); the term is somewhat confusing, given that bacteria often are associated with pits connecting cells. 'Cavitation' bacteria cause diamond-shaped cavities inside cell walls, possibly involving the production of diffusible enzymes (Kim & Singh 2000). Bacteria are often associated with pits between wood cells, and decomposition activity in these areas can greatly increase the permeability of the wood (Greaves 1969).

2.7. Bacterial-fungal community interactions

2.7.1 Community competition and co-operation

It is not difficult to envisage why fungal-bacterial co-existence in wood could lead to conflict. Both may compete for the same substrates; bacteria may remove the products of fungal extracellular enzyme decomposition (effectively microbial kleptoparasitism); and either group may regard the other as a food resource. Certainly wood-decay fungi have an arsenal of competitive strategies capable of deployment (Boddy 2000). Bacteria described as closely associated with *Phanerochaete chrysosporium* were all proficient at utilising lignin breakdown products *in vitro*, supporting the idea that they gained nutrition from fungal activity (Seigle-Murandi *et al.* 1996).

Studies show that the introduction of *Hypholoma fasciculare* can alter the abundance and community composition of bacteria within wood (Folman *et al.* 2008; de Boer *et al.* 2010), and rapid pH change has been suggested as a possible mechanism (de Boer *et al.* 2010). Whether this is representative of a natural situation is uncertain: bacteria were very abundant in more decayed *H. fasciculare*-colonised wood in a field scenario, which could be due to bacterial recovery over time, or the proliferation of fungus-tolerant bacteria (Valásková *et al.* 2009). Inoculation with *Phlebiopsis gigantea* lowered bacterial species richness in stumps after 12 months, although the effect had disappeared by 6 years post-inoculation (Sun *et al.* 2013). It is possible that direct fungal-bacterial competition occurs in wood similar to that observed in soil, where experimental inhibition of bacteria results in accelerated fungal growth indicative of competitive release (Rousk *et al.* 2008; Rousk *et al.* 2010). Intriguingly, the competitive outcome appears to depend on the ambient pH: fungi prevail at low pH, bacteria at higher pH (Rousk *et al.* 2010). This is salient in light of the major pH modification – typically lowering – that fungi effect in wood (de Boer *et al.* 2010).

There is much *in vitro* evidence for antagonism amongst saproxylic micro-organisms, both of fungi against bacteria (Janes *et al.* 2006; Popova *et al.* 2009) and of bacteria against fungi (Murray & Woodward 2003; de Boer *et al.* 2007; Caldeira *et al.* 2008; Boaisha 2012).

This is not necessarily evidence for antagonism *in situ*, given that the effect can depend on the culture medium (Murray & Woodward 2003; Boaisha 2012). In addition, soil bacteria with little or no anti-fungal activity on their own (*Pedobacter* sp. and *Pseudomonas* sp.) can show considerable fungal inhibition when in combination, either as a collaborative effort or a by-product of antagonism towards each other (de Boer *et al.* 2007).

Conclusive evidence of fungal-bacterial mutualism requires demonstration of a benefit to both partners. Although this has been demonstrated in a variety of habitats (Frey-Klett *et al.* 2011), there is a shortage of clear examples in terrestrial dead-wood. There are reports of basidiomycetes gaining more biomass and decaying wood faster in the presence of yeasts and nitrogen-fixing bacteria than in their absence (Blanchette & Shaw 1978). Moreover, SEM revealed close physical association between the mycelial fungi, yeasts and bacteria (Blanchette & Shaw 1978). *H. fasciculare* and *Resinicium bicolor* decompose wood significantly faster in the presence of bacteria than alone; *Heterobasidion annosum* displayed the same effect only if bacteria were added after the fungus had become established (Murray & Woodward 2003). However, in other instances, bacteria had no effect on *H. fasciculare* decomposition (Weißhaupt *et al.* 2013). Such variability could be influenced by bacterial community composition, fungal intra-specific variation, or environmental conditions. Increased decomposition may be attributable to bacterial nutrient provision (*e.g.* vitamin production (Ghignone *et al.* 2012) or N-fixation), or up-regulation of fungal enzymes due to the removal of breakdown products (Murray & Woodward 2003; de Boer *et al.* 2005). Whilst the latter scenario would represent facilitation in ecosystem process terms, the benefit to the fungus is questionable, depending on whether faster decomposition translates to increased fungal growth, or simply to decreased efficiency due to bacterial consumption of breakdown products. This could be particularly disadvantageous to fungi with an ecological strategy that involves slowly decomposing wood over a long period, as with some xylariacious ascomycetes (Boddy *et al.* 1989).

There are examples of bacterial-fungal interactions that benefit at least one party with (currently) no evidence of harm to the other, suggesting at least a commensal association. There is also *in vitro* evidence for growth enhancement, which should be regarded with the same caveats as *in vitro* antagonism. For example, a bacterium of the *Burkholderia cepacia* complex, isolated from a *Pleurotus ostreatus* fruit body, showed increased growth in the presence of *P. ostreatus* mycelium (Yara *et al.* 2006). A *Curtobacterium* sp. from dead wood promoted growth of *Stereum* sp. – although it was the only one out of 24 culturable strains to do so (Kamei *et al.* 2012). Notably, *Streptomyces* from

woodland soil showed negative or neutral influences on mycorrhizal and pathogenic fungi, yet all consistently and markedly promoted growth of the white-rot fungus *Phanerochaete chrysosporium*; it may be salient that none of these *Streptomyces* strains showed ligninolytic activity themselves (Bontemps *et al.* 2013).

The fungal-migratory bacterium *Paraburkholderia terrae* BS001 (see Section 2.4.3) has been demonstrated to protect a (non-wood-decay) fungus from inhibition by the fungicide cycloheximide or metabolites from the antagonistic bacterium *Pseudomonas fluorescens* CHA0 (Nazir *et al.* 2014). This raises the possibility of a true mutualism, whereby *P. terrae* BS001 gains access to resources and in return affords protection to its fungal host. The level of protection depended on the identity of the fungus, and, for reasons unknown, the timing of bacterial arrival (Nazir *et al.* 2014).

2.7.2 Bacterial endosymbiosis and intimate hyphal associations

Bacteria co-exist endosymbiotically with arbuscular mycorrhizal fungi (Bonfante & Anca 2009), but such an association has yet to be conclusively demonstrated for wood-decay fungi. It is likely that bacteria do occur within the hyphae of wood-decay fungi, as they have also been found inside ectomycorrhizal hyphae (Bertaux *et al.* 2005), plant-pathogenic fungi (Partida-Martinez & Hertweck 2005), a range of endophytic fungi (Hoffman & Arnold 2010) and the soil saprotroph *Mortierella elongata* (Sato *et al.* 2010). Intrahyphal existence does not necessarily indicate true endosymbiosis, which implies active interaction between living cells (Lumini *et al.* 2006). Rather, bacteria may enter compromised or senescent hyphae opportunistically and without vertical transmission (de Boer *et al.* 2005; Lumini *et al.* 2006). This distinction is not always made clearly in the literature on fungal 'endosymbionts', nor is it always recognised that bacteria may associate intimately but extracellularly with mycelium.

Where true endosymbiosis does occur, the extent of its implications are illustrated by the well-characterised association between the plant-pathogenic fungus *Rhizopus microsporus* and its bacterial endosymbiont, *Paraburkholderia rhizoxinica* (Partida-Martinez & Hertweck 2005). The toxin rhizoxin forms a key part of *R. microsporus* pathogenicity, yet is synthesised not by the fungus but by the bacteria within it. Vertical transmission of the bacteria is guaranteed, as *R. microsporus* has lost the ability to sporulate in the absence of *P. rhizoxinica* (Partida-Martinez *et al.* 2007b). *P. rhizoxinica* is also highly competent to colonise *R. microsporus* hyphae from the outside by localised chitinase activity that does not cause fungal lysis (Moebius *et al.* 2014). Colonisation relies on both the type II and type III secretion systems (Lackner *et al.* 2011a; Moebius *et al.* 2014). Pertinently, these secretion systems

have been implicated in other fungus-bacteria interactions (Nazir *et al.* 2017), such as mycorrhiza formation, described as the 'helper bacteria effect' (Cusano *et al.* 2011); mycophagy (Mela *et al.* 2012); co-migration (Warmink & van Elsas 2009; Nazir *et al.* 2012; Nazir *et al.* 2013; Nazir *et al.* 2014; Haq *et al.* 2016; Yang *et al.* 2016); and an undefined bacteria-fungus association (Warmink & van Elsas 2008).

Aside from true endosymbiosis, there is evidence that wood-decay fungi form intimate mycelial associations with bacteria (Seigle-Murandi *et al.* 1996; Lim *et al.* 2003; Yara *et al.* 2006). For example, bacteria have been observed to co-exist with ten strains of *Phanerochaete chrysosporium* (Seigle-Murandi *et al.* 1996), although Janse *et al.* (1997) failed to isolate bacteria from five strains of the same fungus, including one described by Seigle-Murandi *et al.* (1996). Thirty-two other wood-rot fungi tested negative, but where bacteria were present on *P. chrysosporium*, pure cultures of the fungus could not be established even from conidiospores, suggesting that bacteria may be within the hyphae and vertically transmitted (Seigle-Murandi *et al.* 1996). Similarly, *Paraburkholderia sordidicola* was isolated from two strains of *Phanerochaete sordida*, and bacteria-free fungal cultures could not be established (Lim *et al.* 2003).

2.7.3 Mycophagy and predation

From mycelial associations, it is a small step to bacterial mycophagy (fungus-eating): the active utilisation of living fungal matter for bacterial growth (Leveau & Preston 2008). Given that bacteria are smaller than fungi, and do not kill the entire host organism, bacterial mycophagy is more analogous to parasitism than predation. There is also potential for mutualistic mycophagy, where bacteria 'pay their way' by provision of specific nutrients or degrading toxins (Leveau & Preston 2008). Endosymbiosis can be regarded as a specialised form of mycophagy (Leveau & Preston 2008).

An increasing body of evidence suggests that glycerol is a favoured carbon source for many mycophagous bacteria, although so far none of this evidence is derived directly from wood. In liquid culture *Paraburkholderia terrae* BS001 stimulates glycerol release by the fungus *Lyophyllum* sp. strain Karsten, and the glycerol is apparently consumed by the bacteria (Nazir *et al.* 2013). Whilst this ability has not yet been tested in an ecologically realistic situation, the *P. terrae* BS001 genome encodes glycerol transporters that are unique among the *Paraburkholderia*, possibly linked to its fungus-associated lifestyle (Haq *et al.* 2014). *Paraburkholderia rhizoxinica*, the *Rhizopus* endosymbiont, likewise possesses genes involved in glycerol metabolism and can utilise glycerol as a carbon source (Partida-Martinez *et al.* 2007a; Lackner *et al.* 2011b). The mycophagous bacterium *Collimonas fungivorans* is

also capable of metabolising glycerol (de Boer *et al.* 2004). In several *Burkholderia/Paraburkholderia species,* glycerol induces production of antibiotics, including the antifungal pyrrolnitrin (Depoorter *et al.* 2016).

True mycophagy can be difficult to demonstrate, as many bacteria may feed saprotrophically on dead hyphae or passively on fungal exudates, whilst others may lyse hyphae for reasons other than nutrition (Leveau & Preston 2008). Evidence for mycophagy in wood is limited. A strain of *Streptomyces violaceusniger* isolated from bark inhibited fungi by endochitinase production, but only after being 'conditioned' by exposure to chitin (a major component of fungal cell walls) (Shekhar *et al.* 2006). This is an example of probable mycophagy where it has not yet been demonstrated that the bacteria fulfil the criterion of using fungal material for growth. Bacteria closely related to the mycophagous *Collimonas fungivorans* have been found on mycelial cords of the white-rot fungus *R. bicolor* (Folman *et al.* 2008). *Collimonas* is able to use fungal hyphae as a sole carbon source, and apparently uses the fungal exudate oxalic acid as a signal molecule to locate hyphae (Rudnick *et al.* 2015). The abundance of *Collimonas* cells has been observed to be higher in forest soils than in either grassland or ex-arable soils (Höppener-Ogawa *et al.* 2007). The further observation that collimonads can alter the fungal community composition in soil microcosms indicates their potentially far-reaching importance (Höppener-Ogawa *et al.* 2009).

Conversely, there is also evidence for wood-decay fungi feeding on bacteria; for example, the physical disappearance of bacteria cells following fungal inoculation, and/or the appearance of bacterial nutrients in mycelium under starvation conditions (Folman *et al.* 2008; Weißhaupt *et al.* 2011). Wood decay fungi have been observed to lyse bacterial colonies in culture, including consumption of the bacteria that were decomposing dead nematodes (Tsuneda & Thorn 1994). Again, such associations could be mutualistic if the fungus, despite consuming some bacterial cells, provided bacteria with nutrition and/or habitat. This seems to be the case for the soil saprotroph *Morchella crassipes*, which showed reciprocal carbon exchange with *Pseudomonas putida* but lysed some of the bacteria to feed the nutrient-intensive process of sclerotia formation: a situation that has been described, controversially, as fungal farming of bacteria (Pion *et al.* 2013).

2.8. Conclusions and future perspectives

Despite the many gaps in our knowledge of wood-dwelling bacteria, a picture emerges of a diverse and dynamic community, intimately linked to their physical habitat and the fungi they share it with. The complexity of these potential interactions, and the

challenges associated with wood as a study system, mean that gaining a clear understanding of this environment will require the assembly of many 'jigsaw pieces' of information. The ultimate goal in researching fungus-bacteria interactions in wood is a functional understanding of how fungi and prokaryotes interact, in terms of outcome for each partner, mechanisms of interaction, and effects on the process of wood breakdown. Before such questions can be addressed, it is first necessary to ascertain which organisms are present and their activities within the dead-wood environment. Outstanding questions include:

- What is the origin of bacteria in wood; how and over what time-scale does colonisation occur?
- What are the major biotic and abiotic determinants of bacterial communities, and by what mechanisms do these operate?
- Are interactions with fungi predominantly beneficial or antagonistic? Does one partner consistently benefit at the expense of the other?
- How, and to what extent, do bacteria influence ecosystem-level flows of carbon and nitrogen in the context of dead wood?

One of the major features that emerges with regard to fungal-prokaryote interactions is just how hard it can be, in any given case, to distinguish the exact identity of the association. If fungal growth increases in the presence of a bacterium (or *vice versa*), is it mutualism, commensalism or parasitism? Do we truly see mycophagy rather than saprotrophy, endosymbiosis rather than opportunism? When fungi alter the bacterial community, are they selecting for their specific symbionts or simply unable to out-compete the remaining bacteria – or a mixture of the two? Such associations can be deceptively hard to disentangle.

Happily, a suite of methods is coming of age that will hopefully assist in answering such questions. Metagenomics gives a snapshot not just of the taxonomic identities of the community, but also of their genomic potential (although it still has limited ability to marry the two). Metatranscriptomics and metaproteomics offer insight into which of these potential abilities are realised in a particular situation. Metabolomics explores the complete metabolic signature of a microbial community under given conditions. At the same time, new culture methods offer hope for isolating key community players, allowing physiological characterisation and manipulative experiments (Ling *et al.* 2015; Oberhardt *et al.* 2015; Kielak *et al.* 2016a). Each of these techniques comes with associated limitations, pitfalls and

benefits, and it will require judicious use of these approaches, combined with appropriate statistical and mathematical methods, to pick apart fungal-prokaryote associations.

The overwhelming conclusion regarding the current state of knowledge is that, despite the work already done on saproxylic bacteria and their interactions with fungi, we have still barely scratched the surface. Results can be disparate or even contradictory depending on the environmental conditions, identity of the organisms involved, or methods employed, frustrating the chance of drawing together a robust theoretical framework. With so much ground still to cover, the microbiota of dead wood remains a lively and under-explored area of ecological research, but one that is likely to be highly rewarding and will be furthered by deployment of modern genomic and post-genomic approaches.

Chapter 3. Bacterial qPCR primers co-amplify fungi

3.1 Abstract

Quantitative PCR (qPCR) depends on primer specificity in order to draw meaningful conclusions. Universal 16S rRNA gene bacterial primers used for qPCR and other applications were screened *in silico* against a fungal database and found to match perfectly against sequences therein. Two pairs of qPCR primers were also tested *in vitro* on mixed bacterial and fungal DNA; both amplified fungal DNA, sometimes to the exclusion of a bacterial product. These results demonstrate that universal bacterial primers targeting 16S rRNA gene sequences are not specific in samples with a high fungal abundance. They underline the importance of validating primer specificity in a given environment before use.

3.2 Introduction

Molecular methods have revolutionised the field of microbial ecology, enabling studies to move beyond the culturable fraction and encompass the so-called 'microbial dark matter' (Solden *et al.* 2016). Consequently, traditionally challenging environments and communities have become amenable to study, such as the bacterial component of dead-wood microbiota (Rinta-Kanto *et al.* 2016). Explicitly recognising the assumptions and limitations of these powerful tools is an ongoing challenge (*e.g.* Orgiazzi *et al.* 2015).

Quantitative PCR (qPCR) is a widely used and valuable tool in microbial ecology. It uses a fluorescent reporter to quantify the accumulation of amplicons during a PCR: either a dye that intercalates with double-stranded DNA, or a probe that fluoresces when bound to an amplicon. By monitoring the fluorescent signal, the progress of the reaction can be followed in real time. Extrapolating this information back in time produces an estimate of how many copies of the target region were present in the starting material. However, qPCR has frequently suffered from use of poor methodology, and a lack of transparency in reporting of methods (Bustin *et al.* 2009; Bustin *et al.* 2013). The introduction of the MIQE guidelines (Minimum Information for publication of Quantitative real-time PCR Experiments) has increased awareness of the importance of proper method validation and reporting, but the majority of papers still do not adhere to the guidelines (Bustin *et al.* 2013). Primer specificity is of critical importance as, unlike in endpoint PCR, the amplicons are not subject to identification methods such as DGGE or sequencing.

One common use for qPCR is quantifying total bacterial abundance in mixed microbial samples. To that end, a multiplicity of universal bacterial primers, mostly targeting the 16S rRNA gene, is used to capture diversity. An idealised set of universal bacterial primers would amplify all bacterial taxa whilst excluding archaea and eukaryotes. However, there are reports of universal bacterial primers co-amplifying eukaryotic DNA when it is present in large quantities (*e.g.* in host tissue samples) (Huws *et al.* 2007; Galkiewicz & Kellogg 2008). Here, evidence is presented that commonly-used universal 16S rRNA gene bacterial also amplify fungal DNA and, therefore, may not be reliable in quantifying bacteria in environments where fungi are relatively abundant.

3.3 Materials and methods

Nine commonly-used universal bacterial 16S primer sets (Table 3.1) were screened *in silico* using Primer-BLAST (Ye *et al.* 2012) implemented in R by the package *primerTree* (R Development Core Team 2011; Hester 2016), with database '*refseq representative genomes*'

and organism '*Fungi*' (NCBI Resource Coordinators 2016; O'Leary *et al.* 2016). These primer sets are used for a variety of applications, including amplicon sequencing, DGGE and qPCR. Where primers included degeneracies, all possible forward and reverse combinations were screened. To confirm that the results were supported *in vitro*, primer set 519F-907R (Schwieger & Tebbe 1998) was selected for further testing.

Fungal DNA was extracted from pure cultures of *Hypholoma fasciculare*, *Trametes versicolor* and *Vuilleminia comedens* (Cardiff University Culture Collection). Mixed-community DNA was obtained from soil samples taken from three locations in the Cardiff University grounds: flowerbed, lawn and under a tree (*Quercus* sp.). Finally, to represent a fungus-rich environment, DNA was obtained from *Fagus sylvatica* wood colonised with the above wood decay basidiomycetes and field-exposed for 1 yr (Hiscox *et al.* 2016; Chapter 4). DNA was extracted from all samples using the PowerSoil® kit (MO BIO, Carlsbad, USA), incorporating a bead-beating step (3 x 20 sec at 4 m sec^{-1} in a MP FastPrep®-24 bead beater).

PCR with fungal primers gITS7F-ITS4R (targeting the internal transcribed spacer region of the rRNA gene; Ihrmark *et al.* 2012) revealed very low fungal DNA content in the soil extractions. PCR with bacterial primers was conducted in 50 µl reactions containing 1 µl DNA, 400 nM each primer (MWG Eurofins, Ebersberg, Germany), 5 µg BSA (Promega, WI, USA), 0.025 U μl^{-1} Taq polymerase (PCR Biosystems, London, UK) in supplied buffer, amplified in a Dyad DNA Engine Peltier thermal cycler (Bio Rad, Herts, UK) for 95°C for 5 min, 30 x [95°C for 30 s, 55°C for 1 min, 72°C for 1 min], and then 72°C for 7 min. To confirm that the bands in fungus-only samples derived from non-specific amplification rather than hyphal-associated bacteria, these bands were cut out, purified with the QIAQuick gel extraction kit (Qiagen, Hilden, Germany), and Sanger sequenced by MWG Eurofins.

To try to quantify thresholds for non-specific amplification, dilution series were prepared for DNA from *H. fasiculare* DNA and the flowerbed soil DNA; three 'gradient' series were then prepared and amplified with the universal bacterial 16S rRNA gene primer sets 519F-907R (Schwieger & Tebbe 1998) and 338F-518R (Fierer *et al.* 2005). In gradient one, fungal DNA decreased from 2.9 µg ml^{-1} to 2.9 x 10^{-7} µg ml^{-1} in tenfold dilution increments, whilst bacterial DNA remained constant at 25.8 µg ml^{-1}. In gradient two, bacterial DNA decreased from 25.8 µg ml^{-1} to 2.58 x 10^{-6} µg ml^{-1} in tenfold dilution increments, whilst fungal DNA remained constant at 2.9 x 10^{-2} µg ml^{-1}. In gradient three, fungal DNA decreased whilst bacterial DNA increased according to the above dilution series. Products were analysed by

Table 3.1 Universal bacterial primer pairs used in the Primer-BLAST against the fungal database.

Primer set	**Source**	**Sequence**	**Notable use**
27F-1492R	Weisburg *et al.* 1991	AGAGTTTGATCCTGGCTCAG TACCTTGTTACGACTT	Very widely used universal bacterial primers
27F-534R	Shakya *et al.* 2013	AGAGTTTGATYMTGGCTCAG TYACCGCGGCTGCTGG	Captured a mock community better than other primers (Shakya *et al.* 2013)
338F-518R	Fierer *et al.* 2005	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Used to quantify bacteria in fungus-decayed wood (Rinta-Kanto *et al.* 2016)
357F-518R	Muyzer *et al.* 1993	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	
357F-926R	Jumpstart Consortium Human Microbiome Project (HMP) 2012	CCTACGGGAGGCAGCAG CCGTCAATTCMTTTRAGT	Human Microbiome Project
515F-806R*	Caporaso *et al.* 2011/ Walters *et al.* 2016	GTGYCAGCMGCCGCGGTAA GGACTACNVGGGTWTCTAAT	Earth Microbiome Project
519F-907R	Schwieger and Tebbe 1998	CAGCAGCCGCGGTAATAC CCGTCAATTCCTTTGAGTTT	
968F-1378R	Heuer *et al.* 1999	AACGCGAAGAACCTTACC CGGTGTGTACAAGGCCCGGGAACG	
1048F-1175R	Maeda *et al.* 2003	GTGSTGCAYGGYTGTCGTCA ACGTCRTCCMCACCTTCCTC	Recommended for coverage (Horz *et al.* 2005)

** N.B. The exact sequences from Walters et al. (2016) are given as they appear in the Earth Microbiome Project official protocol, and differ slightly from the originals given in Caporaso et al. (2011).*

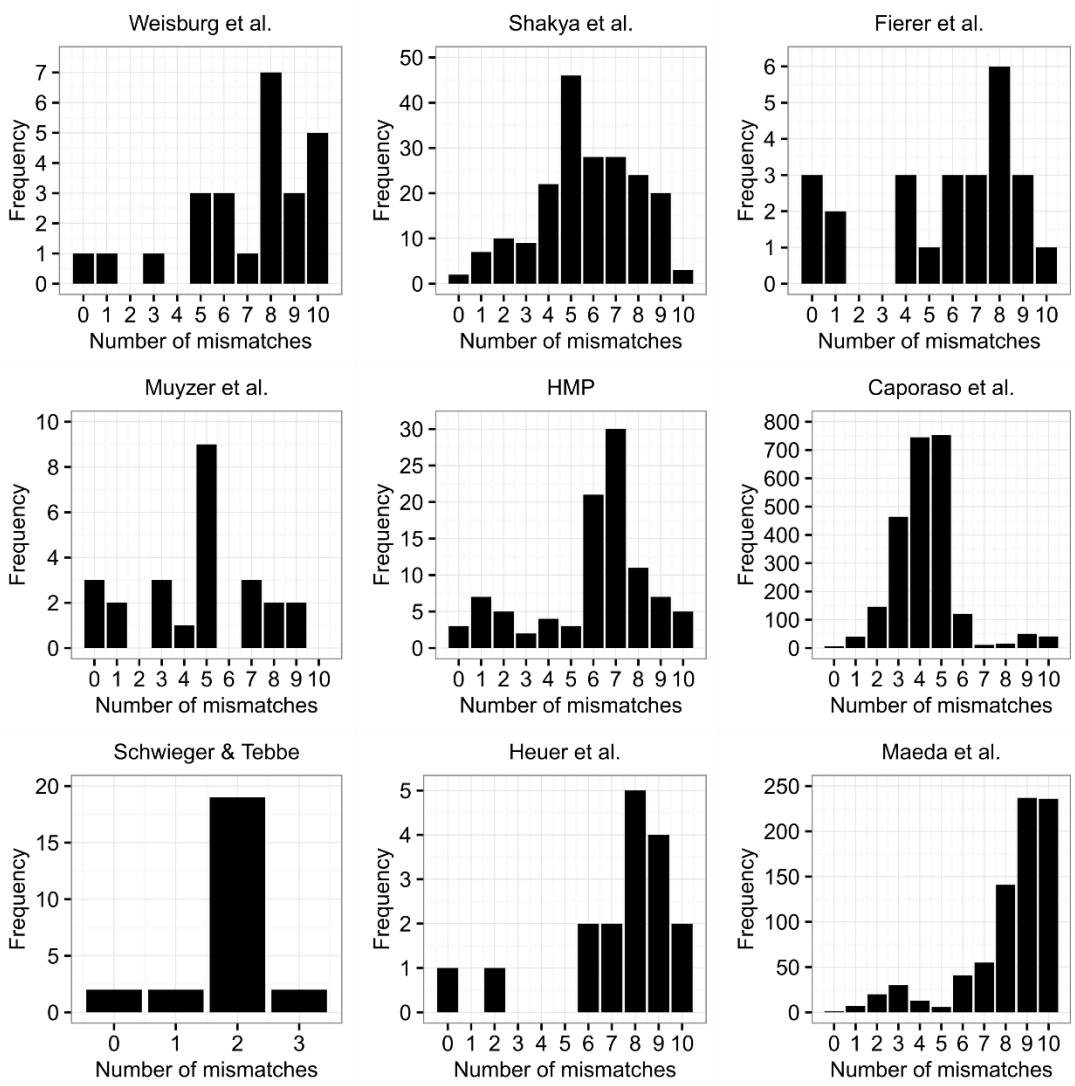

Figure 3.1 Distribution of the number of mismatches for each of the primer sets in Table 3.1 when searched against the fungal database. The top 25 hits were considered from each search; therefore, total hits for degenerate primer sets = 25 * number of possible combinations.

electrophoresis on a 1.2% agarose gel with Sybr®Safe dye (ThermoFisher, Waltham, MA, USA) for 40 min at 90 V.

3.4 Results and Discussion

BLAST screening showed that every one of the 16S rRNA gene primer pairs under consideration hit against at least one fungus with no mismatches (Fig 3.1). Four individual primers from these pairs (338F, 357F, 518R and 907R) were also screened by Huws *et al.* (2007), who likewise identified perfect hits against fungal sequences. Particularly notable are the 338F-518R primers, which have been described as fungal-exclusive (Fierer *et al.* 2005). The discrepancy probably arises because Fierer *et al.* (2005) tested the primers with soil samples, which typically have a relatively low fungal content, and sequenced only 20-25 clones. For 27F-1492R, similar non-target amplification has been seen from coral (Galkiewicz & Kellogg 2008). Across the range of primers tested, hits occurred both against mitochondrial and genomic fungal DNA. The results presented here are not exhaustive, as the number of alignments returned was restricted to 25 and additional search criteria were applied. Also, the database resources are evolving; over time, new records may be added and existing records may be revised. The analysis presented here reflects content available for query on 5th January 2017.

One possible source of perfect matches is bacterial contamination of some fungal genomes in the database (Laurence *et al.* 2014). If this were the case, a discrete cluster of hits would be expected with no mismatches, and all other hits with a large number of mismatches. Contrary to this, there was a continuous distribution of mismatches for most primer sets (Fig 3.1), suggesting that genome contamination alone is not sufficient to explain these results. Fungi represented multiple phyla, with both yeast and mycelial lifestyles. Just considering hits with ≤ 2 mismatches, 28 fungal species were represented, and increasing this up to 10 mismatches hit 177 species.

All samples tested showed a PCR product when amplified with 519F-907R; the fungal cultures produced a band of approximately 600 base pairs, larger than the approximately 400 base pair band in the bacterial samples (Fig 3.2a). Both bands were present in the wood samples. Sequencing of bands from the fungal samples, and using BLASTn to search against the NCBI database, returned sequences from *Hypholoma* sp., *Trametes versicolor* and *Vuilleminia comedens*; this confirmed that these products are derived from the fungi. Fungi are notoriously unreliable qPCR targets due to their polynucleate cells and dynamic

movement of nuclei in the mycelium, ruling out the possibility of using a correction factor to compensate (Baldrian *et al.* 2013).

The results of the admixture gradients showed that, under these conditions, 519F-907R were specific for bacteria when the ratio of bacterial : fungal DNA was ≥ 1000:1. At ratios between 1000:1 and 1:1, dual bands were produced, and whenever fungal DNA was in excess, the bacterial product disappeared (Fig 3.2b). The 338F-518R primers produced a bacterial product slightly larger than 200 base pairs and a fungal product slightly smaller than 200 base pairs. This fungal product was present at all the concentrations tested, and excluded the bacterial products at bacterial : fungal ratios ≤ 1:1 (Fig 3.2c). The first three lanes in gradient three ($1:10^6$-$1:10^2$) had a second band, but this appears to be an artefact present when fungal DNA is in a large excess (*c.f.* positive control).

One way to improve primer specificity for qPCR might be to use reporter probes rather than an intercalating dye. Given the extent of non-specific amplification at high fungus : bacteria ratios, it is dubious whether probes would be sufficient to deal with the problem. Even so, it remains an avenue for exploration, particularly if the target samples were close to the threshold.

The results presented here indicate that many common bacterial primers are also capable of amplifying fungal DNA. If fungal abundance is low, even a few mismatched bases may prevent noticeable amplification, but higher fungal presence or perfect matches to the primers will result in amplification. Therefore, primers may be specific for bacteria in one environment but not another.

For many applications, modest amounts of co-amplification are tolerable but for qPCR, target specificity is essential. These findings suggest that universal 16S rRNA gene based primers for qPCR are not reliable in samples containing a relatively high abundance of fungi, and underline the importance of checking the specificity of qPCR primers for each environment under investigation.

In conclusion, many universal bacterial primers also closely match fungal sequences. This can result in non-target amplification in samples with a high proportion of fungal DNA, sometimes even to the exclusion of a bacterial product. Therefore, prior to qPCR in mixed-community samples, primers should be carefully checked by running products on a gel and comparing to both bacterial and fungal positive controls.

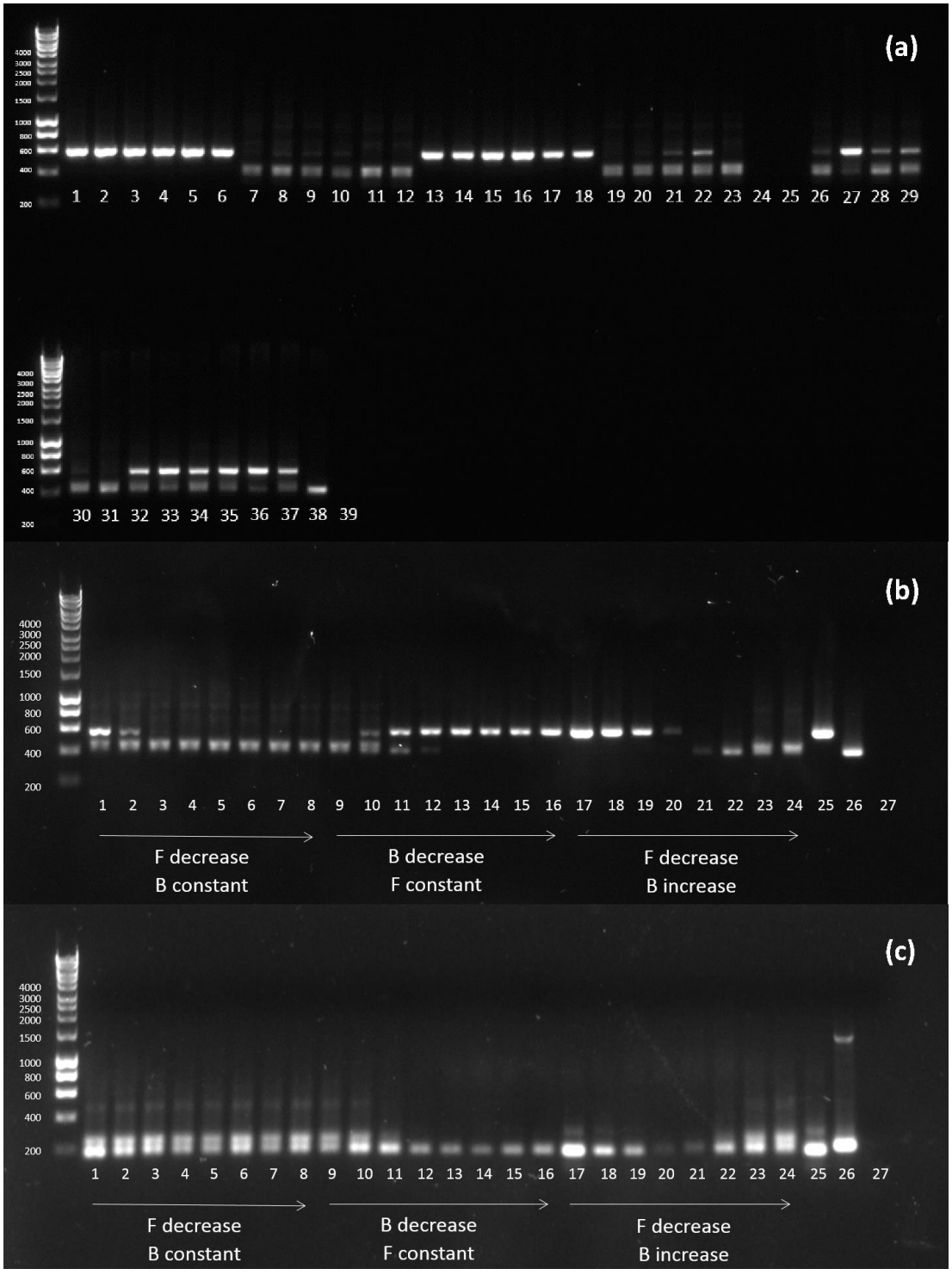
(a)
4000
3000
2500
2000
1500
1000
800
600
400
200
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
30 31 32 33 34 35 36 37 38 39
(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
F decrease
B constant
B decrease
F constant
F decrease
B increase
(c)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
F decrease
B constant
B decrease
F constant
F decrease
B increase

Figure 3.2 Gel electrophoresis of PCR testing. (a) Amplicon products from the 519F-907R primer set (Schwieger & Tebbe 1998). Lanes 1&2 *H. fasciculare*; 3&4 *T. versicolor*; 5&6 *V. comedens*; 7&8 rhizosphere soil; 9&10 lawn soil; 11&12 flowerbed soil. Lanes 13-24 contain the same samples at a 1:10 dilution. Lane 25 is an extraction kit negative control. Lanes 26-37 contain wood samples with mixed fungal and bacterial colonisation. Lane 38 is the positive control (*Escherichia coli*), lane 39 the negative. Ladder is Hyperladder 1Kb (Bioline, London, UK). (b) Three concentration gradients of fungal and bacterial (soil) DNA amplified with the 519F-907R primer set. In gradient one (lanes 1-8), fungal DNA decreases, whilst bacterial DNA remains constant. In gradient two (lanes 2-16), bacterial DNA decreases, whilst fungal DNA remains constant. In gradient three (lanes 17-24), fungal DNA decreases whilst bacterial DNA increases. Lane 25 is the fungal positive control (*H. fasciculare*), lane 26 the bacterial positive control (*E. coli*), and lane 27 the negative control. (c) Concentration gradients as in (b), amplified with the 338F-518R primer set.

Chapter 4. Highly competitive fungi manipulate bacterial communities in decomposing wood

4.1 Abstract

The bacterial communities in decomposing wood are receiving increased attention, but their interactions with wood-decay fungi are poorly understood. This study is the first to examine bacterial communities in wood in a field context whilst experimentally manipulating the fungi initially present. *Proteobacteria* and *Acidobacteria* were proportionally dominant, as in previous studies. Pre-colonising wood with decay fungi had a clear effect on the bacterial community, apparently via direct fungal influence; the bacterial and fungal communities present at the time of collection were strongly correlated with each other. Site was less important than fungal influence in determining bacterial communities. Wood pH was also a strong bacterial predictor, but was itself under considerable fungal influence. Certain bacterial families showed directional responses against the trend of the bacterial community as a whole.

4.2 Introduction

Wood-decay fungi are the major terrestrial agents of wood decomposition, and known for their highly territorial and competitive ecological strategies (Boddy 2000; Boddy *et al.* 2017). Despite the extensive literature on inter-fungal interactions, very little is known about how they interact with bacteria in wood (de Boer *et al.* 2005). The interactions between fungi and bacteria in dead wood are likely to have ramifications for ecosystem processes, particularly the rate of wood decomposition. Given that different fungi decompose wood at very different rates, anything that affects fungal community composition will have knock-on impacts on the regulation of this nutrient source (Crowther *et al.* 2011). Although direct bacterial contributions to decomposition are likely to be modest, bacteria could potentially have a far greater indirect effect on decomposition rates by consuming the breakdown products of fungal enzymatic activity, thus preventing enzyme down-regulation via feedback inhibition (de Boer *et al.* 2005; Johnston *et al.* 2016).

It is only with the rise of high-throughput sequencing that accurate bacterial surveys have become possible, as the culturable fraction of dead-wood-inhabiting bacteria can be as low as 1% (Folman *et al.* 2008). The surveys performed so far indicate a diverse suite of bacteria within decomposing wood (Zhang *et al.* 2008; Hoppe *et al.* 2014; Sun *et al.* 2014; Hoppe *et al.* 2015; Kielak *et al.* 2016; Rinta-Kanto *et al.* 2016). Many physical properties of wood, such as pH, state of decay, moisture content and C:N ratio, influence the bacterial community composition (Hoppe *et al.* 2015). Each of these can be altered by fungi activity, varying depending on species. This underlines the direct and indirect influence that fungi could exert over bacteria in wood. Microcosm studies already indicate that wood-decay fungi modify bacterial communities within their resource (Folman *et al.* 2008; Hervé *et al.* 2014), and there is evidence that certain bacterial and fungal taxa associate non-randomly in the field (Hoppe *et al.* 2014; Kielak *et al.* 2016).

Bacterial diversity in wood appears to be highly heterogeneous within and between sites (Sun *et al.* 2014; Hoppe *et al.* 2015). Soil type (Sun *et al.* 2014), and the surrounding forest management regime (Hoppe *et al.* 2015) are important predictors of inter-site variability. At the landscape scale, pH is the major driver of soil bacterial communities and bacterial richness increases as pH does (Fierer & Jackson 2006; Griffiths *et al.* 2011). If, as is usually assumed, soil is the primary source of wood-inhabiting bacteria, then local soil pH would be expected to constrain the pool of potential colonists. Bacteria entering the wood would undergo a second round of selection by pH, as wood usually represents an acidic environment and many wood-decay fungi dramatically lower the pH of their environment

(de Boer *et al.* 2010). Under this scenario, it would be expected that bacterial diversity in wood would be negatively correlated with wood pH, and that this effect would be most obvious at sites with a high soil pH (where there is a more diverse pool of colonists).

Fungal decomposition of wood is a dynamic process, carried out by a successional series of fungi. Each fungus has a particular chemical signature of decomposition (Schilling *et al.* 2015), and also varies idiosyncratically in its ability to hold territory against invaders (Boddy 2000; Boddy *et al.* 2017; Hiscox *et al.* 2017). These factors lead to priority effects in wood: distinctive patterns of successor species dictated by the identity of former colonists (Hiscox *et al.*, 2015; Hiscox *et al.* 2016). It may be more meaningful to think of bacteria associating with a particular fungal community, rather than a particular fungus, with that community shaped by both the currently dominant fungus, and a succession of predecessors. There is also the possibility that priority effects operate on bacteria directly as well, due to the biochemical legacy of fungi that have been replaced.

This study surveyed the bacterial community in decomposing wood at six UK woodland sites, with explicit reference to the fungi present. It tests three predictions: (1) that the bacterial community would vary depending on the identity of the original fungal colonist; (2) that the bacterial community would be significantly correlated with the identity of the fungal community present at time of sampling; and (3) that there would be inter-site differences in the bacterial community. In addition to testing these formal hypotheses, exploratory analysis was conducted to further characterise the drivers of bacterial community composition.

Table 4.1 Fungal species used to colonise disks.

Name	**Strain**	**Family**	**Ecological strategy**	**Acronym**
Hypholoma fasiculare	HfDD3	Strophariaceae	Late stage secondary/ tertiary colonist; cord former	Hf
Trametes versicolor	TvCCJH1	Polyporaceae	Early-mid stage secondary colonist	Tv
Vuilleminia comedens	VcWVJH1	Corticiaceae	Primary colonist	Vc

All fungi are white-rot wood decay basidiomycetes from the Cardiff University Culture Collection.

4.3 Materials and Methods

4.3.1 Overview

Wood disks were lab-colonised with wood-decay fungi, and exposed for 1 yr on the floor at six woodland sites across the southern UK. After collection, bacterial and fungal communities were characterised by amplicon sequencing. The fungal analysis has been published elsewhere (Hiscox *et al.* 2016), and is only dealt with here insofar as it pertains to the bacterial data.

4.3.2 Field experiment

Branches from beech trees (*Fagus sylvatica*) were felled and cut into sections approx. 2 cm thick and 10-20 cm diameter. Wood disks were frozen after cutting, and sterilised by autoclaving three times in a 72-hr period. Disks were colonised for 3 months by single wood-decay fungi (Table 4.1) on 0.5% malt agar (5 g l^{-1} malt, 15 g l^{-1} agar no. 2, LabM, UK) in 400 ml plastic tubs (Cater4you, UK). 25% of disks were kept sterile and frozen at -20°C as uncolonised controls. In autumn 2012, disks were scraped free of surface mycelium, and disks colonised by each of the three fungal species, with uncolonised controls, (9 replicates) were placed on the forest floor at random positions on a grid at each of six sites across the southern UK (Table 4.2). All field sites were situated in mixed deciduous woodland containing predominantly *F. sylvatica* but were otherwise unmatched, in order to encompass greater site diversity.

Disks were collected after 1 yr (autumn 2013) and transported back to the lab individually in sealed plastic bags. A soil sample was taken directly below each disk for pH analysis. Each disk was surface-sterilised with 10% household bleach, before drilling with a sterile drill bit at random points spaced evenly across the face of the disk. Swarf was flash-frozen in liquid N_2 and stored at -80°C. At six points on each face of the disk, chips of wood were removed aseptically and re-isolated onto 2% malt agar to assess pre-coloniser persistence. DNA was extracted from 0.3 g swarf using the MoBio PowerSoil® kit (Carlsbad, CA USA), replacing the vortex step with 3 x 20 s bead-beating at 4 m s^{-1} in a MP FastPrep®-24. For each disk, 0.5 g from a second aliquot of swarf was added to 5 ml distilled water and mechanically shaken for one hr; pH was measured using a Hanna Instruments pH20 pH meter. Soil pH readings were taken by the same method.

4.3.3 Molecular analysis

Joint fungal-bacterial community analysis was performed on four disks per treatment per site, with the exception of disks initially colonised with *H. fasciculare* at Bagley.

In this case, replicates were lost during the field exposure (probably due to mammal activity), and the treatment had to be excluded from the analysis.

The fungal ITS2 region was amplified using gITS7 (GTGARTCATCGARTCTTG) and ITS4 (CCTCCGCTTATTGATATGC); the ITS4 reverse primer was modified to incorporate 8-base identification tags (HPLC-purified, Integrated DNA Technologies, Inc, Belgium) (Ihrmark *et al.*, 2012). PCRs were carried out in 50 µl reactions (2.5 µl template, 300 nM tagged ITS4, 500 nM gITS7, 0.025 U HS Taq polymerase (PCRBiosystems, UK), 10 µl supplied buffer) in a Dyad DNA Engine Peltier thermal cycler. The initial incubation was 5 min at 94°C; followed by 22–30 × (30 s at 94°C; 30 s at 56°C; 30 s at 72°C) and 7 min at 72°C Triplicate PCRs per sample were pooled equimolarly based on image analysis using ImageJ software (Rasband, 1997-2014), purified with the QIAQuick gel extraction kit (Qiagen, Hilden, Germany), and quantified with the Quant-iT PicoGreen dsDNA assay kit (Life Technologies Ltd, UK). Samples were sequenced on a Roche 454 GS FLX+ (Hoffman La-Roche Ltd., Germany) by the NERC Biomolecular Analysis Facility, Centre for Genomic Research, Liverpool, UK.

PCR and sequencing of the bacterial 16S rRNA gene region from the same samples was carried out by the Institute of Applied Biotechnologies, Prague, Czech Republic. Triplicate PCRs were performed using primers S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC) (Klindworth *et al.* 2013), and the products pooled. This primer pair targets the V3-V4 region of the 16S rRNA gene, and shows excellent taxonomic coverage (Klindworth *et al.* 2013). Samples were sequenced on an Illumina MiSeq (v3, 2 x 300 base-pair reads) (Illumina, Inc., San Diego, USA) with Nextera XT assay chemistry.

4.3.4 Sequence analysis

Fungal sequence data were processed as described in Hiscox *et al.* (2016). For bacterial sequences, paired-end reads were joined and demultiplexed. The sequences were filtered to retain only those with complete, error-free primer regions, and the primers and barcodes were removed. USEARCH v9.0.2132 (Edgar 2010) was used to exclude sequences with less than 400 base pairs or more than two expected errors, before downstream analysis with QIIME (Caporaso *et al.* 2010). Chimeric sequences were identified in QIIME using USEARCH 61 and removed. Sequences were clustered into operational taxonomic units (OTUs) by open reference picking against the Greengenes 16S rRNA gene database (DeSantis *et al.* 2006) at 97% sequence similarity. Singletons (OTUs occurring only once) were removed at this stage. To check for fungal sequence contamination, OTU picking was repeated against the SILVA_119 16S/18S rRNA gene database (Pruesse *et al.* 2007; Quast *et al.* 2013). No

sequences were assigned to fungi, so the Greengenes OTUs were used for subsequent analysis. Relative abundance plots were produced in QIIME with *summarize_taxa_through_plots.py*. Fungal sequence data are archived at NCBI SRA (accession no. SRP052547). Bacteria sequence data are archived at the European Nucleotide Archive (ENA) under accession number PRJEB22364.

4.3.5 Statistical analysis

Analysis was performed in R (R Development Core Team 2011) using RStudio (RStudio Team 2016) and packages *dplyr* (Wickham & Francois 2016), *ggplot2* (Wickham 2009), *metacoder* (Foster 2016) and *vegan* (Oksanen *et al.* 2016). All R code to reproduce the analyses is available in Appendix 2 as an R markdown file (Protocol S4.1; Allaire *et al.* 2016).

The nature of sequencing technology means that raw amplicon data vary hugely in sequence numbers (creating unequal sample sizes). This is normally dealt with by rarefying (randomly subsampling data to an equal numbers of observations). The practice has come under valid criticism, but lacks robust alternatives (McMurdie & Holmes 2014; Weiss *et al.* 2017). After careful data exploration, it was decided not to rarefy the current dataset, for the following reasons. (1) Sequencing depth did not co-vary with any of the factors of interest. (2) NMDS of the bacterial data revealed that variation due to sequencing depth could be separated out on a single axis, independently of other predictors. (3) For OTU richness assessment, modelling and residual-based analysis provide a more robust and explicit way to deal with sequencing depth. (4) Where abundances were required for plotting or Procrustes analysis, proportions were used in place of raw read counts. Amplicon data are inherently compositional, so proportions simply scale the data to make it comparable (Lovell *et al.* 2010).

4.3.6 Community analysis

Permutation ANOVA (PERMANOVA; 999 permutations) was used on a Bray-Curtis distance matrix for formal significance testing of pre-coloniser and site effects (Anderson 2001). Owing to the lack of *post hoc* tests for PERMANOVA, the dataset was then broken down into pairwise combinations and PERMANOVA run separately on each (Hiscox *et al.* 2016). Pairwise tests were conducted for species differences between sites (not site differences between species) to limit the number of tests. *P*-values for the pairwise tests were subjected to the Benjamini-Hochberg false discovery rate (FDR) correction for multiple testing (Benjamini & Hochberg 1995).

Table 4.2 Sites used in the experiment.

Name	**County**	**Grid reference (lat., long.)**	**Tree species present**	**Predominant local soil type**	**Soil pH**	**Ground cover**	
						%	**Species**
Gwaelod-y-garth	Cardiff	51.535784, -3.277605	*F. sylvatica*, *Quercus sp.*, *Ilex aquifolium*	Freely draining, slightly acid but base-rich soil	4.70	<5	Fern, sedge, bramble
Usk	Monmouthshire	51.672524, -2.953690	Predominantly *F. sylvatica*, some *Fraxinus excelsior*, *Castanea sativa* and *Acer psuedoplatanus*	Freely-draining, slightly acid, loamy soil	4.68	<5	Woodrush, fern, ivy
Tintern	Monmouthshire	51.711529, -2.684246	Predominantly *Quercus sp*, some *F. sylvatica* and *I. aquifolium*	Freely-draining, slightly acid, loamy soil	5.67	95	Bluebells, bramble, ferns, sedge
Whitestone	Monmouthshire	51.725378, -2.690553	*F. sylvatica*, *Quercus sp*, *A. psuedoplatanus*	Freely-draining, slightly acid, loamy soil	4.95	75	Bluebells, ivy, ferns, bramble, ivy, wood anemone
Wytham	Oxfordshire	51.768902, -1.344022	Predominantly *F. sylvatica*, some *Corylus avellana*	Shallow lime-rich soil over chalk or limestone	6.58	<5	Sedge, moss
Bagley	Oxfordshire	51.720134, -1.266750	*F. sylvatica*, *C. avellana*, *I. aquifolium*	Slowly permeable, seasonally wet, acid loamy and clayey soil	4.52	25	Fern, bramble, sedge

All sites were wooded, with F. sylvatica present. This table is adapted from Hiscox et al. (2016). Soil type information from Cranfield University 2015. The Soils Guide. Available: www.landis.org.uk. Cranfield University, UK. Last accessed 09/06/2015

To relate the whole bacterial and fungal communities to each other, Procrustes analysis was used to superimpose the two OTU tables (999 permutations). Subsequently, the Procrustean association metric (PAM) was extracted and regressed individually against pre-coloniser, site, wood pH and soil pH using one-way ANOVAs (Lisboa *et al.* 2014). The response variable was natural log-transformed to meet parametric assumptions.

The bacterial community composition between samples was visualised using non-metric dimensional scaling (NMDS) on a Bray-Curtis distance matrix. Given that overdispersion in the data can introduce artefacts in distance metrics (Warton *et al.* 2012), the ordination was validated by qualitative comparison with principal components analysis using a Hellinger transformation for compositional data (Fig S4.1). To compare community composition simultaneously at multiple taxonomic levels, heat trees were plotted for the different treatments (Foster *et al.* 2017). The taxonomic composition of the samples is displayed in a tree format, with node size and colour dictated by the relative abundance of each taxon.

4.3.7 Richness

OTU richness was strongly correlated with sequencing depth in the raw data. To correct for this without discarding data by rarefying, a linear model was run with sequencing depth as the sole predictor. The response variable was natural log-transformed to meet parametric assumptions. Back-transformed residuals were calculated and plotted instead of the raw data, to control for sequence depth. To further characterise drivers of richness, a general linear model was run with sequencing depth, pre-coloniser, site, wood pH and soil pH as predictors. The response variable was again *ln*-transformed. Coefficients from the model were extracted and back-transformed to quantify the relative importance of the different predictors.

4.3.8 Exploration of selected taxa

OTUs from three bacterial families (*Acetobacteraceae*, *Acidobacteriaceae* and *Burkholderiaceae*) were selected for further exploration based on their apparent association with fungal pre-colonisers. OTU richness within each of these families was modelled by the same process as for overall richness (see above), with the exception that no data transformation was necessary.

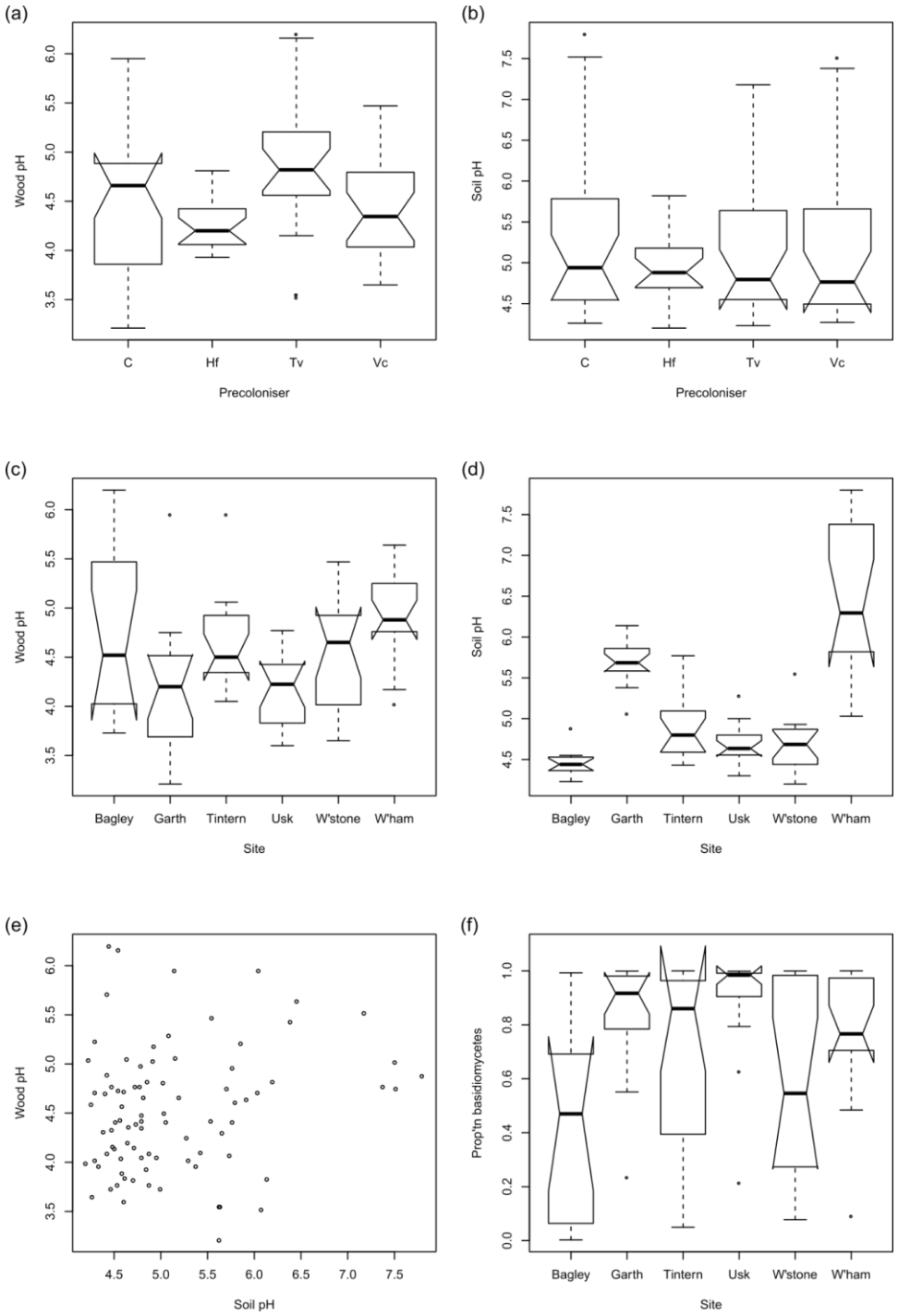

Figure 4.1 Relationships between predictors of the bacterial community. (a) wood pH broken down by pre-coloniser identity; (b) soil pH broken down by pre-coloniser identity; (c) wood pH broken down by site of origin; (d) soil pH broken down by site of origin; (e) relationship between wood pH and soil pH; and (f) the proportion of reads assigned to basidiomycetes on each site (N.B. the Hf treatment was absent at Bagley). Notches on boxplots represent 95% confidence intervals; where these extend beyond the quartiles, 'hinges' appear on the plot. Abbreviations are control (C), *Vulleminia comedens* (Vc), *Trametes versicolor* (Tv), *Hypholoma fasciculare* (Hf).

4.3.9 Relationships between predictors

The relationships between abiotic factors, and between the fungal community and abiotic factors, were explored graphically. Because wood-decay fungi are known to manipulate pH, one-way ANOVAs were used to assess how much of the variation in wood pH could be attributed to past or current fungal activity.

4.4 Results

4.4.1 Preliminary analysis of sequencing data

Of the bacterial paired-end reads, 2 710 316 passed quality filtering and were grouped into 7 380 OTUs. One sample (a *T. versicolor* pre-colonised disk from Tintern) was excluded from subsequent analysis due to concerns that it had been mislabelled.

4.4.2 Relationships between predictors

Soil pH varied strongly between sites. Wood pH also varied (to a lesser extent) between pre-colonisers and sites, but there was no relationship between wood pH and soil pH (Fig 4.1). Pre-coloniser identity explained 8.5% of the variation in wood pH, whereas the genus of the dominant fungal OTU at time of sampling explained 65.5% (adjusted R^2, one-way ANOVA).

4.4.3 Pre-coloniser and site effects on bacterial community composition

Axis 1 of the NMDS separated samples by pre-coloniser (specifically, *H. fasciculare* samples clustered separately to other treatments), indicating this was the most important source of variation (Fig 4.2a). The second axis was explained by sequencing depth (Fig S4.2). There was little patterning by site, other than limited clustering of samples from the Usk site (Fig 4.2b). Neither soil pH nor wood pH showed a clear pattern in the NMDS (Fig 4.2c-d).

The overall PERMANOVA revealed highly significant effects of pre-coloniser and site, and a highly significant interaction between them ($F_{14,68}$ = 1.793, P = 0.001). None of the pairwise tests were significant at P = 0.05 after FDR correction, but most of the results closest to significance separated *H. fasciculare* samples from other treatments at Tintern, Usk, Whitestone and Wytham sites (Table 4.3, Fig S4.3).

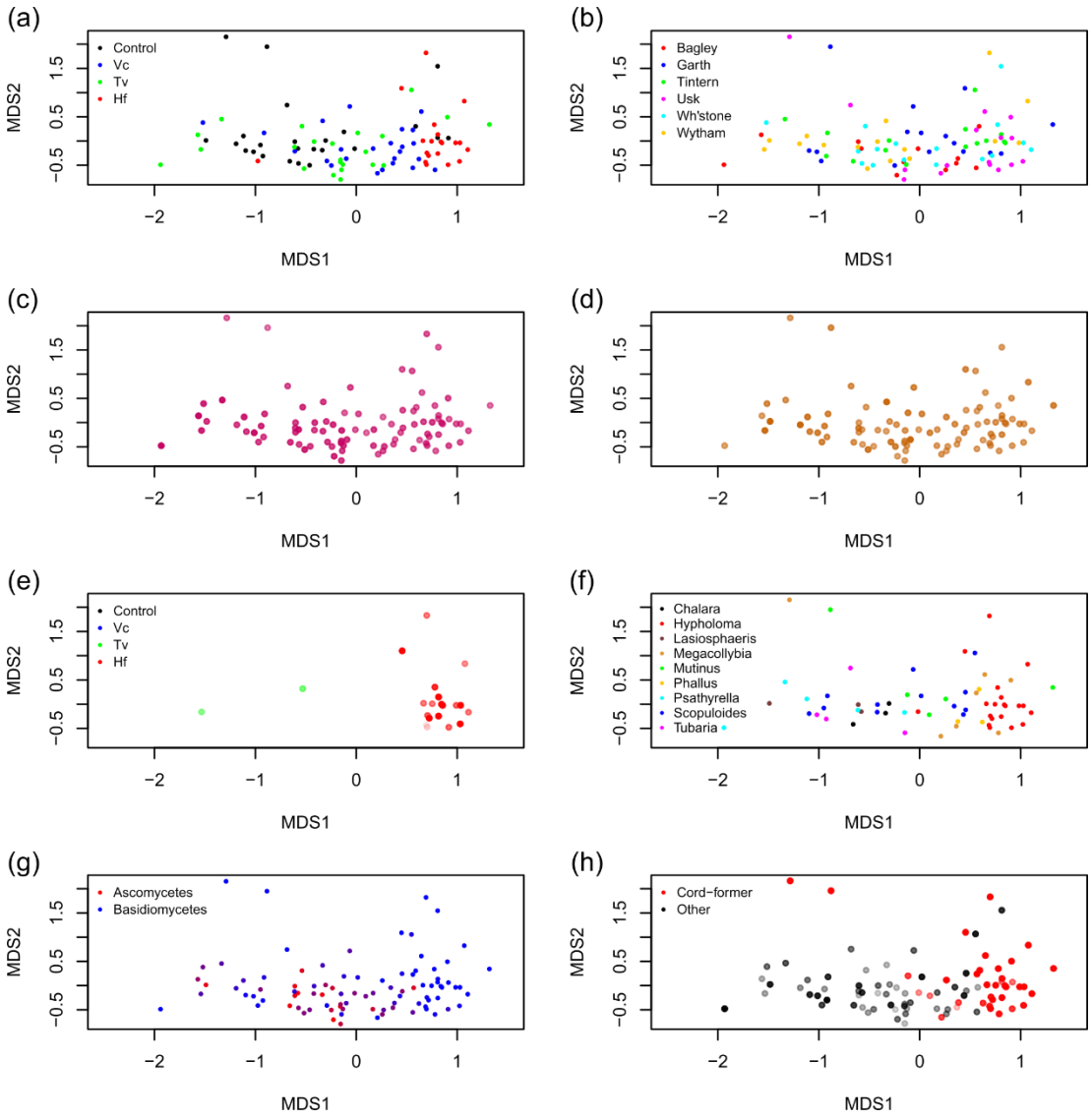

Figure 4.2 NMDS ordination of the bacterial community in fungus-colonised wood disks. Points are coloured by (a) pre-coloniser identity; (b) site of origin; (c) gradient of wood pH; (d) gradient of soil pH; (e) pre-coloniser persistence (colour dictated by identity, transparency by persistence); (f) genus of the dominant fungal OTU (only genera with three or more records are shown); (g) relative proportions of ascomycete and basidiomycete reads; and (h) whether the dominant fungal OTU belonged to a cord-forming genus (transparency dictated by % of disk held by the dominant OTU). Abbreviations are control (C), *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf).

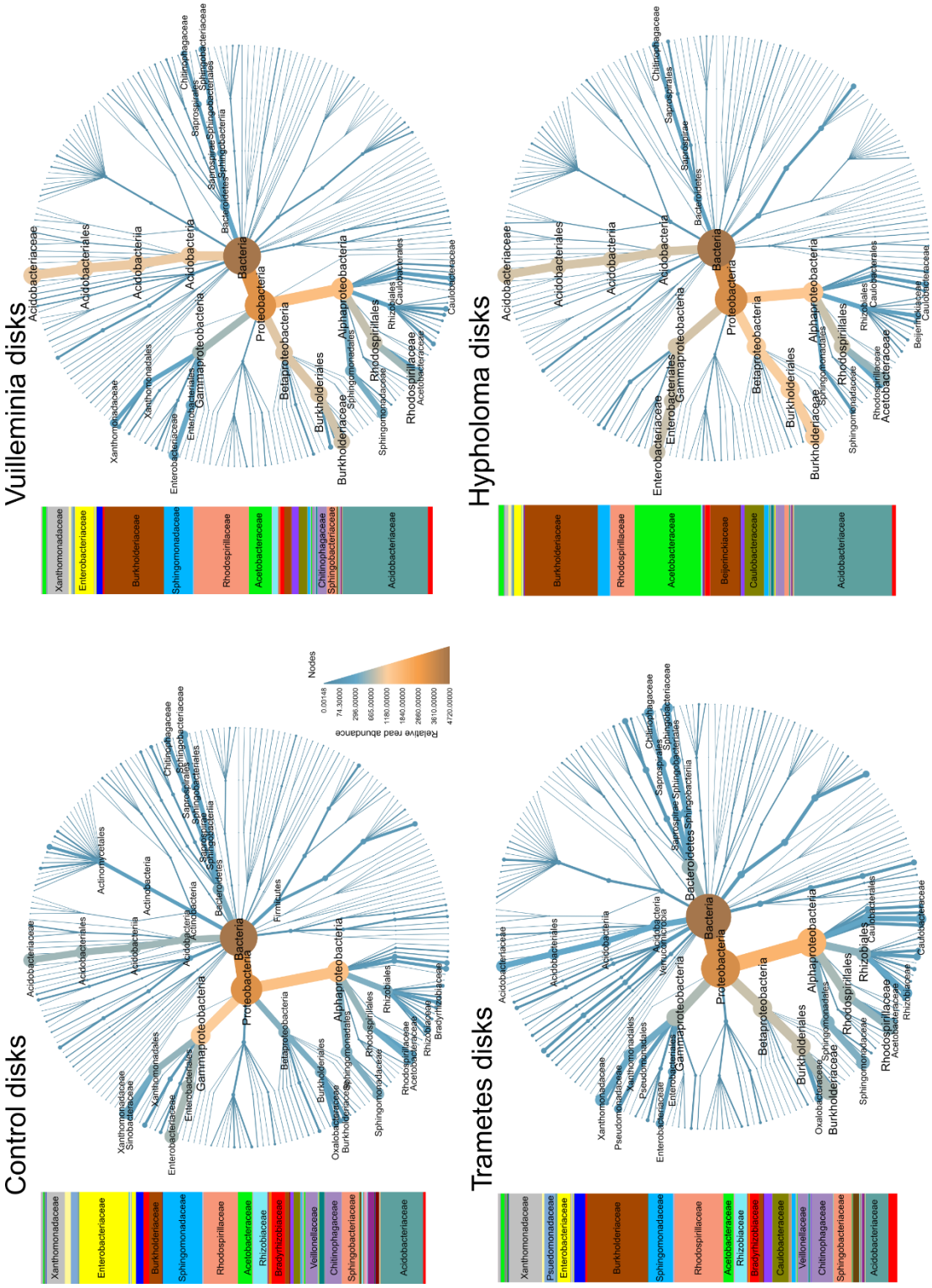

Figure 4.3 Taxonomic composition of the bacterial community in fungus-colonised wood disks, broken down by treatment. Stacked bar charts of family relative abundance are presented alongside heat trees. Node colour and size on the heat trees represent relative abundance for that taxon; relative read abundance is on an arbitrary scale where each sample sums to 100. OTUs unassigned at the Domain/Kingdom level are excluded for ease of visualisation.

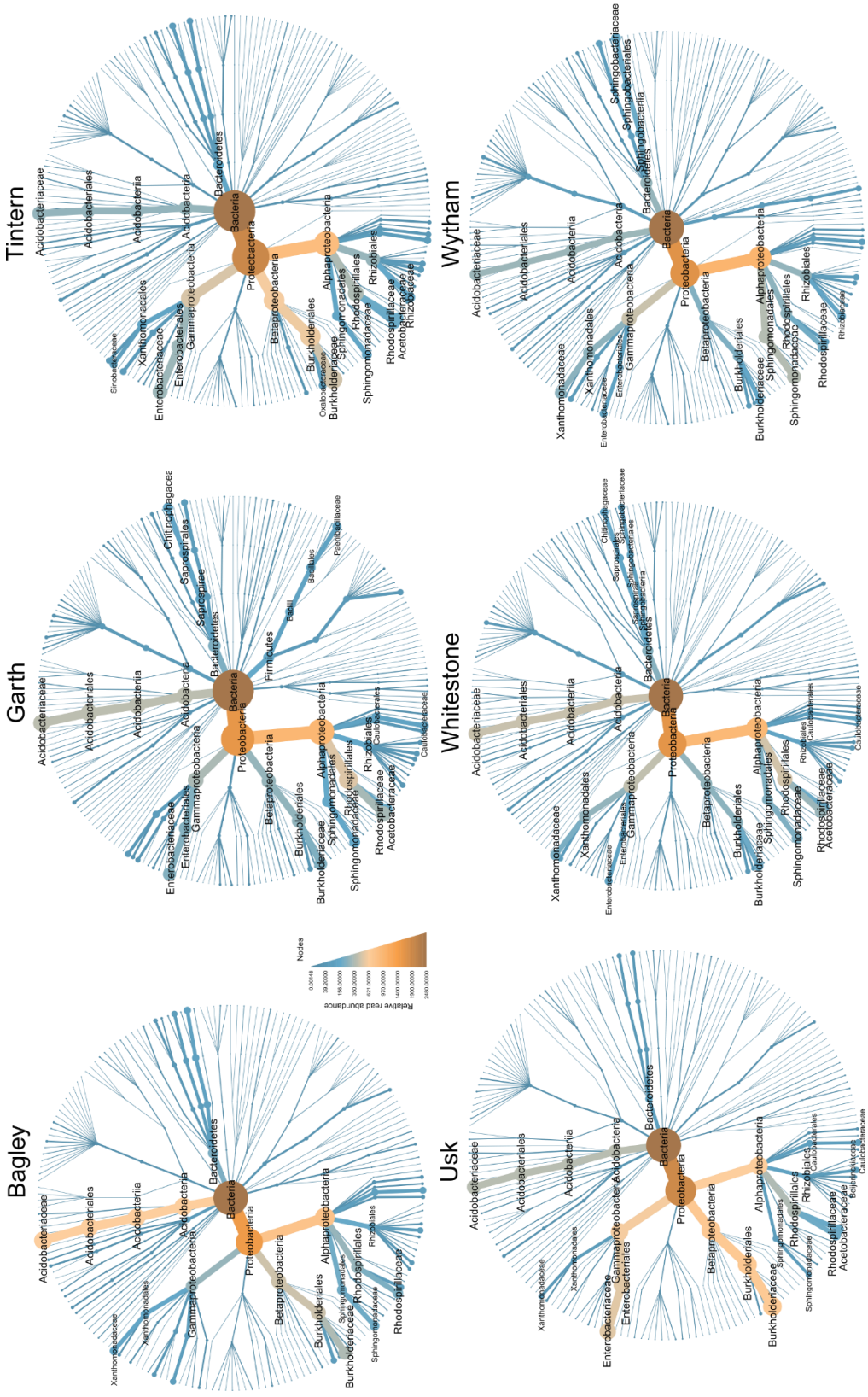

Figure 4.4 Taxonomic composition of the bacterial community in fungus-colonised wood disks, broken down by site. Node colour and size represent relative abundance for that taxon. Relative read abundance is on an arbitrary scale where each sample sums to 100. OTUs unassigned at the Domain/Kingdom level are excluded for ease of visualisation.

Table 4.3 Adjusted *P*-values from pairwise PERMANOVA comparisons of bacterial community composition.

Comparison	Bagley	Garth	Tintern	Usk	Whitestone	Wytham
Control-Vc	**0.064**	**0.064**	0.092	0.711	0.291	**0.064**
Control-Tv	0.092	**0.064**	***0.064***	0.257	**0.064**	0.073
Control-Hf	-	0.112	**0.064**	**0.064**	**0.064**	**0.064**
Vc-Tv	***0.064***	0.092	0.323	0.323	0.308	0.221
Vc-Hf	-	0.128	**0.064**	***0.064***	**0.064**	**0.064**
Tv-Hf	-	0.088	**0.064**	***0.064***	**0.064**	**0.064**

All numbers are given to three decimal places. Values in italics should be regarded with caution, as the between-group dispersions were unequal for those subsets of the data. Values in bold are those closest to significance.

Proteobacteria were dominant in all treatments (Fig 4.3). The overall bacterial community showed notable similarity between *V. comedens* and *H. fasciculare* disks. Both showed enrichment in *Acetobacteraceae* and *Acidobacteriaceae* relative to the other treatments (Fig 4.3). All three pre-coloniser treatments were enriched in *Burkholderiaceae*, and showed a decrease in *Actinobacteria* and a slight reduction in *Firmicutes*. Also noteworthy was the prominence of *Chitinophagaceae* in all treatments. A number of taxa also showed difference in relative abundance between sites (Fig 4.4): Bagley had proportionately more *Acidobacteria* and fewer *Gamma-proteobacteria* than the other sites, whilst Garth was the only site where *Firmicutes* were prominent. Most sites showed reciprocal abundance between *Enterobacteriaceae* and *Xanthomonadaceae*.

4.4.4 Fungal community effects on bacterial community composition

Of all the fungal pre-colonisers, only *H. fasciculare* could still consistently be re-isolated after 1 yr (Fig 4.2e). A diverse range of fungi colonised the disks; considering just the dominant OTU within each disk, 18 identifiable genera were represented across the experiment, of which nine were dominant in three or more disks each (Fig 4.2f). The relative proportion of ascomycetes *versus* basidiomycetes within each disk did not produce a discernible pattern in the bacterial community (Fig 4.2g). The clearest separation in bacterial communities arose between samples with a dominant fungal OTU belonging to a genus of known cord-formers, compared to those where the dominant OTU was not a cord-forming species (Fig 4.2h).

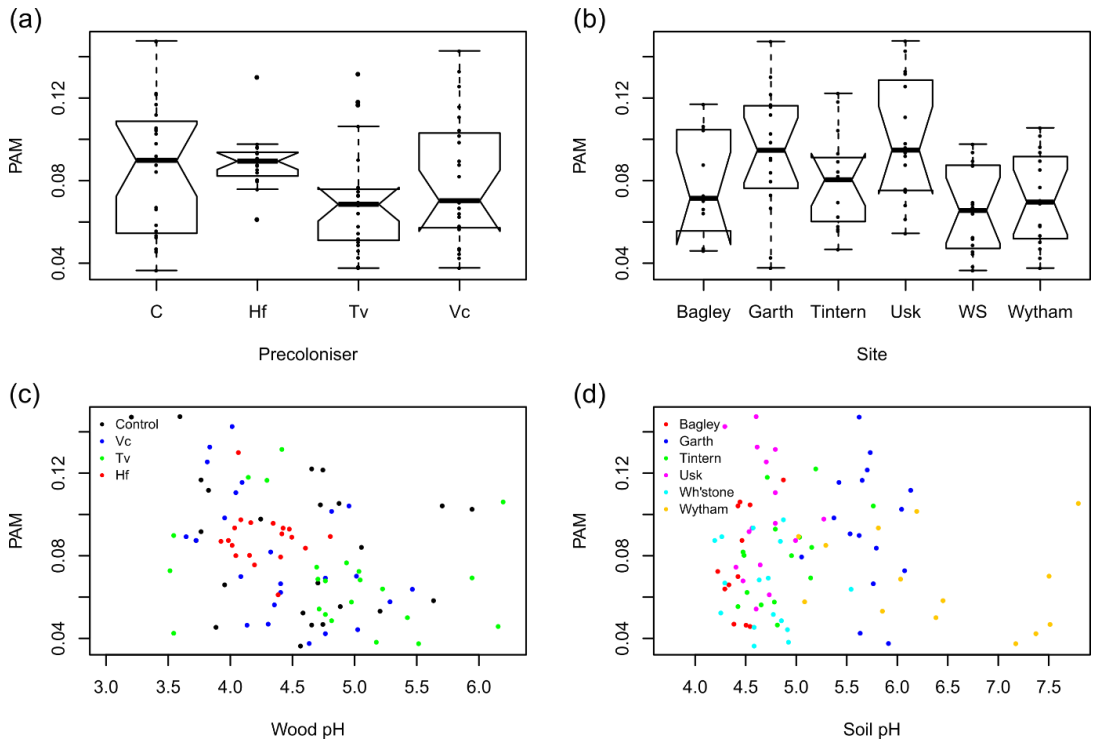

Figure 4.5 Procustean association metric (PAM) between the fungal and bacterial communities. PAM shows the residual variation plotted against (a) pre-coloniser identity; (b) site of origin; (c) wood pH (coloured by pre-coloniser); and (d) soil pH (coloured by site). Individual data points are overlaid on boxplots. Notches on boxplots represent 95% confidence intervals; where these extend beyond the quartiles, 'hinges' appear on the plot. Abbreviations are control (C), *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf), Whitestone (WS).

Procrustes analysis produced a correlation of 0.571 between bacterial and fungal communities (Procrustes sum of squares = 0.674, P = 0.001). Of the residual variance in bacterial-fungal occurrence (Fig 4.5), 5.0% was explained by pre-coloniser identity, 15.7% by site and 11.8% by wood pH (adjusted R^2, one-way ANOVAs). Soil pH had no explanatory power in fungal-bacterial co-occurrence.

4.4.5 Drivers of bacterial richness

Bacterial OTU richness was higher in the control than in pre-colonised disks (Fig 4.6a; Table 4.4). The only site to show a marked difference in bacterial richness was Usk, which had on average fewer bacterial OTUs than the other sites (Fig 4.6b). There was an upward trend in richness with increasing wood pH, but no effect of soil pH (Fig 4.6c-d). Soil pH had a model coefficient of 1.0981, and wood pH of 1.395, *i.e.* a one-unit increase in soil pH corresponded to a 10% increase in OTU richness, compared to a 39% increase in richness for

the same change in wood pH (Table 4.4). Therefore, when other factors were controlled for, wood pH was almost four times as important as soil pH in dictating bacterial richness. Bacterial richness was lower in basidiomycete-dominated disks compared to ascomycete-dominated disks (Fig 4.6e), and decreased substantially when the dominant fungal OTU belonged to a cord-forming genus (Fig 4.6f).

4.4.6 Focus on taxa of interest

Burkholderiaceae showed an increased OTU richness and relative abundance in the pre-colonised samples compared to the control (Fig 4.3; Fig 4.7a). *Acidobacteriaceae* showed the same pattern, but only for the *V. comedens* and *H. fasciculare* pre-colonised samples (Fig 4.7d). *Acetobacteraceae* was notable for its dramatic increase in *H. fasciculare* pre-colonised samples (Fig 4.7g). Richness for each of these families varied slightly and idiosyncratically between sites (Fig 4.8). All three showed a distinct negative trend with increasing wood and soil pH, although wood pH was twice as important as soil pH for *Acidobacteriaceae* and four times as important for *Burkholderiaceae* (Fig 4.7; Table 4.4). Statistical modelling showed that the pre-coloniser relationships for each family held true even when pH was controlled for (Table 4.4). None of these three bacterial families showed any trend based on the relative proportion of basidiomycetes in the disk, but all had higher richness when the dominant fungal OTU was a cord-former (Fig 4.9).

4.5 Discussion

This is the first study to examine fungus-bacteria associations in wood whilst experimentally manipulating the fungal colonisers in the field. It revealed that the bacterial community is dependent on the ecological strategy of the dominant fungus, with competitive secondary colonisers reducing bacterial diversity and driving community shifts. This controlling effect of the dominant fungus was a more important determinant than either resource history or geographical location. The strong effect of wood pH hints that pH manipulation may be a key means by which wood-decay fungi exert their influence.

Forest soils are dominated by *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* (Lladó *et al.* 2017). The relative dominance of *Proteobacteria* and *Acidobacteria* in the present study is consistent with previous studies of bacteria in wood (Rinta-Kanto *et al.* 2016; Chapter 2). *Firmicutes* were poorly represented in the wood and particularly in the fungal-pre-colonised samples, perhaps because they are more associated with mineral rather than organic soil horizons (Lladó *et al.* 2017). However, Hoppe *et al.* (2015) found both *Firmicutes* and *Actinobacteria* were noticeable components of wood-

Table 4.4 Estimates from general linear models on OTU richness.

Coefficient	Overall richness	Burkholderiaceae	Acidobacteriaceae	Acetobacteraceae
Control-Vc	0.896‡	12.8	9.34	0.754
Control-Tv	0.831‡	14.3	-4.67	-2.61
Control-Hf	0.776‡	13.6	10.5	3.85
Vc-Tv	0.928‡	1.44	-14.0	-3.36
Vc-Hf	0.866‡	0.814	1.21	3.10
Tv-Hf	0.933‡	-0.627	15.2	6.46
Bagley-Garth	0.933‡	-17.7	-0.725	7.16
Bagley-Tintern	1.118‡	-10.7	3.52	6.04
Bagley-Usk	0.801‡	-10.4	-2.73	4.66
Bagley-Whitestone	1.0978‡	-15.1	-3.19	3.74
Bagley-Wytham	0.967‡	-9.89	4.03	8.01
Garth-Tintern	1.199‡	7.00	4.24	-1.12
Garth-Usk	0.859‡	7.27	-2.01	-2.51
Garth-Whitestone	1.177‡	2.52	-2.47	-3.42
Garth-Wytham	1.0371‡	7.75	4.76	0.846
Tintern-Usk	0.716‡	0.275	-6.25	-1.39
Tintern-Whitestone	0.982‡	-4.48	6.71	-2.30
Tintern-Wytham	0.865‡	0.758	0.516	1.97
Usk-Whitestone	1.371‡	-4.75	-0.462	-0.911
Usk-Wytham	1.208‡	0.483	6.76	3.35
Whitestone-Wytham	0.881‡	5.23	7.23	4.27
Wood pH	1.395‡	-16.8	-11.3	-4.52
Soil pH	1.0981‡	-3.60	-5.67	-4.51
Sequencing depth	1.0000176‡	0.000526	0.000495	0.000312

For the overall richness model, estimates were obtained by backtransforming coefficients from the model (marked ‡). Values represent the ratios of geometric means when moving between levels (categorical predictors, i.e. Vc has 89.6% richness of the control) or for a one unit increase in the predictor (continuous predictors, i.e. a one-unit increase in wood pH corresponds to a 39.5% increase in OTUs). The individual family models did not require transformation, so each estimate simply represents the average increase in OTU numbers between levels (categorical predictors) or the average increase in OTU numbers for a one-unit increase in the predictor (continuous predictors). All numbers are given to three significant figures.

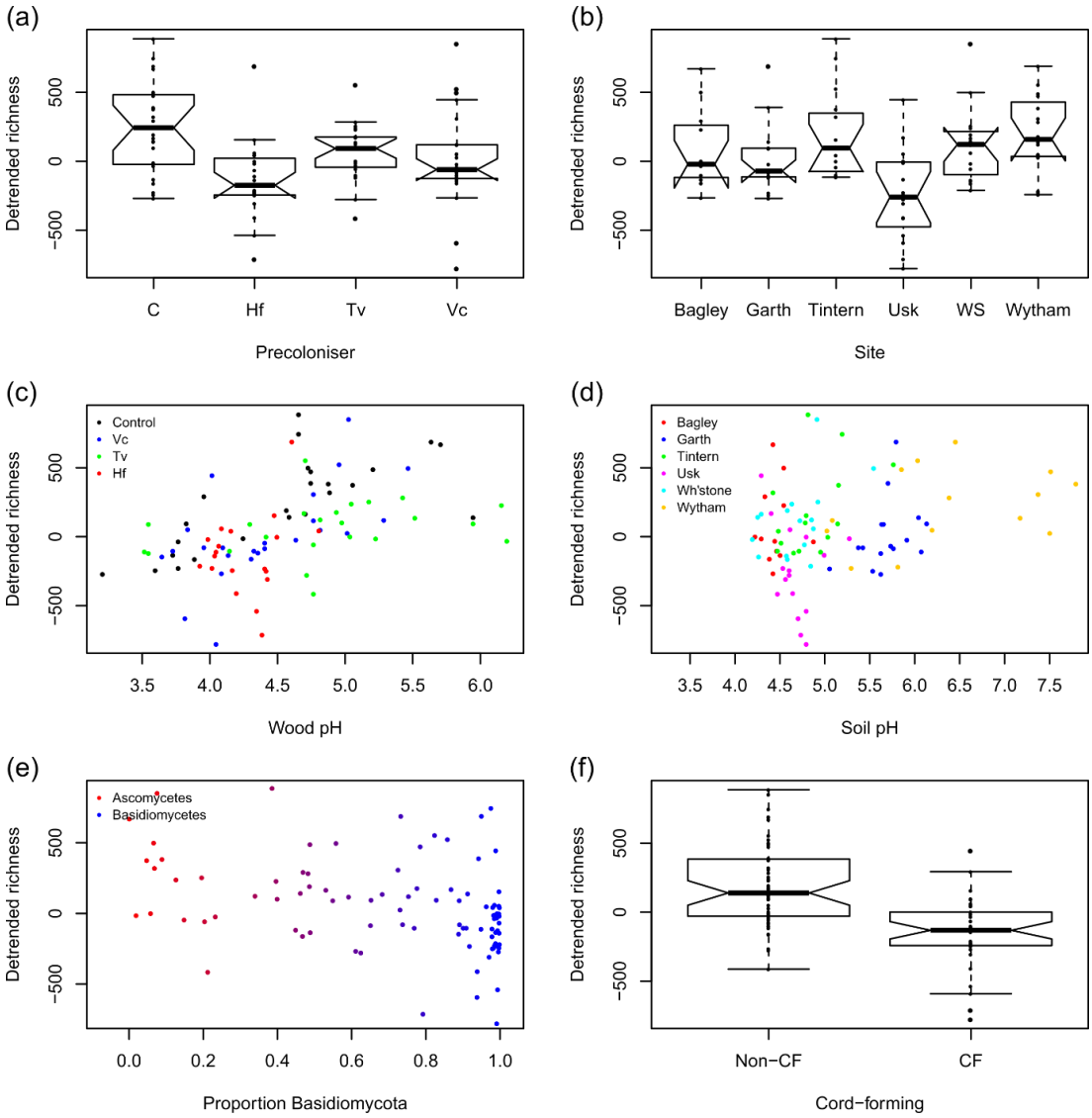

Figure 4.6 Overall bacterial OTU richness in fungus-colonised wood disks. Residuals from a model to correct for sequencing depth (the *y*-scale is therefore arbitrary), broken down by (a) pre-coloniser identity; (b) site of origin; (c) wood pH (coloured by pre-coloniser); (d) soil pH (coloured by site); (e) relative proportions of ascomycete and basidiomycete reads; and (f) whether the dominant fungal OTU belonged to a cord-forming genus. Individual data points are overlaid on boxplots. Notches on boxplots represent 95% confidence intervals; where these extend beyond the quartiles, 'hinges' appear on the plot. Abbreviations are control (C), *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf), Whitestone (WS), cord-former (CF).

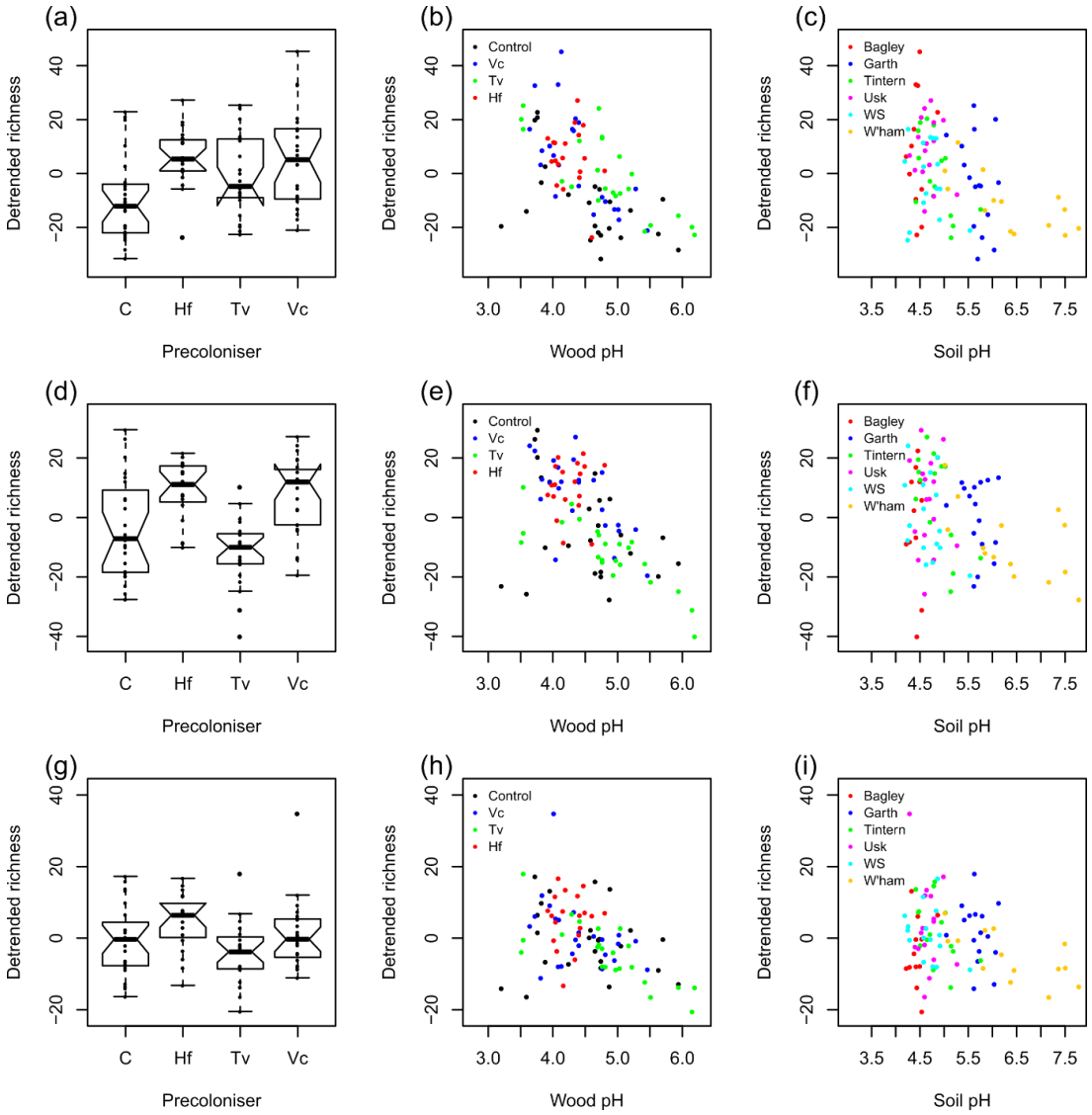

Figure 4.7 OTU richness for selected bacterial families in fungus-colonised wood disks. Residuals from models to correct for sequencing depth (the *y*-scale is therefore arbitrary). (a) *Burkholderiaceae* broken down by pre-coloniser identity; (b) *Burkholderiaceae* broken down by wood pH (coloured by pre-coloniser); (c) *Burkholderiaceae* broken down by soil pH (coloured by site); (d)-(f) *Acidobacteriaceae* broken down by the same predictors; and (g)-(i) *Acetobacteraceae* broken down by the same predictors. Notches on boxplots represent 95% confidence intervals; where these extend beyond the quartiles, 'hinges' appear on the plot. Abbreviations are control (C), *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf).

inhabiting taxa. The low abundance of *Actinobacteria* in the present study is indicative of the differences between soil and wood communities. Their absence may be related to their preference for higher-pH environments (Lladó *et al.* 2017). Among the *Bacteroidetes* present, *Chitinophagaceae* was a major component: given the abundance of chitin in fungal cell walls, this hints at bacterial predation or decomposition of fungal biomass.

4.5.1 Fungal community composition is more important than resource history

The fungal and bacterial communities within the wood showed a strong correlation, explaining nearly 60% of the covariance between them. Significant co-occurrence patterns between fungi and bacteria have previously been observed in decomposing wood (Hoppe *et al.* 2014; Rinta-Kanto *et al.* 2016). Of the three possible explanations (fungi dictate bacteria; bacteria dictate fungi; both are dictated by the same environmental factors), all are likely to operate to a greater or lesser degree. This study addressed the first by manipulating the fungus initially present. *H. fasciculare* remained in the disks across the whole study period, so correlation with the bacterial community could only be fungus-driven. By retaining its territory for the whole year, *H. fasciculare* also had the longest opportunity to select bacteria. The results confirmed that bacterial selection by *H. fasciculare* occurs not only in the lab (Folman *et al.* 2008; de Boer *et al.* 2010) but also in the field. The other pre-coloniser fungi were competitively replaced over the course of the experiment, and the bacterial communities for these treatments could not be separated from each other or the control disks. This indicates that it is the fungi currently present that shape the bacterial community, rather than the resource history. This nonetheless leaves room for a more subtle effect of previous colonisers, as they can influence the path of subsequent succession via priority effects (Hiscox *et al.* 2015; Hiscox *et al.* 2016).

One possibility is that *only* extremely combative fungi have the capacity to determine the bacterial community; this cannot be addressed at present as the only pre-coloniser to retain its territory was the cord-forming *H. fasciculare*. Chapter 5, carried out over a shorter time span, addresses this problem by including wood where less combative pre-colonisers are still present at the time of collection.

4.5.2 Fungal succession simplifies the bacterial community

All pre-coloniser treatments reduced bacterial OTU richness relative to the control: the later the successional position of the pre-coloniser, the greater the reduction in richness. It is important to note that after a year in the field the control disks were completely

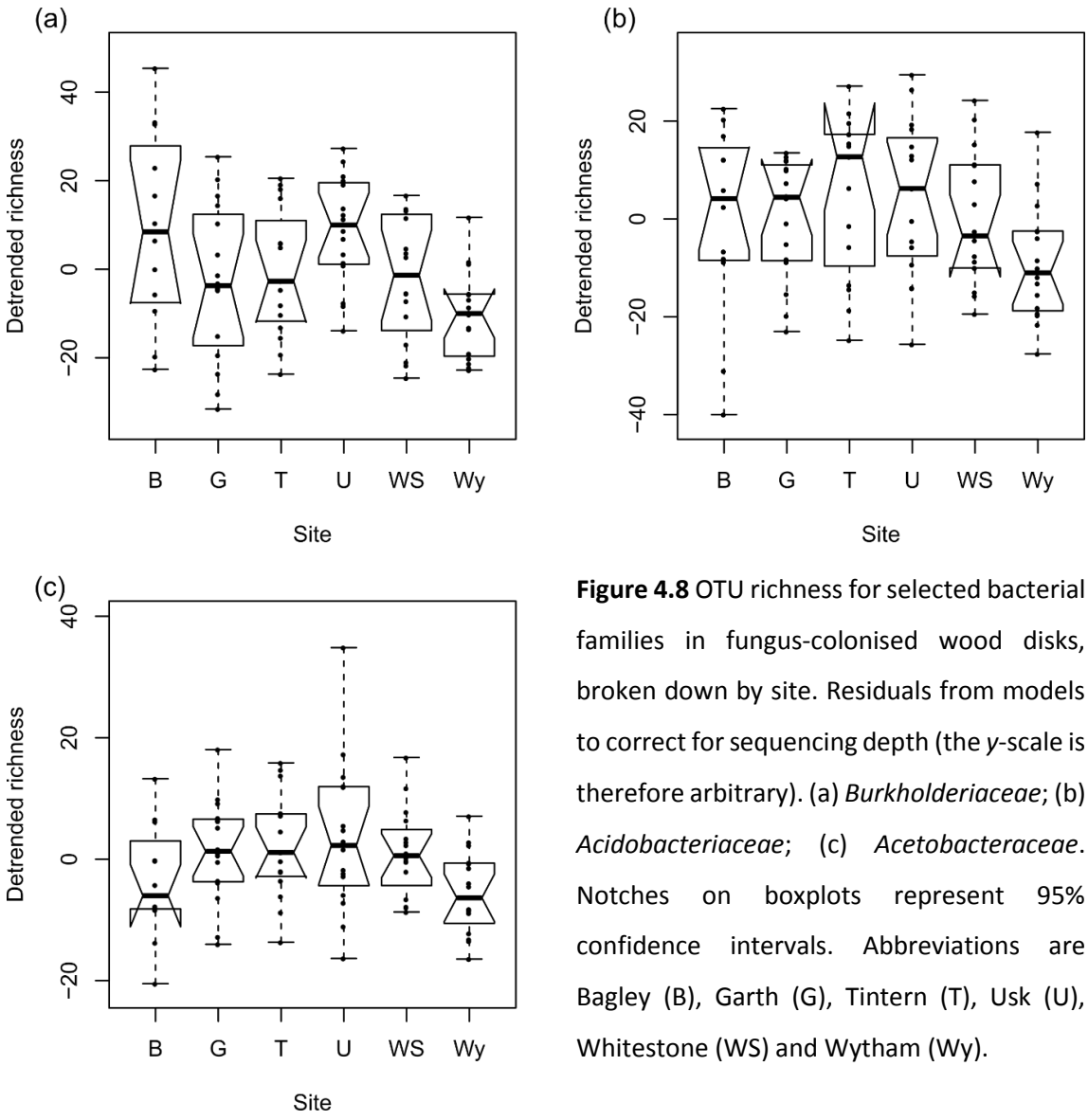

Figure 4.8 OTU richness for selected bacterial families in fungus-colonised wood disks, broken down by site. Residuals from models to correct for sequencing depth (the *y*-scale is therefore arbitrary). (a) *Burkholderiaceae*; (b) *Acidobacteriaceae*; (c) *Acetobacteraceae*. Notches on boxplots represent 95% confidence intervals. Abbreviations are Bagley (B), Garth (G), Tintern (T), Usk (U), Whitestone (WS) and Wytham (Wy).

colonised by fungi, but at an earlier stage of fungal community development than the pre-colonised disks. Wood-decay fungi have been previously observed to reduce the number and diversity of bacteria in their resource (Folman *et al.* 2008). This simplification of the overall bacterial community occurred concurrently with enrichment of 'fungus-tolerant' bacteria such as *Burkholderiaceae*. Surveys of naturally-decaying wood have found that bacterial richness and abundance increased with decay stage (Sun *et al.* 2014; Hoppe *et al.* 2015; Rinta-Kanto *et al.* 2016; Kielak *et al.* 2016). There are two possible explanations as to why richness may increase with decay, despite fungal succession (presumably) occurring with colonisation by progressively more competitive fungi: firstly, environmental factors (*e.g.* water content, which tends to increase with decay) may counteract the negative effects of fungal activity; secondly, some of the above studies may have included wood at a very late

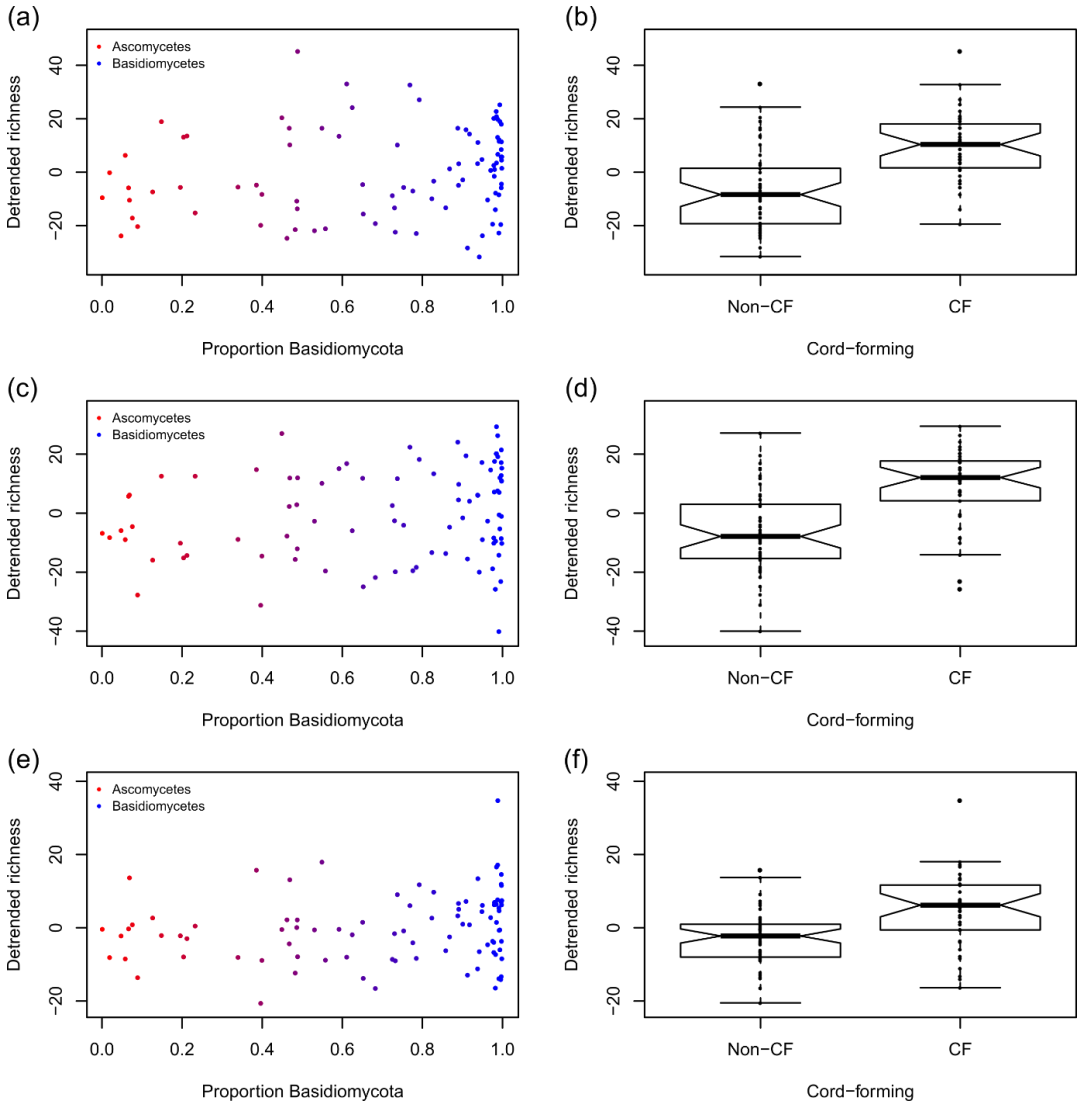

Figure 4.9 OTU richness for selected bacterial families in fungus-colonised wood disks in relation to the fungi present. Residuals from models to correct for sequencing depth (the *y*-scale is therefore arbitrary). (a) *Burkholderiaceae* broken down by the relative proportions of ascomycete and basidiomycete reads; (b) *Burkholderiaceae* broken down by whether the dominant fungal OTU belonged to a cord-forming genus; (c)-(d) *Acidobacteriaceae* broken down by the same predictors; and (d)-(f) *Acetobacteraceae* broken down by the same predictors. Notches on boxplots represent 95% confidence intervals. Abbreviations are control (C), *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf), Whitestone (WS), cord-former (CF).

stage of decay, when the highly competitive fungi have been replaced by stress-tolerant species (Boddy & Hiscox 2016).

4.5.3 Fungal ecology is more important than identity

The clearest separation between bacterial communities was driven by the ecological strategy of the dominant fungus. The ability to form mycelial cords is peculiar to certain wood decay basidiomycetes, and is often associated with high competitive ability and a late secondary position in the successional hierarchy (Boddy 1993). Therefore, it is unsurprising that cord-forming fungi are adept at manipulating the bacterial community. More surprising is that this trait appears to be more important than the identity of the fungus concerned. Even at the phylum level, there was no clear effect of fungal taxonomy on bacterial community composition. It has been suggested that wood-inhabiting bacteria respond to abiotic changes in wood (proximate cause) rather than fungi directly (ultimate cause) (Kielak *et al.* 2016). The cord-formers had a greater chance to produce a discernible effect, because they generally occupied more of the disk and so had a greater influence over the sampling unit. This territory effect was not in itself sufficient to explain the separation in bacterial communities. However, it underlines the importance of single-species dominance in fungal communities within a woody resource.

4.5.4 pH is an important means of fungal resource control

Wood pH was an important determinant of bacterial richness, but in turn, was itself heavily influenced by the identity of the dominant fungus in the wood. This supports the idea that pH is an important means by which fungi control the wood environment, and specifically the bacterial community within it (de Boer *et al.* 2010). Counterintuitively, there was a negative relationship between PAM values and wood pH, suggesting that at low pH there is less concordance between bacterial and fungal communities. This is likely due to the influence of fungal richness, which was negatively correlated with PAM: the disks containing the most competitive fungi tended to have lowest pH, lowest fungal and bacterial richness, and therefore fewer OTUs to be correlated.

4.5.5 Site is a less important determinant than fungal influences

Whilst sites did not show a clear clustering on the ordination plot, site nonetheless showed significant influence as a predictor. This may have been mediated by an altered fungal community between sites, leading to an altered pattern of succession (Hiscox *et al.* 2016). Support for this explanation comes from the clear separation between pre-coloniser treatments at Wytham, where Hiscox *et al.* (2016) found the most distinct fungal successor communities following each pre-coloniser. The Usk site showed differences to the other

sites, both in its tendency to form a cluster in the ordination, and in its markedly lower bacterial richness. This is most likely due to the localised dominance of *Megacollybia platyphylla*, a highly competitive cord-forming basidiomycete, which left its 'signature' on the bacterial community at the whole site. Given that site explained 16% of the residual variation in fungal-bacterial community correlation, the inter-kingdom relationship may be influenced by location (although confidence intervals overlapped for all sites).

4.5.6 Patterns in taxa

The three bacterial families selected for further exploration all showed responses different to and often opposing the behaviour of the community as a whole, underlining the value of exploring individual taxa (Warton 2008). Of particular note is that all three decreased in richness with increasing pH; this is contrary to the usual pattern for soil bacteria, which are competitively disadvantaged compared to fungi at low pH (Rousk *et al.* 2010), and indicates that these taxa are adapted both to fungal presence and to environments more amenable to fungal growth. It is possible that pH operates a two-stage filter on bacterial colonisation of wood: soil pH constrains the pool of colonists available to enter the resource, and wood pH constrains which of those are then capable of colonising the resource. This was not visible at the whole-community level, but did apply for these families.

Burkholderiaceae are outstanding among bacteria for their ability to form fungal associations (Johnston *et al.* 2016; Chapter 2), and in the pre-colonised wood they were markedly and consistently higher both in richness and relative abundance compared to in the controls. This affinity for co-occurring with fungi may be mediated partially by their tolerance for low pH environments (Stopnisek *et al.* 2015); within soil, *Burkholderia* are most plentiful in slightly acidic environments around pH5-6, but are still abundant in soils of pH 3-4, more similar to wood decay environments (Stopnisek *et al*. 2014). *Acidobacteriaceae* are a relatively newly-described and underexplored group of heterotrophic soil bacteria (Kielak *et al.* 2016). The present study agrees with the limited pre-existing knowledge of this family, in that they show an affinity for low pH, low nutrient environments. At least some forest soil *Acidobacteria* have the capacity to metabolise chitin and the cellulose breakdown product, cellobiose (Lladó *et al.* 2015). It has been suggested that members of the phylum *Acidobacteria* are K-strategists (Kielak *et al.* 2016), which may make them well-suited to the low-nutrient environment in dead wood. *Acetobacteraceae* are also known for their acid tolerance and ability to metabolise a range of low-molecular weight carbon sources (Mamlouk & Gullo 2013). Intriguingly, given the low nitrogen content of wood, this family includes some diazotrophs (Reis & Teixeira 2015).

4.5.7 Conclusions

Overall, this study underlines the importance of wood-decay fungi in controlling the dead-wood environment. In territory held by a highly competitive fungus, the bacterial community shifts towards acid-tolerant, metabolically versatile taxa adapted to the fungal environment. This study demonstrates for the first time that fungi drive bacterial communities in the field. This relationship is particularly pronounced when the dominant fungus is a cord-former. Several bacterial families, notably *Burkholderiaceae*, show a marked positive association with fungal-colonised wood.

Chapter 5. Fungal control of early-stage bacterial community development in decomposing wood

5.1 Abstract

The earliest stages of bacterial wood colonisation have received little attention, particularly with respect to how the colonisation process may be affected by the presence of wood-decay fungi. This study examined the bacterial community in wood that had been incubated in the field for 14 or 84 days, in the presence and absence of three fungal pre-colonisers (*Vuilleminia comedens, Trametes versicolor* and *Hypholoma fasciculare*). All three fungal species significantly delayed bacterial colonisation of the wood. *V. comedens* and *H. fasciculare* also reduced bacterial OTU richness and altered bacterial community composition, increasing the relative abundance of *Burkholderiales* and reducing the proportion of *Enterobacteriaceae* and *Bacteroidetes*. Wood that had not been pre-colonised showed seasonal differences between autumn and spring, but these only became apparent after 84 days. *Archaea* were also detected in nearly a third of samples, but with no apparent pattern and always at low abundances.

5.2 Introduction

Wood-decay fungi follow a well-characterised successional path, from the R (ruderal) and S (stress-tolerant) species present during early decay, through a series of increasingly competitive decomposer species (Boddy 2001). At the very latest stages of decay, these highly competitive fungi are in turn often replaced by stress-tolerant species. Bacterial succession in wood is much less well understood, although there have been a number of correlative studies determining the bacterial community in wood of different decay classes (Hoppe *et al.* 2015; Rinta-Kanto *et al.* 2016; Kielak *et al.* 2016b). The very earliest stages of wood colonisation by bacteria have rarely been examined (Sun *et al.* 2014).

Bacteria are often said to be the earliest colonisers of wood, influencing which fungi can subsequently establish (Greaves 1971; de Boer & van der Wal 2008; Sun *et al.* 2014), but it is likely that bacteria colonising wood almost always encounter a fungal community that has already developed. Wood-decay fungi are latently present in functional sapwood, and rapidly colonise wood once the water content drops to a favourable level (Boddy 2001). Consequently, fungal decay begins in the canopy, and wood is usually well colonised before it falls to the forest floor. Whilst bacterial saprotrophs may likewise live endophytically in wood, a large component of the bacterial community in dead wood probably originates from soil (Chapter 2), and thus the bacteria are secondary colonisers. There is evidence that wood-decay fungi exert active selection over bacteria, raising the possibility that fungi act as 'gatekeepers' for which bacteria may enter a resource (Chapter 4). The time-scales over which this operates are unknown (Chapter 2).

In a study in which wood pre-colonised by specific fungi decayed on the forest floor (Chapter 4), distinct bacterial communities were associated with cord-forming basidiomycete fungi, which are highly competitive late-secondary colonisers. However, of the original fungal pre-colonisers, only one retained its territory over the 1-yr duration of the experiment; the rest were replaced by other fungi through natural succession. This meant that, although bacteria were often correlated with particular fungi, whether the relationship was causative largely could not be determined. It is an open question as to whether non-cord-forming, less competitive fungi are also able to manipulate bacterial communities. These less competitive, earlier-stage colonisers are typical of the fungal community bacteria would encounter in newly-fallen wood.

In addition to biotic determinants, microbial communities are highly influenced by seasonal variation and accordingly show distinct patterns of community composition and activity (López-Mondéjar *et al.* 2015; Žifčáková *et al.* 2016). Seasonality is particularly

pronounced in temperate climates, where water availability, temperature and daylight are highly influenced by the time of year. Little is known of seasonal effects on bacteria in wood, but there are indications that season may be important: a model of nitrogen fixation in wood predicted considerable seasonal changes, with different abiotic factors becoming limiting at different times of year (Hicks *et al.* 2003a). Forest litter and soil also show seasonal differences in bacteria : fungus ratios, functional activity and horizon-specific bacterial community composition (López-Mondéjar *et al.* 2015; Žifčáková *et al.* 2016).

The present study investigates the influence of fungal pre-colonisation and season on bacterial community composition in decomposing wood, during the very earliest stages of colonisation. A manipulative field experiment was designed to examine bacterial colonisation of wood pre-colonised with fungi, in both spring and autumn. Four predictions were tested: (1) the fungal species within the wood would alter both the diversity and composition of the bacterial community, with competitive fungi hosting a less diverse bacteria community; (2) this effect would become increasingly apparent over time; (3) the fungal influence would be most prominent in the autumn, when many fungi are active; and (4) within-season variability would be evident within the bacterial community, but that this stochastic variation would be secondary to the fungal and seasonal effects above.

5.3 Materials and Methods

5.3.1 Overview

Wood blocks were colonised with wood-decay fungi in the laboratory and then placed on the forest floor for 14-84 days to allow bacterial colonisation. The experiment was repeated at three starting dates in both spring and autumn, *i.e.* 6 times. Bacterial communities were then characterised by amplicon sequencing.

5.3.2 Field experiment

Blocks of kiln-dried beech wood (*Fagus sylvatica*; 3 x 3 x 3 cm) were sterilised by triple autoclaving, leaving at least 24 hrs between runs. The sterilised blocks were sealed in 1000 ml plastic tubs (Cater4you, UK) containing a base covering of malt agar colonised by either *Hypholoma fasciculare* (HfGTWV2), *Trametes versicolor* (TvAW-HxFP) or *Vuilleminia comedens* (VcWVJH1). Blocks were left to colonise for a minimum of 3 months in the dark at 20°C. 25% of blocks were kept sterile as controls, and stored at -20°C until placed in the field.

Before being placed in the field, each block was scraped free of surface mycelium, and paired with another colonised by the same fungus with vessel ends touching. Control (uncolonised) blocks were likewise paired with each other. Pairs were held together with plastic-coated wire (Fig S5.1). Blocks were placed in a random-position grid in mixed

woodland, with *F. sylvatica* the dominant species (Whitestone Woods, Tintern, lat. 51.72 long. -2.69). Blocks were set out in 2015, at 2-week intervals on three dates in spring (4th May, 18th May and 1st June) and three dates in autumn (15th Oct, 29th Oct and 12th Nov). Set-outs were staggered within seasons in order to capture stochastic variability in time, creating three 'sets' per season. Each 2 x 2 m grid square contained two block pairings of each treatment, secured with a tent peg through a loop in the wire. Half the samples from each square were collected after 2 weeks, and the remainder after 12 weeks (4 replicates per treatment per time point). Blocks were returned to the lab and processed within a few hours of collection. Each block was split into quarters with a sterile chisel, wrapped in sterile foil and flash-frozen in liquid N_2 for storage at -80°C. One quarter of each block was left unfrozen for fungal re-isolation to determine pre-coloniser persistence: four small chips were removed from the interior of each block (*i.e.* 8 per 2-block sample) under aseptic conditions and isolated onto 2% malt agar. Outgrowing mycelium was identified as pre-coloniser or non-pre-coloniser by morphology.

5.3.3 Molecular analysis

Frozen blocks were drilled with a 4 mm bit under aseptic conditions to create swarf for DNA extraction. 0.3-0.5 g swarf from each block was immediately added to a PowerSoil® bead tube and re-frozen at -20°C. Extraction negative controls were performed by running the drill over an open tube. DNA was extracted following the MoBio PowerSoil® kit protocol (Carlsbad, CA USA), with the vortex step replaced by 3 x 20 sec in a MP FastPrep®-24 bead beater at 4 m sec^{-1}.

The presence of bacterial DNA was verified by PCR with primers 27F (AGAGTTTGATCMTGGCTCAG) (Weisburg *et al.* 1991) and 907R (CCGTCAATTCCTTTGAGTTT) (Lane *et al.* 1985). Each 50 µl reaction contained 1 µl DNA extraction, 200 nM each primer (MWG Eurofins, Ebersberg, Germany), 5 µg BSA (Promega, WI, USA) and 0.025 U $µl^{-1}$ Taq polymerase (PCR Biosystems, London, UK) in 10 µl supplied buffer. Amplification was performed in a Dyad DNA Engine Peltier thermal cycler (Bio Rad, Herts, UK) (95°C for 2 min, 35 x [94°C for 30 s, 52°C for 30 s, 72°C for 1.5 min] increasing by 1 s $cycle^{-1}$, 72°C for 5 min). Samples that failed to amplify were retested at 1:10 and 1:100 dilutions to ascertain that the reaction was not affected by inhibitors.

For samples that successfully amplified, PCR and sequencing of the bacterial 16S rRNA gene region were performed by the Earlham Institute (Norwich, UK). Each sample was amplified in duplicate for 30 cycles with primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso *et al.* 2011; Kozich *et al.* 2013) and the products

pooled. This primer pair targets the V4 region of the 16S rRNA gene and is widely used for environmental community characterisation, notably in the Earth Microbiome Project (Caporaso *et al.* 2011; Walters *et al.* 2016). Amplicons were sequenced on an Illumina MiSeq (v2, 2 x 250 bp) with Nextera XT assay chemistry.

5.3.4 Sequence analysis

Paired-end reads were merged using QIIME 1.9.1 (Caporaso *et al.* 2010). Low-quality sequences were removed with USEARCH v9.0.2132 (1 max expected error, minimum length 250 bases) (Edgar 2010). Chimeras were identified and removed using USEARCH 61 implemented in QIIME. A number of over-length sequences were still present after chimera removal, so any sequences longer than 257 bases were removed prior to open reference OTU picking at 97% similarity against the Greengenes 16S rRNA gene database (DeSantis *et al.* 2006). Singleton OTUs (*i.e.* those containing only one sequence) were excluded. To check for fungal amplification, OTU picking was repeated again the SILVA_119 16S/18S rRNA gene database (Pruesse *et al.* 2007; Quast *et al.* 2013). No fungal sequences were detected, so the Greengenes OTUs were used for subsequent analysis. Sequences are archived at the European Nucleotide Archive (ENA) under accession number PRJEB87091.

5.3.5 Statistical analysis

All analysis was performed in R (R Development Core Team 2011) using RStudio (RStudio Team 2016) and packages *dplyr* (Wickham & Francois 2016), *lsmeans* (Lenth 2016), *MASS* (Venables & Ripley 2002), *metacoder* (Foster 2016) and *vegan* (Oksanen *et al.* 2016). All R code to reproduce the analyses is available in Appendix 3 as an R markdown file (Protocol S4.1; Allaire *et al.* 2016).

A Bernoulli GLM (conditional log-log link) was used to model the presence/absence of bacteria in the samples versus season, pre-coloniser and time in the field. Bacteria were counted as present if the original DNA extraction produced a visible amplification product after 35 cycles.

Although the experimental design was fully crossed, the variable success in bacterial amplification meant that many treatments were absent from the sequencing dataset or had <3 replicates. Therefore, it was decided to break the data into subsets which were analysed separately to test particular hypotheses. Although this approach is not ideal as it increases the number of models run, it was considered preferable to attempting a single, highly imbalanced and nested model. Only the control samples contained sufficient replicates to test the effects of season and length of time in the field; therefore, the 'time' subset consisted of all control samples, in both spring and autumn, at 14 and 84 days. Each of the

pre-coloniser treatments could only be analysed at 84 days and in one season: for *T. versicolor* and *H. fasciculare* that season was spring, whilst for *V comedens* it was autumn. Given that the time dataset showed significant differences between seasons (see results), pre-colonised samples from different seasons were not combined. *T. versicolor* and *H. fasciculare* were compared to 84-day control samples from the spring ('spring' subset), and *V comedens* to 84-day control samples from the autumn ('autumn' subset). The autumn subset was the only dataset where comparison could be made between three different set-outs. Sequencing data was not rarefied prior to analysis for the reasons discussed in Chapter 4. In the current dataset, depth was not homoscedastic among predictors, but it was nonetheless deemed better to take this into account explicitly during the analysis rather than implicitly by rarefying.

Hypotheses pertaining to bacterial community composition were tested by permutation ANOVA (PERMANOVA; 999 permutations) on a Bray-Curtis distance matrix (Anderson 2001). *Post hoc* tests were approximated by subsetting the dataset into pairwise combinations and running PERMANOVA on each (Hiscox *et al.* 2016). In this instance, no *P*-adjustment was performed due to the limited number of comparisons involved. The time dataset was analysed with sequencing depth as a continuous predictor and season, day and the *season : day* interaction as categorical predictors. Pairwise comparisons tested for a difference between seasons at 14 days and at 84 days, as dispersions were equal for these comparisons but not *vice versa*. The spring and autumn dataset analyses contained depth and pre-coloniser as predictors; the autumn dataset also included set and the *pre-coloniser : set* interaction.

To visualise community differences, the data were ordinated using 4 axes of non-metric dimensional scaling (NMDS) on a Bray-Curtis distance matrix. Sequencing depth could be isolated on axis 2 (Fig S5.2a). To check for artefacts introduced by overdispersion (Warton *et al.* 2012), qualitative comparison was made with principal components analysis using a Hellinger transformation for compositional data (Fig S5.3). The taxonomic composition of each treatment was also visualised down to family level using heat trees (Foster *et al.* 2017).

Differences in OTU richness were modelled using negative binomial generalised linear models, to account for overdispersion in the data. A square root link was added for the time model to improve parametric assumptions. The model for each subset contained the same predictors as the PERMANOVA. Where interactions were included, likelihood ratio tests were used to assess their overall significance. To plot richness whilst controlling for the influence of sequencing depth, the square-root transformed richness values were regressed

against sequencing depth. The residuals were back-transformed and plotted in the place of raw richness values.

Table 5.1 Results of Bernoulli GLM on the presence of detectable bacteria in fungus-colonised wood.

	Coefficient	**Std. error**	***z***	***Pr(>|z|)***
Intercept	0.539	0.293	1.84	0.066
Season: spring	-0.398	0.286	-1.39	0.164
Species: Hf	**-3.59**	**0.682**	**-5.26**	**<0.001**
Species: Tv	**-4.38**	**0.738**	**-5.94**	**<0.001**
Species: Vc	**-3.03**	**0.655**	**-4.62**	**<0.001**
Day: 84	**2.81**	**0.609**	**4.61**	**<0.001**

Null deviance: 252.89 on 187 degrees of freedom; residual deviance: 140.48 on 182 degrees of freedom; R^2=0.555. Reference levels are autumn for season; control for species; and day 14 for day. Pre-colonisers are V. comedens (Vc), T. versicolor (Tv) and H. fasciculare (Hf). All numbers are given to three significant figures. Significant differences are shown in bold.

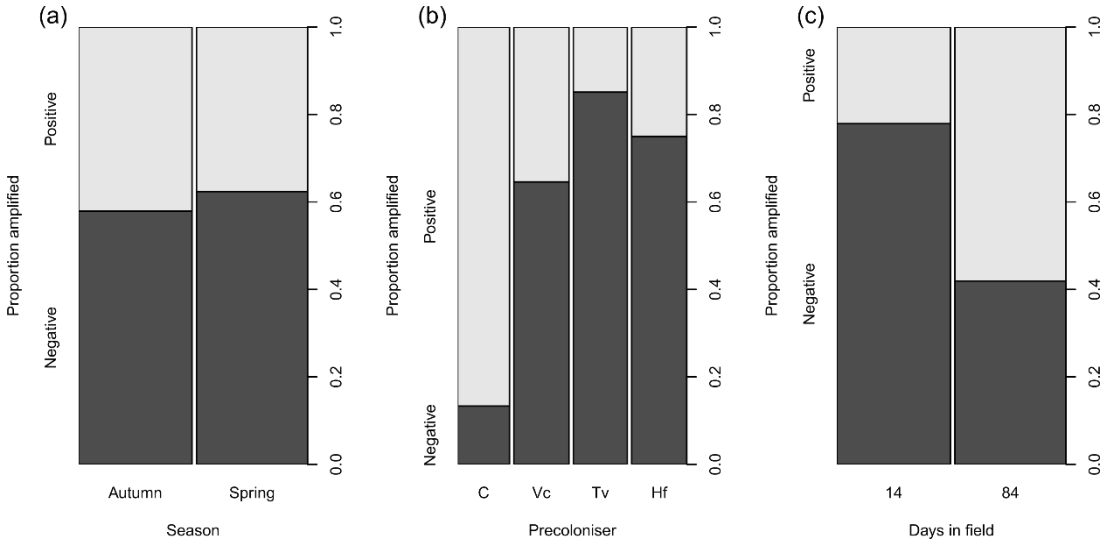

Figure 5.1 Detection probability of bacterial presence (measured by PCR amplification) in fungus-colonised wood blocks incubated in the field, separated by: (a) season of incubation; (b) pre-coloniser identity; or (c) length of time in field. Pre-colonisers were *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf) or uncolonised controls (C).

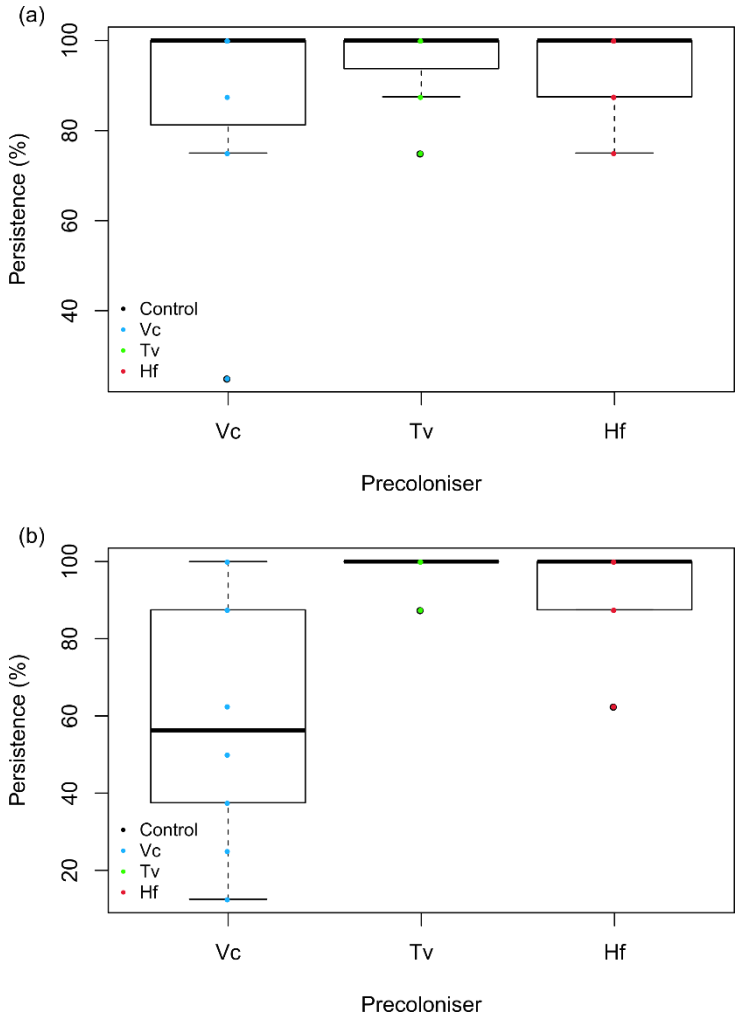

Figure 5.2 Persistence of fungal pre-colonisers in field-incubated wood blocks after (a) 14 days or (b) 84 days. Persistence was measured as the % of the 8 isolation points per sample where the pre-coloniser could be successfully re-isolated. Individual data points are overlaid on boxplots. Data were only available for the autumn samples. Pre-colonisers were *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf) or uncolonised controls (C). Each boxplot represents 10-12 replicates.

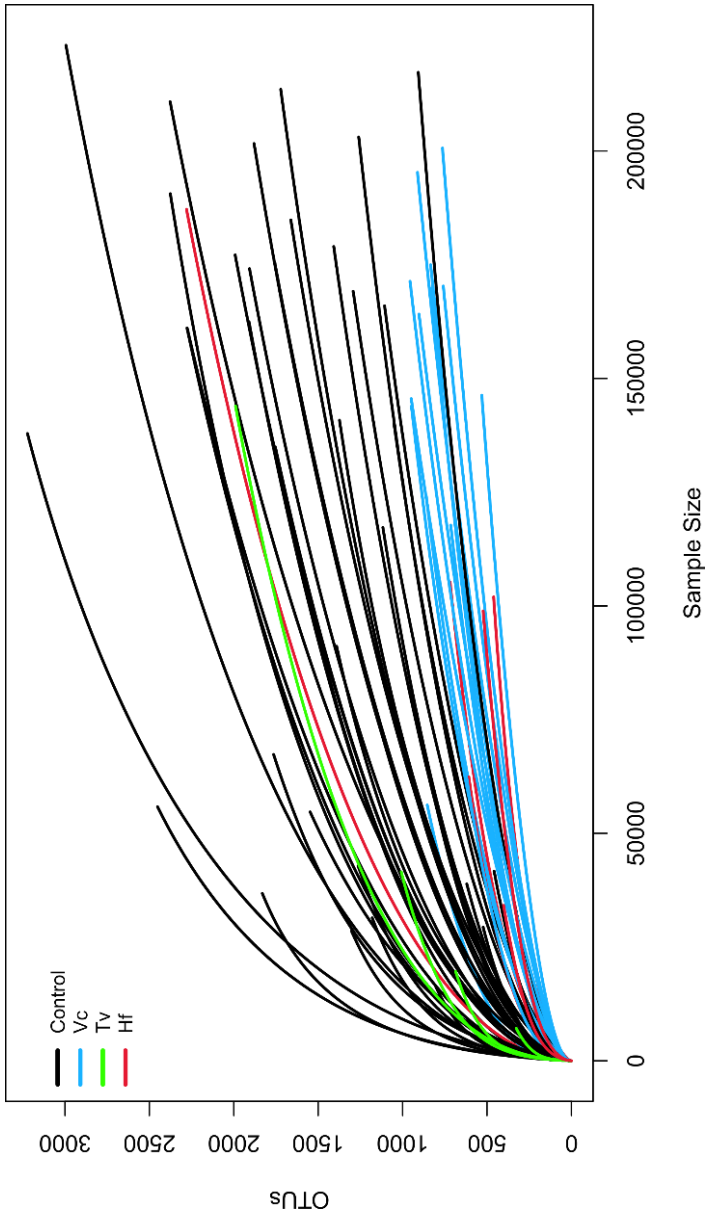

Figure 5.3 Species accumulation (rarefaction) curves for bacterial OTUs in fungus-colonised wood blocks. Curves are coloured by the identity of the fungal pre-coloniser: *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf) or uncolonised controls (C).

5.4 Results

5.4.1 Patterns in bacterial presence/absence

Bacteria were amplified from 75 out of 188 original DNA extractions. Detectable bacterial presence was significantly less common in pre-colonised wood samples than in controls, and in 14-day exposures compared to 84 days (Fig 5.1; Table 5.1; Table S5.1). Season had no effect on the probability of bacterial detection. All the fungal pre-colonisers decreased the likelihood of bacterial presence, but *T. versicolor* had the largest negative effect, followed by *H. fasciculare* and then *V. comedens*. All fungal pre-colonisers retained a strong presence in the wood blocks as determined by re-isolation (Fig 5.2), although *V. comedens* was starting to lose territory by 84 days (Fig 5.2b). Isolations from the control blocks displayed a mixture of fungi and bacteria in all samples.

5.4.2 Preliminary analysis of sequence data

7 561 110 reads passed quality filtering, and were grouped into 15 830 OTUs. The three extraction kit negative controls retained 3, 5 and 6 reads, respectively, so were excluded from further analysis. One 84-day *T. versicolor* pre-colonised sample from the spring yielded only 7 reads, and one 84-day *V. comedens* pre-colonised sample from the spring yielded only 507 reads; both were also excluded. All remaining samples contained more than 3 700 reads. There was no clustering by extraction kit lot number (Fig S5.1b). Rarefaction curves showed that OTU diversity almost saturated for many of the samples, particularly those pre-colonised with *H. fasciculare* or *V. comedens* (Fig 5.3). 15 251 OTUs were assigned to *Bacteria*, 19 to *Archaea* and 560 were unassigned at the Domain/Kingdom level. Although subsequent analysis refers to 'bacteria' for simplicity, all OTUs were included.

5.4.3 Presence of Archaea in wood

Archaea were detected in 20 wood block samples (30%), apparently distributed randomly across treatments. Archaeal reads never accounted for more than 0.8% of the read counts for any given sample. 13 of the 19 archaeal OTUs were *Euryarchaeota*, and of these 6 were *Methanomicrobia* and a further 6 were *Methanobacteria*. However, the most abundant archaeal OTU, both in read counts and number of observations, was assigned to the *Parvarchaeota*.

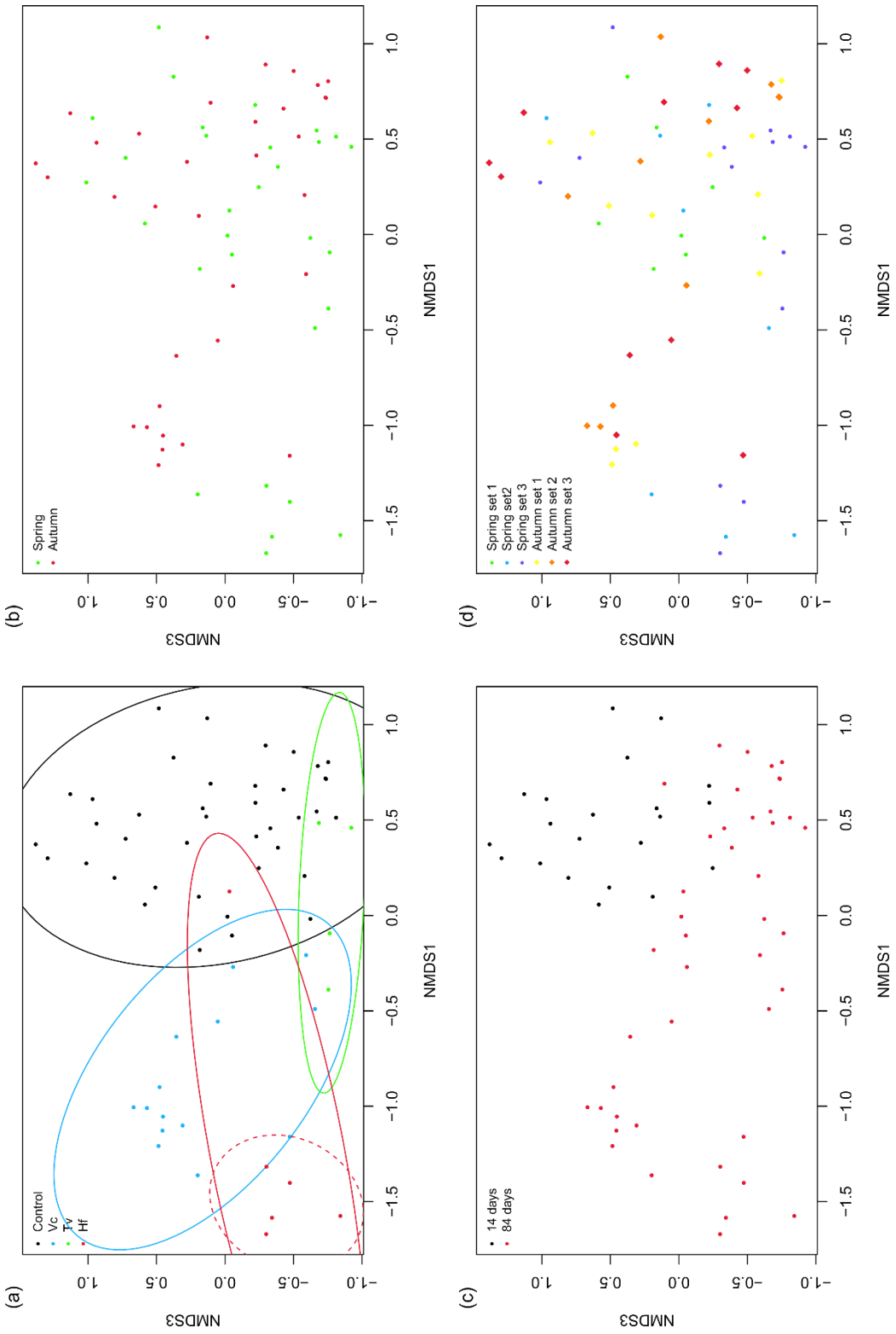

Figure 5.4 NMDS ordination of the bacterial community in fungus-colonised wood blocks. Points are coloured by: (a) fungal pre-coloniser *V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf) or uncolonised controls (C); (b) season of field incubation; (c) length of time in field; or (d) set-out date. In (a), 95% confidence ellipses are shown for each pre-coloniser (solid lines), and for *H. fasciculare* excluding one outlying point (dashed line).

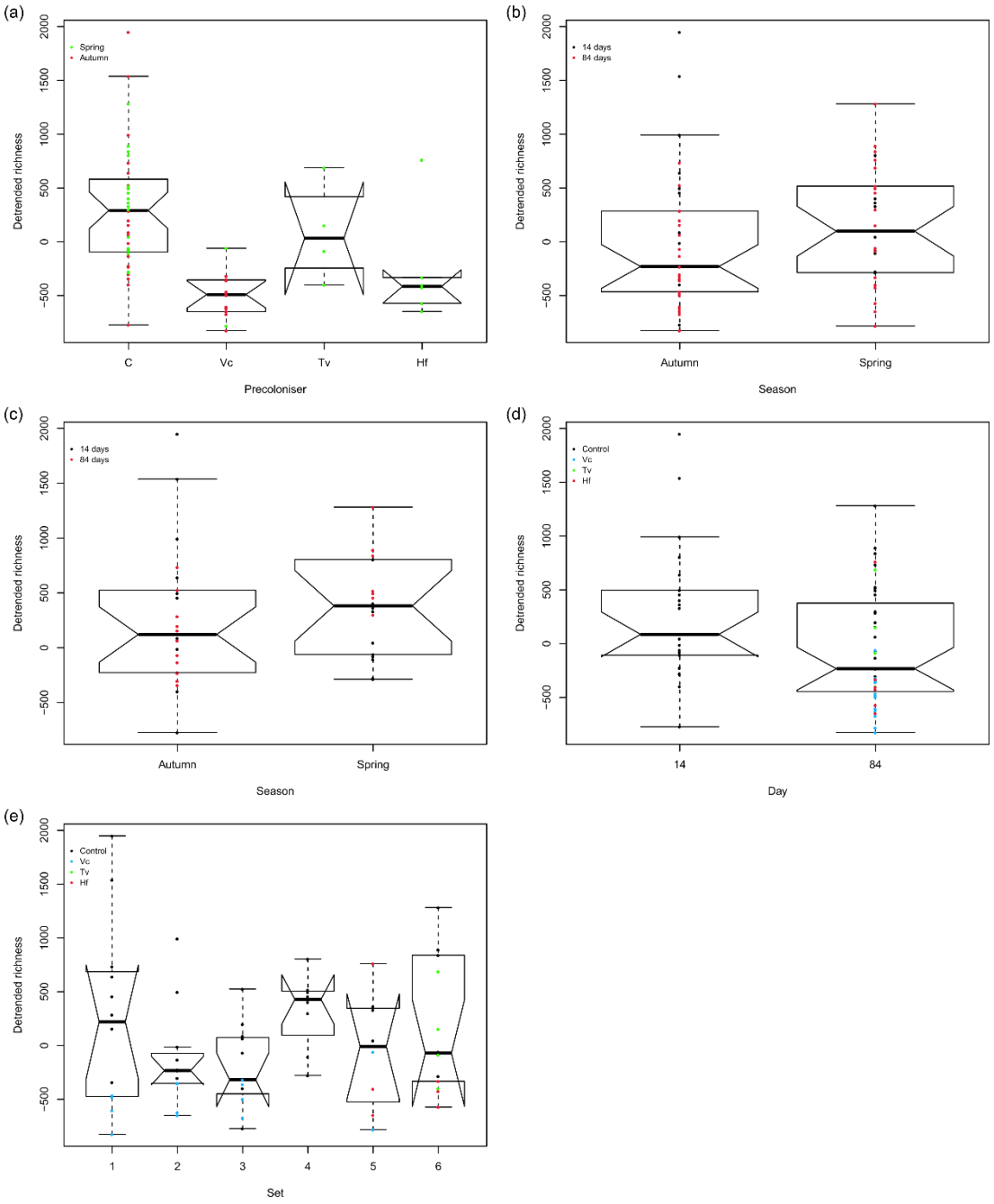

Figure 5.5 Overall bacterial OTU richness in fungus-colonised wood blocks. Residuals from a model to correct for sequencing depth (the *y*-scale is therefore arbitrary), broken down by: (a) pre-coloniser identity (*V. comedens* (Vc), *T. versicolor* (Tv), *H. fasciculare* (Hf) or uncolonised controls (C)), coloured by season; (b) season of field incubation, coloured by length of time in the field; (c) control samples only, split by season of field incubation, coloured by length of time in the field; (d) length of time in the field, coloured by pre-coloniser; (e) set-out date, coloured by pre-coloniser (1-3 represent autumn set-outs, 4-6 are spring). Individual data points are overlaid on boxplots. Notches on boxplots represent 95% confidence intervals; where these extend beyond the quartiles, 'hinges' appear on the plot.

5.4.4 Temporal effects on the bacterial community development in wood

Comparisons between time points could only be made among the control samples (Fig 5.4). PERMANOVA showed a significant interaction between season and length of time in the field. Pairwise comparisons revealed that the seasons were significantly different at 84 days (F=18.3, term R^2= 0.515, *P*=0.001), but not at 14 days (F=1.25, term R^2=0.051, *P*=0.202) (Fig 5.4b,c). OTU richness likewise had a significant interaction between season and field duration (LR=7.14, *Pr*=0.008); richness was higher at 84 days than at 14 days in the spring, but not in the autumn (Fig 5.5b-d).

Proteobacteria dominated at all time points. *Pseudomonadaceae* were dominant in the 14-day samples, along with *Burkholderiales* (particularly *Oxalobacteraceae*) (Fig 5.6). The 84-day samples were enriched in *Bacteroidetes*, particularly in the spring, whilst the autumn samples were heavily dominated by *Enterobacteriaceae*. The spring 84-day samples showed increased abundance of *Alpha-proteobacteria* and *Actinobacteria*.

5.4.5 Fungal effects on the bacterial community

Comparisons between the bacterial communities in the control wood blocks and those pre-colonised by *T. versicolor* or *H. fasciculare* could only be made among the 84-day spring samples, while comparison between *V. comedens* pre-colonised wood and the controls could only be made among the 84-day autumn samples. PERMANOVA showed a significant difference between wood with different pre-coloniser treatments in both subsets (F=2.73, term R^2=0.199, *P*=0.003 for the spring data, F=18.5, term R^2=0.401, *P*=0.001 for the autumn data) (Fig 5.4a). Pairwise comparisons among the spring dataset revealed a significant difference between the bacterial community in *T. versicolor* and *H. fasciculare* pre-colonised wood (F=3.18, term R^2=0.251, *P*=0.001), but neither the bacterial community associated with *T. versicolor* (F=0.815, term R^2=0.05, *P*=0.507) nor the bacterial community associated with *H. fasciculare* (F=1.426, term R^2=0.067, *P*=0.200) was significantly different from that in the controls. The autumn dataset revealed no significant influence of set-out date (set), nor any interaction between wood block set-out date and pre-coloniser (Fig 5.4e).

Bacterial OTU richness in the spring samples was significantly lower in *H. fasciculare* pre-colonised blocks than in the controls ($z_{14,17}$=-2.46, *Pr(>|z|)*=0.014), but there was no difference in bacterial community richness between *T. versicolor* pre-colonised samples and the controls ($z_{14,17}$=1.26, *Pr(>|z|)*=0.207) (Fig 5.5a). In the autumn samples, there was a significant interaction between pre-coloniser species and set-out date (LR=8.94, *Pr*=0.011) (Fig 5.5e). OTU richness in *V. comedens* pre-colonised wood was significantly lower than the

controls in sets 1 and 3, but not in set 2; the set 2 controls were also significantly different from the controls in sets 1 and 3 (Table 5.2).

H. fasciculare and *V. comedens* pre-colonised wood blocks were both enriched in *Burkholderiales*, particularly *Burkholderiaceae*, compared with the controls (Fig 5.7). *T. versicolor* pre-colonised wood showed little difference in bacterial community composition to the controls, apart from a slight reduction in *Beta-proteobacteria* and *Bacteroidetes*. The relative abundance of *Acidobacteria* showed little variation across any of the treatments.

Table 5.2 *Post-hoc* comparisons of bacterial OTU richness between wood blocks colonised with *V. comedens* (Vc) and control blocks, at 84 days across three set-outs staggered 2 weeks apart.

Contrast		**Coefficient**	**Std. error**	***z***	***P***
Set 1 control	**Set 2 control**	**0.348**	**0.106**	**3.28**	**0.013**
	Set 3 control	0.043	0.097	0.439	0.998
	Set 1 Vc	**0.706**	**0.098**	**7.24**	**<0.001**
	Set 2 Vc	**0.606**	**0.099**	**6.11**	**<0.001**
	Set 3 Vc	**0.645**	**0.108**	**5.99**	**<0.001**
Set 2 control	Set 3 control	-0.305	0.100	-3.06	0.027
	Set 1 Vc	**0.359**	**0.100**	**3.57**	**0.005**
	Set 2 Vc	0.258	0.100	2.57	0.104
	Set 3 Vc	**0.297**	**0.102**	**2.92**	**0.042**
Set 3 control	**Set 1 Vc**	**0.664**	**0.093**	**7.17**	**<0.001**
	Set 2 Vc	**0.563**	**0.093**	**6.08**	**<0.001**
	Set 3 Vc	**0.602**	**0.096**	**6.30**	**<0.001**
Set 1 Vc	Set 2 Vc	-0.101	0.093	-1.08	0.890
	Set 3 Vc	-0.062	0.096	-0.639	0.988
Set 2 Vc	Set 3 Vc	0.039	0.095	0.412	0.999

Comparisons are derived from a negative binomial general linear model. Results are given on the log (not the response) scale. P value adjustment: Tukey method for comparing a family of 6 estimates. All numbers are given to three significant figures. Significant differences are shown in bold.

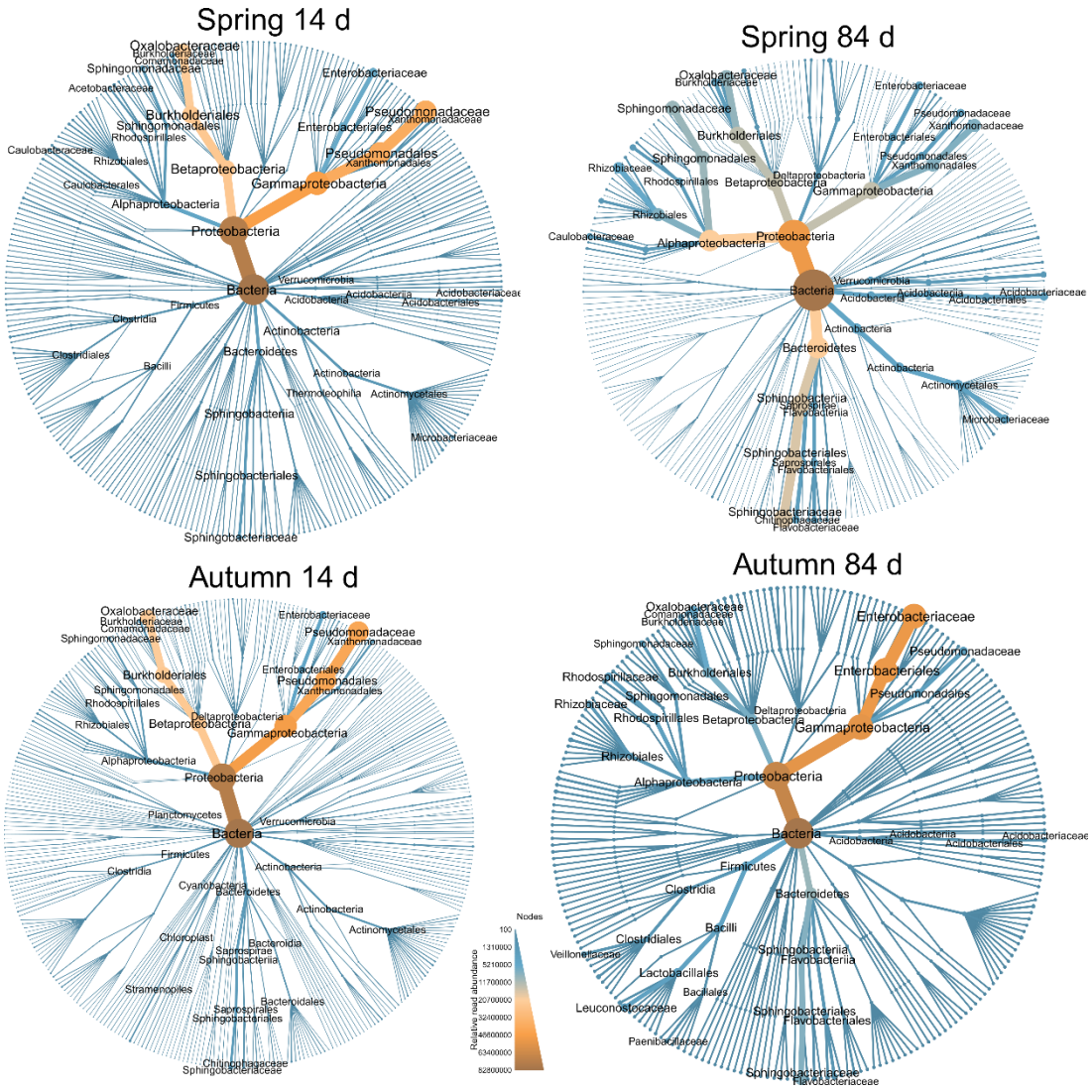

Figure 5.6 Bacterial community composition in control wood blocks (without fungal pre-colonisation), broken down by season and length of time in the field. Node colour and size on the heat trees represent relative abundance for that taxon; relative read abundance is on an arbitrary scale where each sample sums to 100. OTUs assigned to *Archaea* or unassigned at the Domain/Kingdom level are excluded for ease of visualisation.

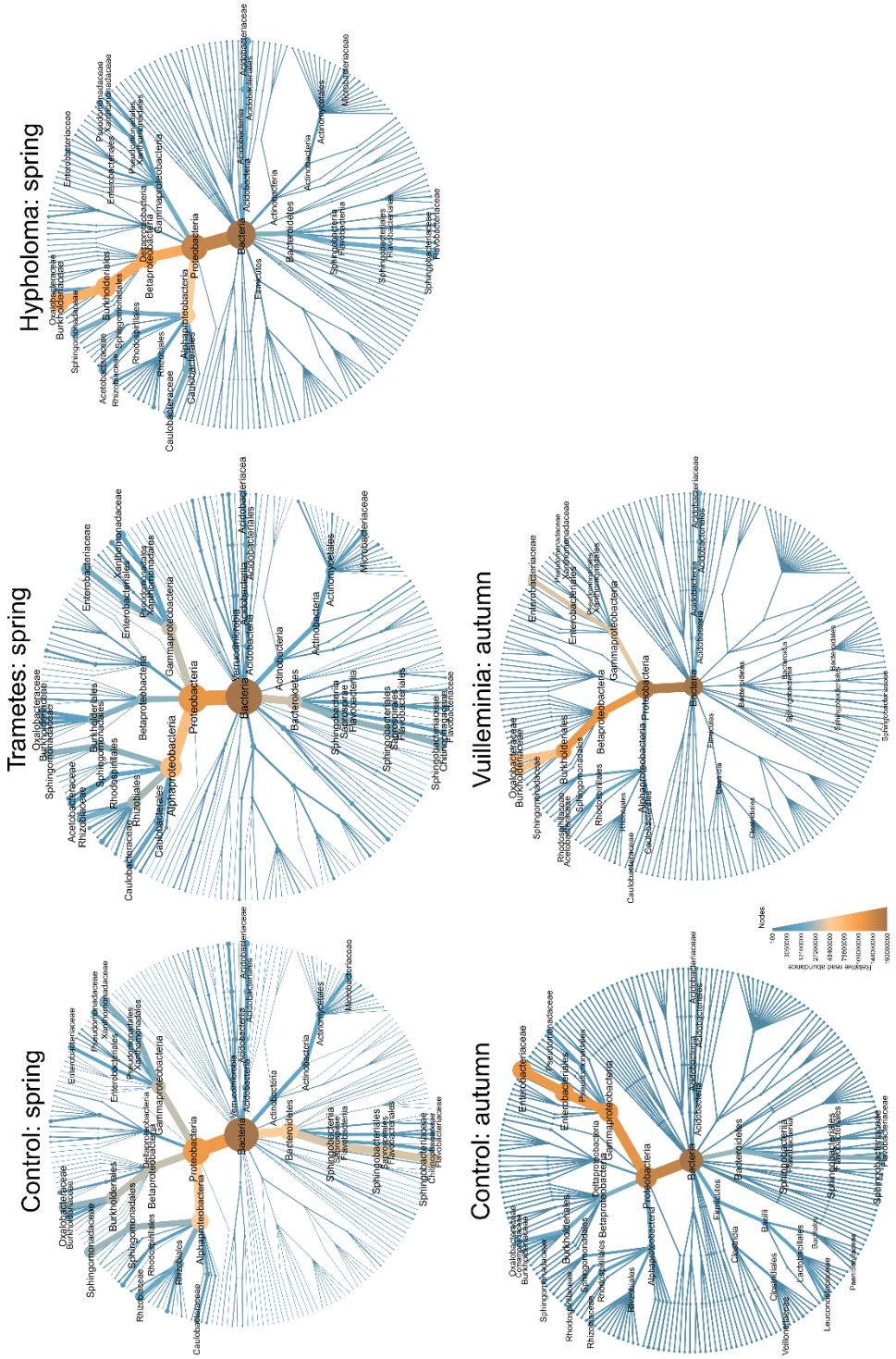

Figure 5.7 Bacterial community composition in fungus-colonised and control wood blocks, broken down by pre-coloniser treatment and season. *T. versicolor* and *H. fasciculare* could only be analysed in the spring, *V. comedens* only in the autumn. All samples were incubated in the field for 84 days. Node colour and size on the heat trees represent relative abundance for that taxon; relative read abundance is on an arbitrary scale where each sample sums to 100. OTUs assigned to *Archaea* or unassigned at the Domain/Kingdom level are excluded for ease of visualisation.

5.5 Discussion

This study demonstrates, for the first time, that wood-decay fungi not only alter bacterial communities but also significantly delay their establishment within wood. When bacteria do colonise the wood, some fungi also affect community composition in a species-specific manner. This study also provides the first indications of the timescale over which the earliest stages of bacterial colonisation operate in wood arriving on the forest floor.

5.5.1 Wood-decay fungi can exclude bacteria from their resource

Detectable bacteria were strikingly higher in the control samples than pre-colonised samples. In most of the pre-colonised wood samples, bacteria had not colonised to a detectable level after 14 days, and detection remained patchy even at 84 days. By contrast, bacteria were consistently present in the controls, albeit at low levels. This indicates that wood-decay fungi can delay bacterial colonisation, probably due to the selection pressure that they exert over the bacterial community (Folman *et al.* 2008; Hervé *et al.* 2014; Chapter 4). Negative PCR results do not necessarily indicate that bacteria were truly absent but rather that they were too scarce to detect, as bacteria were obtained by isolation from samples that were negative by PCR. The control samples were not fungus-free, but the fungal colonisers were poorly established, with high representation of fast-growing, R-selected, non-lignocellulytic fungi rather than wood decayers. Folman *et al.* (2008) also reported difficulty in obtaining 16S rRNA gene PCR products from fungus-colonised wood in microcosms, although with no effect of increasing time. This was corroborated with microscopy and plate counts, which demonstrated that the fungi reduced both the number of bacterial cells present in the wood and the proportion which could be obtained by cultivation (Folman *et al.* 2008).

5.5.2 Primary colonising fungi manipulate bacterial communities

The distinct bacterial community in *V. comedens* pre-colonised wood blocks showed that primary colonising, early-decay fungi can nonetheless influence which bacteria can establish. This was not consistent with the suggestion in Chapter 4 that only highly competitive cord-forming fungi select bacteria, and raises the question why distinct bacterial communities were not apparently associated with the less competitive fungi in the results reported in Chapter 4. One possibility is that cord-forming fungi all select for similar bacteria, whilst less competitive fungi have more varied bacterial communities. A more likely explanation is that fungal influence on bacterial community development is linked to the length of time that the fungus occupies the resource. Highly competitive fungi can hold a resource for a long time and clearly establish their influence over it, whereas a resource that

frequently changes fungal coloniser is likely to exhibit a patchwork of legacy effects (Hiscox *et al.* 2015; Leopold *et al.* 2017).

In the present study, the effects of pre-colonisation by the highly competitive cord-former *H. fasciculare* did not significantly alter bacterial communities compared to the control, but this was probably due to the influence of one outlier, as all the other *H. fasciculare* samples formed a tight cluster (Fig 5.5a). Despite differences in their competitive ability, pre-colonisation by *V. comedens* and *H. fasciculare* reduced the observed bacterial community OTU richness to a similar extent. Given that *T. versicolor* was the fungus that most successfully prevented bacteria colonisation, it is surprising that the bacterial community associated with it was not different in richness or structure compared to the controls. One possible explanation is that it reflects the mechanism of bacterial exclusion. *T. versicolor* forms dense, rubbery mycelium around the outside of wood blocks. Although *T. versicolor* also produces a wide range of enzymes (Hiscox *et al.* 2010), if it mainly excluded bacteria via a physical, rather than chemical, barrier, this may explain why there was less discrimination between taxa.

The difference between control and pre-colonised wood samples demonstrated the important distinction between fresh and canopy-decayed wood, with several important implications. Firstly, experiments using sterile, undecayed wood to study bacterial colonisation should recognise that this represents a very small subset of wood naturally reaching the forest floor. Secondly, senescent wood from unmanaged forests will follow a different path of bacterial colonisation than wood from felled trees and branches, typical of wood inputs in commercial forestry. Lower bacterial diversity and altered community composition have been recorded for *F. sylvatica* logs in comparisons of managed and unmanaged forests (Hoppe *et al.* 2015). Different successional paths have also been observed for fungal communities in fresh and pre-colonised wood, although very early decay ascomycetes complicate the situation (Hiscox *et al.* 2015; Hiscox *et al.* 2016).

Flattening of the rarefaction curves for pre-colonised samples indicated that the bacterial community was comprehensively sampled, and further evidences community simplification in the presence of fungi. This agrees with previous observations of reduced bacterial diversity in wood and soil environments under fungal influence (Chapter 4; Boersma *et al.* 2009). Hervé *et al.* (2014) recorded completely saturated bacterial rarefaction curves for wood even in the absence of fungi, but it is likely that their microcosms exerted selection effects on the bacterial community. Sun *et al.* (2014) reported highly unsaturated bacterial communities after 2 and 4 months of wood decay, but this discrepancy is likely

because that study did not investigate pre-colonised wood, and had far lower read numbers than the present study.

5.5.3 The early-colonising bacterial community differs from later stage communities

The timescale of bacterial wood colonisation is poorly explored (Sun *et al.* 2013; Sun *et al.* 2014; Hervé *et al.* 2014). After 14 days bacteria could be detected throughout a 3 cm cube of fresh sapwood, indicating that they were able to colonise wood rapidly under favourable conditions. Although overall bacterial OTU richness appeared to decrease at 84 days, this was driven by the inclusion of low-diversity pre-colonised blocks among the 84-day samples. In fresh pinewood, bacterial richness and diversity increased between 2 and 4 months, suggesting that not all the available niches had been occupied within that time frame (Sun *et al.* 2014). In contrast, a microcosm experiment found no effect of time or fungal colonisation on bacterial richness, and limited effects on community composition (Hervé *et al.* 2014). This likely reflects the somewhat artificial experimental set-up, where time was not measured on replicates of increasing age, but rather by repeated sub-culturing of the developing community.

Psuedomonadaceae declined through time in both seasons. A decrease in the relative abundance of pseudomonads has also been observed over longer times (Sun *et al.* 2013; Sun *et al.* 2014; Kielak *et al.* 2016b). They were replaced by *Enterobacteriaceae* and *Sphingobacteriaceae* in autumn and spring, respectively. *Enterobacteriaceae* are known to inhabit decaying sapwood (Zhang *et al.* 2008), and some members possess lignocellulytic enzymes (Bugg *et al.* 2011). *Sphingobacteria* have also been previously reported in wood (Sun *et al.* 2013; Sun *et al.* 2014) and are known to associate with fungi (Warmink & van Elsas 2009; Pent *et al.* 2017).

Comparing the present samples to wood pre-colonised by the same fungi but allowed to decay in the field for a year (Chapter 4) revealed *Burkholderiales* were important members of both early and established communities. In contrast, there was a large difference in the relative abundance of *Acidobacteria*. This phylum was one of the largest components of the bacterial community in the one-year samples, yet a minor constituent in the early-decay community, suggesting a slow rate of colonisation. In concordance with this, *Acidobacteria* increased in relative abundance in decomposing wood over a timescale of 2-4 months (Sun *et al.* 2014), and between 1 and 6 years, before decreasing again by year 13 (Sun *et al.* 2013). However, Hoppe *et al.* (2015) recorded no difference in the relative abundance of *Acidobacteria* in logs across multiple decay classes.

5.5.4 Seasonal influences are dependent on stage of bacterial community succession

Regrettably, the scarcity of bacteria-positive samples meant that prediction 3 (greater fungal influence in the autumn) could not be tested. However, the control samples revealed seasonal differences independent of the pre-colonisers. Fungal contribution cannot be ruled out, as the effect could be mediated by different incoming fungal communities between seasons (Hiscox *et al.* 2015). These seasonal differences could only be seen in the 84-day samples. It has been postulated that during the early stages of wood colonisation, bacterial communities are determined by both neutral (stochastic) and niche-based processes, with niche-based processes only becoming dominant at later stages of decay (Kielak *et al.* 2016b). Intra-seasonal differences likewise could not be fully tested, but the autumn 84-day samples showed reduced diversity on one of the three collection dates. This sampling had been preceded by several days of hard frost, which may have adversely affected sections of the bacterial community.

5.5.5 Archaea are a minor but detectable part of the dead-wood prokaryote community

Consistent with previous reports from decaying wood, *Archaea* accounted for <1% of the prokaryote community (Rinta-Kanto *et al.* 2016). *Thaumarchaeota* were not represented, despite being the largest archaeal component in previous surveys of decaying wood (Rinta-Kanto *et al.* 2016). Archaeal PCR on the samples from Chapter 4 also found *Thaumarchaeota* to be the phylum that occurred most frequently (Johnston *et al.*, unpubl. data). Their current absence may be attributable to a known bias against *Thaumarchaeota* in the 515F-806R primer set (Parada *et al.* 2016). *Methanobacteria* inhabit living trees as part of the phenomenon known as bacterial wetwood (Zeikus & Ward 1974; Zeikus & Henning 1975). *Parvarchaeota* are putative acidophiles (Hedlund *et al.* 2014), which may explain their presence in the acidic dead-wood environment (Chapter 4).

5.5.6 Conclusions

In conclusion, this study provides strong evidence that wood-decay fungi act as 'gatekeepers', exerting control over which bacteria can colonise a woody resource. This effect is manifest both in delayed bacterial community development in fungus-colonised wood, and in modified bacterial community composition dependent on the identity of the fungal pre-coloniser. Fungal colonisation significantly delays the process of bacterial establishment, indicating that community development will differ between canopy-decayed and fresh wood. Even early-decay fungi with low competitive ability are capable of modifying the bacterial community. Bacterial community composition in wood varies between seasons, but only once the community is relatively established.

Chapter 6. The influence of migratory *Paraburkholderia* on growth and competition of wood-decay fungi

6.1 Abstract

Certain bacteria are capable of migrating along fungal hyphae, using them as a dispersal mechanism to cross otherwise-prohibitory distances. Many of these bacteria have been assigned to the genus *Paraburkholderia*, recently separated from *Burkholderia*. Three strains of fungal-migratory *Paraburkholderia* were isolated in axenic culture from the mycelium of wood-decay fungi, and inoculated onto ten strains of wood-decay fungi growing on solid medium in the laboratory. With one exception, all bacteria were able to migrate on the hyphae of all fungi, although to differing extents. No bacteria-associated growth inhibition was observed with eight of the ten fungi, but two strains of *Phanerochaete* showed a significant reduction in mycelial extension rate. Bacteria were also introduced into fungus-fungus competitive pairings, where they again significantly reduced the competitive performance of one of the *Phanerochaete* strains. In a subset of combinations, introducing bacteria into competitive interactions also reduced the predictability of the outcome. This is the first time that bacteria have been shown to influence fungal inter-specific competition, and underlines the influence fungal-migratory bacteria can have on their host.

6.2 Introduction

The phenomenon of fungal-migratory bacteria has been recorded for decades (Leben 1984), but has only recently been investigated in detail. Certain bacterial strains have the ability to migrate along fungal hyphae in order to disperse further and in less favourable conditions than would otherwise be possible (Warmink & van Elsas 2009; Nazir *et al.* 2010). Fungal-migratory bacteria have been grouped into two types: single-strain migrators are capable of movement along hyphae on their own, whilst community migrators can only track along hyphae as part of a consortium (Warmink & van Elsas 2009; Warmink *et al.* 2011). It is important to note that migration requires active movement by the bacterium, as fungal hyphae extend apically whilst the rest of the hypha remains stationary in the substratum. Migratory bacteria have been isolated from a variety of soil types, from clay to sandy loam (Nazir *et al.* 2012). Soil type apparently influences migration competence; for a number of bacterial strains, migration success along the same fungal host varies depending on the soil they are in (Nazir *et al.* 2012). A single strain of bacteria can migrate along multiple hosts, but each fungal species appears to have an individual 'carrying capacity' in terms of the bacterial cell count it can support (Nazir *et al.* 2014). Migration is highly directional, with most bacteria moving preferentially towards the hyphal tips (Warmink & van Elsas 2009; Nazir *et al.* 2014), although others move in the opposite direction (Leben 1984; Hover *et al.* 2016).

Many of the single-strain migratory bacteria isolated to date have been assigned to the genus *Burkholderia*, although since the spitting of this genus they have been reassigned *Paraburkholderia* (Sawana *et al.* 2014; Oren & Garrity 2015). This genus seems to be especially predisposed to forming fungal symbioses of various kinds (Stopnisek *et al.* 2015; Johnston *et al.* 2016; Chapter 2). *Paraburkholderia* spp. have relatively large genomes for bacteria, and produce a diverse range of secondary metabolites, some of which have anti-fungal activity (Depoorter *et al.* 2016). Genome analysis of the independent migrator *Paraburkholderia terrae* BS001 revealed motility-related genes for flagellae and walking pili, as well as genes involved in biofilm formation (Haq *et al.* 2014). Intriguingly, when a migratory strain of *Paraburkholderia glathei* was grown axenically it expressed cell motility proteins, but when co-cultured with a fungus many of these proteins were no longer expressed (Stopnisek *et al.* 2015); *P. terrae* BS001 likewise upregulated motility genes when near a fungus, but downregulated them again once hyphal contact had been made (Haq *et al.* 2017). A type III secretion system (T3SS) appears to have a beneficial but non-essential role in these migratory associations (Haq *et al.* 2016; Yang *et al.* 2016; Nazir *et al.* 2017).

The exact nature of this fungus-bacteria relationship remains unknown, in terms of the costs and benefits to each partner. The presence of a fungus allowed migratory bacteria to survive and grow in low pH soil and low-nutrient medium, neither of which could support the bacteria alone (Warmink & van Elsas 2009; Nazir *et al.* 2012; Stopnisek *et al.* 2015). The benefit to the bacteria could be mimicked in absence of the fungus by raising the soil pH and adding glycerol (Nazir *et al.* 2010). There are also indications that these benefits come at a cost: *P. terrae* BS001 carries a number of anti-toxin genes (Haq *et al.* 2014), and *P. glathei* upregulated its stress responses when tracking along a fungus (Stopnisek *et al.* 2015), possibly indicating that these bacteria may experience antibiosis from the fungus. Some community-migratory bacteria are selectively inhibited by the fungus *Lyophyllum* sp. strain Karsten (Warmink & van Elsas 2009). Outcomes for the fungi present a mixed picture. *Paraburkholderia terrae* BS001 protects its hosts from inhibition by pathogenic bacteria and ambient anti-fungal compounds (Nazir *et al.* 2014). Conversely, *Serratia marcescens* migrating over *Rhizopus oryzae* kill the fungus (Hover *et al.* 2016).

Thus far, there has been little exploration of whether migratory bacteria occur with wood-decay fungi: much of the work has focused on soil saprotrophs (*e.g.* Nazir *et al.* 2014; Haq *et al.* 2016; Simon *et al.* 2017), and there has been no exploration of whether wood-decay fungi coexist with migratory bacteria in the field. This relationship would be particularly interesting for cord-forming fungi, which primarily disperse, not as spores, but by forming large networks of mycelial cords across the forest floor (Boddy & Hiscox 2016). These networks would represent a major dispersal opportunity for any bacterium capable of exploiting them, providing a favourable habitat along which to migrate, and direct passage to new resources. *Paraburkholderia terrae* BS001 has been experimentally observed to migrate along the mycelial cords of *Phanerochaete velutina*, albeit less proficiently than with other fungal species (Nazir *et al.* 2014).

Wood-decay fungal communities are driven by inter-specific competition in the form of direct, confrontational interactions (Boddy 2000). Wood-decay fungi frequently encounter one another as mycelia within a woody resource, or cord systems on the forest floor. When this happens, they engage each other via diffusible and volatile compounds; increased enzyme activity; environmental pH manipulation; and gross mycelial contact (Boddy 2000). This has two implications for any bacterial symbionts living with wood-decay fungi. Firstly, these fungi are highly capable of manipulating the microbial community in their surroundings, and there is evidence that this extends beyond other fungi to include bacteria (Chapters 4 & 5). Secondly, there is potential for bacteria in turn to influence fungal

communities by affecting the outcomes of interactions. Wood-decay fungi form competitive hierarchies, but these can be altered by changes in abiotic or biotic conditions. For example, invertebrate grazers can exert top-down control of fungal communities when they preferentially graze the dominant competitor, weakening it such that a less competitive but grazing-resistant fungus is able to take over (Crowther *et al.* 2011). In this manner, the presence of a 'controlling' organism can have knock-on effects throughout the dead-wood environment.

Table 6.1 Fungal strains used to assess the effect of three migratory bacteria on fungal extension rates over agar.

Fungus	Strain	Abbreviation	Phylum	Successional stage	Source
Biscogniauxia nummularia	BxnFF1	Bxn	Ascomycota	Primary	Fruit body isolation
Vuilleminia comedens	VcWVJH1	Vc	Basidiomycota	Primary	Beech wood isolation
Bjerkandera adusta	BaSS1	Ba	Basidiomycota	Early-mid secondary	Fruit body isolation
Stereum hirsutum	ShSS1	Sh	Basidiomycota	Early-mid secondary	Fruit body isolation
Trametes versicolor	TvFPxH	Tv	Basidiomycota	Early-mid secondary	Lab cross
Hypholoma fasciculare	HfABWS1	Hf1	Basidiomycota	Late secondary/ tertiary	Cord isolation
Hypholoma fasciculare	HfGTWV2	Hf2	Basidiomycota	Late secondary/ tertiary	Fruit body isolation
Phanerochaete velutina	Pv29	Pv	Basidiomycota	Late secondary/ tertiary	Beech wood isolation
Phanerochaete sp.	PW271	Psp	Basidiomycota	Late secondary/ tertiary	Cord isolation
Resinicium bicolor	Rb1	Rb	Basidiomycota	Late secondary/ tertiary	University of Aberdeen

All strains are from the Cardiff University Culture Collection unless otherwise stated. Phanerochaete sp. PW271 and H. fasciculare HfABWS1 were both newly isolated during the course of this study.

The present study was designed to investigate the relationship between wood-decay fungi and migratory bacteria. The first objective was to isolate migratory bacteria directly from wood-decay fungi, in a UK mixed deciduous woodland: to date, the best-studied strains originate from soil in the Netherlands. Obtaining these strains allowed three hypotheses to be tested: (1) that the bacteria would be competent to migrate with a range of different wood-decay fungi as hosts; (2) that the presence of migratory bacteria would reduce fungal growth rate; and (3) that the presence of migratory bacteria would affect the competitive ability of wood-decay fungi.

6.3 Methods

6.3.1 Overview

Migratory bacteria were isolated directly from the mycelium of wood-decay fungi. These bacteria were then used in agar-based laboratory experiments to determine their effect on fungal growth and competitive ability.

6.3.2 Isolation and identification of migratory bacteria from the field

Mycelial cords were collected from the forest floor at Whitestone Woods, Monmouthshire, U.K. in the autumn-winter of 2015 (lat. 51.72, long. -2.69; site described Hiscox *et al.*, 2016). Cords were vigorously shaken by vortex-mixer in ten changes of sterile distilled water to remove all but tightly attached bacterial cells, before inoculation onto 2% malt agar (1.5 g l^{-1} LabM agar 1, 2 g l^{-1} malt). Static bacterial colonies were excluded during subsequent subcultures of the mycelium, but bacteria that clearly tracked the hyphal growth were retained. Of the 53 cords collected, migratory bacteria were obtained only from one. Pure cultures were established of both the bacterium (*Paraburkholderia* sp. BCC1884) and fungus (*Phanerochaete* sp. PW271).

Two further strains of migratory bacteria (*Paraburkholderia* sp. BCC1885 and *Paraburkholderia* sp. BCC1886) were obtained from mycelium isolated from decaying beech wood (*Fagus sylvatica*) at the same site. Small chips of wood were taken under aseptic conditions from the interior of 3 x 3 x 3 cm blocks that had been colonised with *Vuilleminia comedens* in the laboratory, and left on the woodland floor for 84 days (Chapter 5). The chips were placed onto 2% malt agar and bacteria isolated as above.

For preliminary identification of bacteria and fungi, DNA was extracted from cultures with the PowerSoil® kit (MO BIO, Carlsbad, USA) (amended to include 20 s at 4 m s^{-1} in a MPBio FastPrep bead beater). Fungal ITS rDNA markers were amplified using primers gITS7F (GTGARTCATCGARTCTTTG) and tagged ITS4R (TCCTCCGCTTATTGATATGC-AGTACGAG)

(Ihrmark *et al.* 2012) in 50 µl reactions containing 2.5 µl template, 300 nM tagged ITS4, 500 nM gITS7, 0.025 U HS Taq polymerase (PCR Biosystems, UK) and 10 µl supplied buffer, in a Dyad DNA Engine Peltier thermal cycler. The initial incubation was 94°C for 5 min, followed by 24 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C, with a final incubation at 72°C for 7 min. Bacterial 16S rRNA gene makers were amplified with primers 27F (AGAGTTTGATCMTTGGCTCAG) (Weisburg *et al.* 1991) and 1492R (TACCTTGTTACGACTT) (Lane *et al.* 1985) in 50 µl reactions containing 1 µl DNA, 200 nM each primer (MWG Eurofins, Ebersberg, Germany), 5 µg BSA (Promega, WI, USA), 0.025 U μl^{-1} Taq polymerase (PCR Biosystems, UK) and 10 µl supplied buffer, in a Dyad DNA Engine Peltier thermal cycler. The initial incubation was 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1.5 min increasing by 1 s $cycle^{-1}$, and a final incubation at 72°C for 5 min. The amplicons were purified with the QIAQuick gel extraction kit (Qiagen, Hilden, Germany), and sent for unidirectional Sanger sequencing by MWG Eurofins. Fungal sequences were assigned against the UNITE ITS database (Kõljalg *et al.* 2013) using BLASTn as a search engine. Bacterial sequences were assigned against the Greengenes database (DeSantis *et al.* 2006) in QIIME using UCLUST as a search engine (Caporaso *et al.* 2010).

6.3.3 Culture preparation and maintenance

All ten wood-decay fungi used in the experiments were taken from the Cardiff Culture Collection (Table 6.1). They were selected to represent a variety of successional stages and, therefore, competitive abilities. The strains represent a range of taxonomic dissimilarity, from inter-phylum to intra-specific. All fungi were maintained on 2% malt agar for the duration of the experiment.

The three migratory bacterial strains were maintained on 2% malt agar plates. Bacterial cultures for long-term storage were frozen at -80°C in 2% malt broth diluted 80:20 with glycerol. Experimental bacterial inoculum was prepared from 48-hr cultures grown at 20°C in 2% malt broth with gentle agitation. The suspension was diluted 1:5 in 2% malt broth on the morning of the set-up, and viable cell numbers determined by dilution series and total viable counting (TVC).

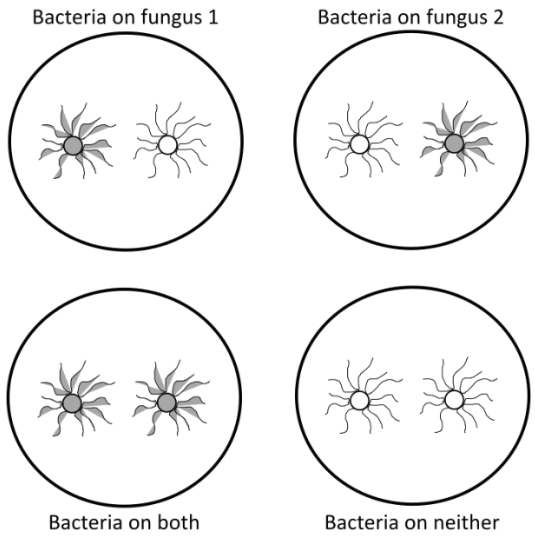

Figure 6.1 Design of the competition experiment. Each pair of fungi was subject to ten treatments in total: no-bacteria controls + (3 bacterial strains x 3 bacterial arrangements).

6.3.4 Mycelial extension rates

6 mm plugs were cut from 7-day old fungal cultures and individually inoculated onto 2% malt agar in 9 cm diameter Petri dishes. The following day, each was inoculated with a 10 µL drop of bacterial suspension (treatment; 2-7 x 10^5 colony-forming units, CFU) or sterile broth (control) directly on top of the inoculum plug. Plates were incubated at 10°C for 7 weeks or 20°C for 4 weeks. Mycelial extension and bacterial migration were measured along four radii extending from the edge of the inoculum plug. Five replicates were prepared per treatment.

6.3.5 Competition experiments

Plates were prepared as for the growth rate experiment, except that two plugs were inoculated onto each plate 3.5 cm apart, to set up competitive interactions. A restricted range of fungi was used due to the large number of potential combinations. *Bjerkandera adusta*, *Stereum hirsutum*, *Trametes versicolor*, *Phanerochaete velutina* and *Phanerochaete* sp. PW271 were paired in all combinations, including self-pairings. For each pairwise combination of fungi, interactions were set up with bacteria on both competitors; bacteria on one competitor but not the other, and *vice versa*; and bacteria on neither competitor (Fig 6.1). Five replicates were prepared per treatment. Bacteria (1-7 x 10^4 CFUs) and sterile broth were added as for the growth rates experiment. Plates were incubated at 10°C for 8 weeks or 20°C for 6 weeks, and the progress of interactions recorded weekly by visual inspection. At the end of the experiment, fungal re-isolations were made from the underside of the agar disk to verify that any observed replacement was not merely overgrowth. Bacterial re-isolations were made by drawing a sterile loop across the surface and streaking onto 2% malt agar.

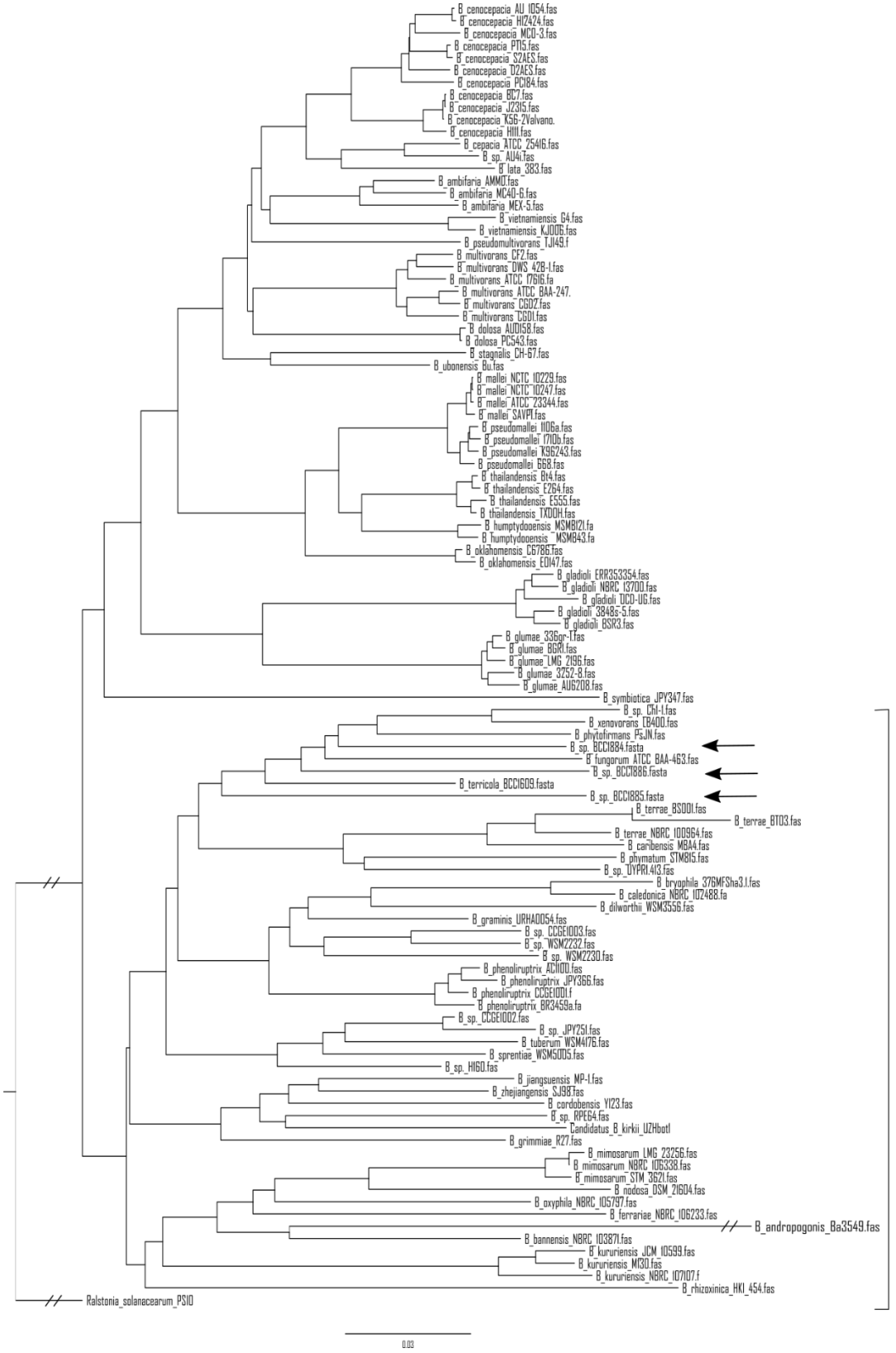

Figure 6.2 MASH whole genome cluster analysis of *Burkholderia* and *Paraburkholderia* genomes. The three strains isolated in this study (BCC1884, BCC1885 and BCC1886) are marked with arrows. The deepest branches have been shortened for ease of visualisation. MASH analysis and tree supplied by Alex Mullins.

6.3.6 Confocal microscopy

Additional interactions between *Phanerochaete* sp. PW271 and *Stereum hirsutum* were inoculated with *Paraburkholderia* sp. BCC1884 and visualised by confocal microscopy. A small slice of agar was cut out at each point to be visualised, and cells were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (ThermoFisher, Waltham, MA, USA). Images were taken of 1 and 7 week-old interactions, both at the interaction zone and on either side of it, using a Leica TCSSP2 confocal microscope.

6.3.7 Statistical analysis

Unless otherwise stated, all analysis was performed in R (R Development Core Team 2011) using RStudio (RStudio Team 2016) and packages *dplyr* (Wickham & Francois 2016), *ggplot2* (Wickham 2009), *lattice* (Sarkar 2008), *lsmeans* (Lenth 2016) and *nlme* (Pinheiro *et al.* 2016). R code to reproduce the analyses is available in Appendix 4 as an R markdown file (Protocol S6.1; Allaire *et al.* 2016).

Mycelial extension rates were modelled using a linear mixed effects model (Zuur *et al.* 2009), with fungal identity, bacterial identity, temperature and time as fixed main effects predicting the length of outgrowing mycelium. *Fungus : time* and *fungus : bacterium* interactions were also included, as each fungus grows at a different rate and may respond differently to the bacteria. The random part of the model consisted of a random intercept for replicate identity and a random slope for *time | replicate identity*, to account for the longitudinal design. A likelihood ratio test established the overall significance of the *fungus : bacterium* interaction, the main term of interest in the model. *Post-hoc* comparisons were done in package *lsmeans* with Tukey adjustment.

The outcomes of competitive interactions were codified as an interaction metric. Each competitor was given a score of 2 for completely replacing the other fungus; 1 for partial replacement; 0 for deadlock; -1 for being partially replaced; or -2 for being completely replaced. The number of weeks that each interaction took to reach that conclusion was recorded, and the metric calculated as *score x (1/time of completion)*. This gave an identical score of opposite sign to each competitor, and created a metric whereby a fast win scored higher than a slow one, and a fast loss scored lower than a slow one. Deadlock always produced a score of 0.

In each interaction, the fungal competitors were arbitrarily assigned as F1 and F2. The outcomes were modelled by regressing the metric score for F1 against a zero-sum contrast matrix in which F1 was coded as positive and F2 as negative. When F1 won, then its positive (winning) scores continued to be counted as positive, and were simultaneously

multiplied by -1 and counted as negative to F2; when F1 lost, its negative (losing) scores continued to be negative, and were simultaneously multiplied by -1 and counted as positive to F2. The model then included a second predictor matrix with a column for each combination of fungus and bacterium, *e.g. Paraburkholderia* sp. BCC1884 on *B. adusta*. If F1 had been inoculated with bacteria, it received a 1 in the relevant column; if F2 had been inoculated, it received -1 in the relevant column. This allowed the effect of each bacterium on each fungus to be estimated irrespective of whether the fungus was F1 or F2. Finally, temperature was included in the model as a simple categorical variable.

6.4 Results

6.4.1 Bacterial strain identity

16S rRNA gene sequencing placed all three strains within the genus *Burkholderia*, as the Greengenes database does not currently reflect the updated *Paraburkholderia* taxonomy. Subsequent whole-genome analysis placed the strains within *Burkholderia* clade 3 (Fig 6.2; A. Mullins, pers. comm.), which has been reassigned to the new genus *Paraburkholderia* (Sawana *et al.* 2014). Strain BCC1884 was isolated from a mycelial cord of *Phanerochaete* sp. PW271, whilst strains BCC1885 and BCC1886 were isolated from *F. sylvatica* wood blocks being decayed by *V. comedens*.

6.4.2 Migratory capability of bacterial isolates

All the bacteria were competent to migrate along at least one fungus, and all fungi successfully hosted at least one bacterium (Fig 6.3). There was considerable inter-fungal variation in the success of bacterial establishment and the extent of migration (Fig 6.3). Bacteria travelled furthest on the two *Phanerochaete* strains, and also migrated very successfully on *S. hirsutum*. In contrast, only one replicate indicated substantial bacterial migration on *R. bicolor*. The two strains of *H. fasciculare* showed an unexpected intra-specific difference: bacteria successfully established on only 5 out of 30 replicates of *H. fasciculare* GtWV2, compared with 19 out of 30 for *H. fasciculare* ABWS1. The only bacterium-fungus combination that completely failed to establish was BCC1885 on *H. fasciculare* GtWV2.

6.4.2 Mycelial extension rates

As expected, fungi showed considerable inter-specific variation in extension rate. At 20°C the fastest species, *B. adusta*, averaged 6.0 mm day^{-1}, whilst the slowest grower, *H. fasciculare* ABWS1, averaged 1.1 mm day^{-1} (Fig 6.4). Primary colonising fungi (*i.e.* early successional species) grew more quickly than did secondary/tertiary-stage colonisers. At

10°C, growth rates were reduced for all fungi, typically 2-3 times slower than at 20°C (Fig 6.5).

None of the three bacterial treatments significantly altered fungal extension rate when considered across all fungi. However, there was a significant interaction between fungal identity and bacterial treatment (LR 136, *P* <0.001). *Post-hoc* comparisons revealed that the interaction was driven by the *Phanerochaete* species, both of which showed significantly slower growth in the presence of bacteria (Table 6.2). The only other significant effect was on *H. fasciculare* ABWS1, where the mycelial extension rate increased by 28% in the presence of *Paraburkholderia* sp. BCC1886.

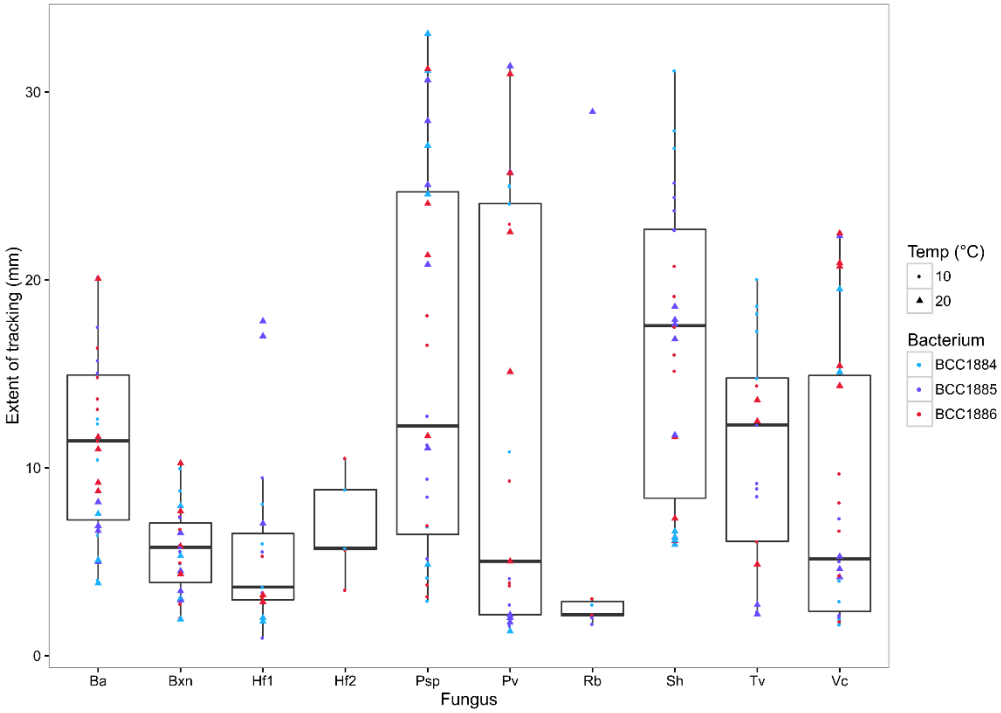

Figure 6.3 Extent of *Paraburkholderia* migration along hyphae of wood-decay fungi on 2% malt agar. Each point represents the maximum extent of bacterial migration on one replicate, at the time the fungus reached the edge of the plate. Shape of points indicates temperature (°C); colour indicates bacterial strain identity.

Table 6.2 *Post-hoc* comparisons of mycelial extension rate for wood-decay fungi inoculated with fungal-migratory *Paraburkholderia*.

Fungus	**Bacterium**	**Coefficient**	**Std error**	**DF**	***t***	***P***
Ba	BCC1884	-0.346	0.508	355	-0.682	>0.999
	BCC1885	-0.050	0.512	355	-0.097	>0.999
	BCC1886	0.453	0.508	355	0.893	>0.999
Bxn	BCC1884	0.084	0.489	355	0.173	>0.999
	BCC1885	-0.226	0.489	355	-0.463	>0.999
	BCC1886	-0.718	0.489	355	-1.47	>0.999
Hf1	BCC1884	-1.59	0.504	355	-3.16	0.395
	BCC1885	-1.21	0.504	355	-2.39	0.930
	BCC1886	**-2.20**	**0.481**	**355**	**-4.58**	**0.004**
Hf2	BCC1884	0.177	0.502	355	0.353	>0.999
	BCC1885	0.042	0.502	355	0.083	>0.999
	BCC1886	0.050	0.516	355	0.097	>0.999
Psp	**BCC1884**	**2.57**	**0.528**	**355**	**4.87**	**0.001**
	BCC1885	**3.21**	**0.543**	**355**	**5.91**	**<0.001**
	BCC1886	**2.67**	**0.548**	**355**	**4.87**	**0.001**
Pv	**BCC1884**	**2.24**	**0.527**	**355**	**4.25**	**0.016**
	BCC1885	**4.10**	**0.533**	**355**	**7.70**	**<0.001**
	BCC1886	**3.75**	**0.570**	**355**	**6.58**	**<0.001**
Rb	BCC1884	0.515	0.610	355	0.845	>0.999
	BCC1885	0.955	0.610	355	1.57	>0.999
	BCC1886	1.35	0.610	355	2.21	0.974
Sh	BCC1884	0.177	0.538	355	0.329	>0.999
	BCC1885	-0.036	0.538	355	-0.067	>0.999
	BCC1886	-0.088	0.538	355	-0.164	>0.999
Tv	BCC1884	-0.155	0.525	355	-0.295	>0.999
	BCC1885	-0.144	0.525	355	-0.274	>0.999
	BCC1886	-1.062	0.525	355	-2.02	0.994
Vc	BCC1884	-0.136	0.533	355	-0.254	>0.999
	BCC1885	-0.324	0.526	355	-0.616	>0.999
	BCC1886	-0.921	0.525	355	-1.75	>0.999

Comparisons are derived from a linear mixed effects model. P value adjustment: Tukey method for comparing a family of 40 estimates (not all comparisons are shown). Significant terms are shown in bold. All numbers are given to three significant figures. Fungal abbreviations are given in Table 6.1.

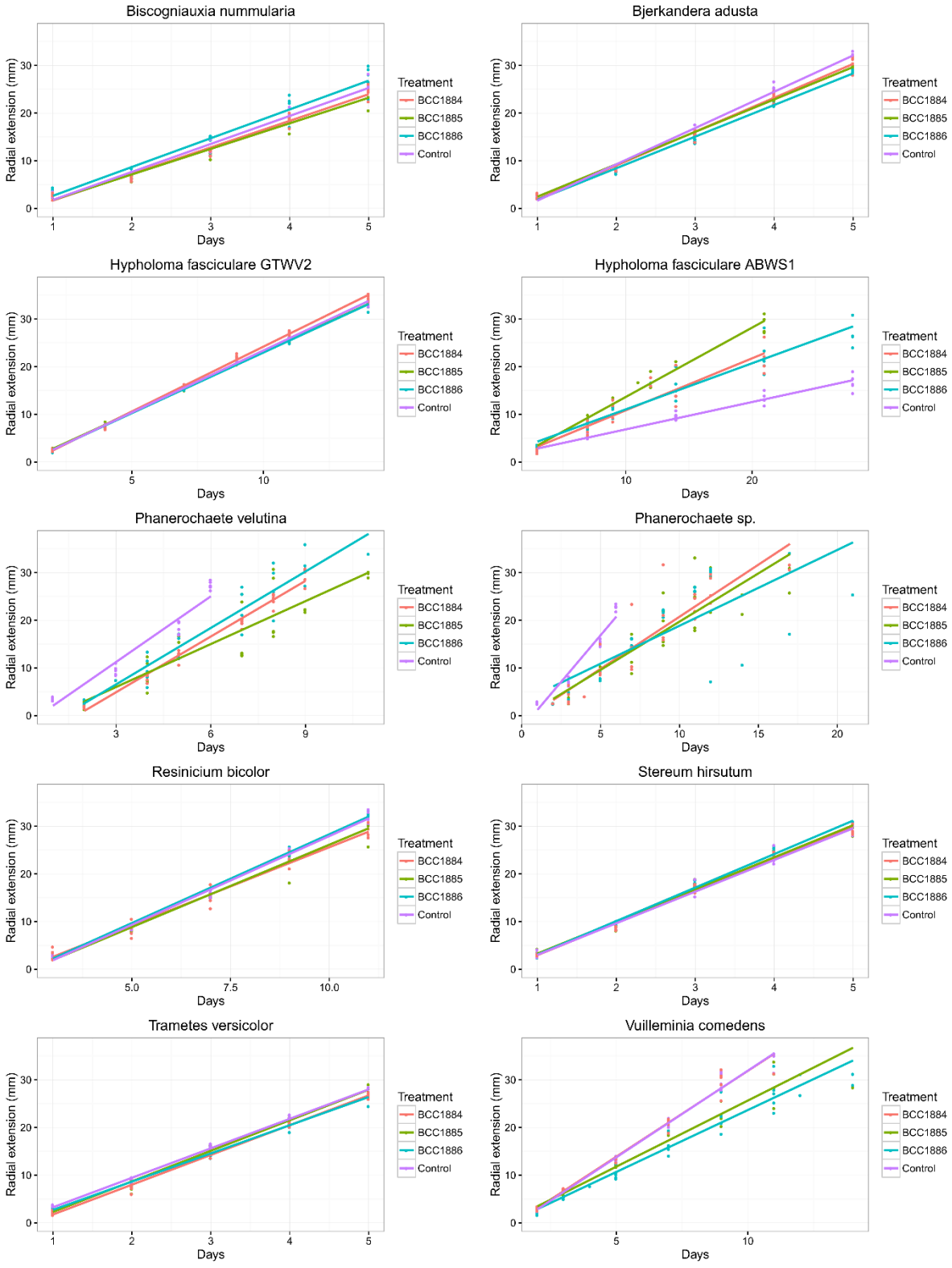

Figure 6.4 Mycelial extension rates of ten wood-decay fungi on 2% malt agar at 20°C in the presence and absence of *Paraburkholderia*. Different colours indicate the three different strains.

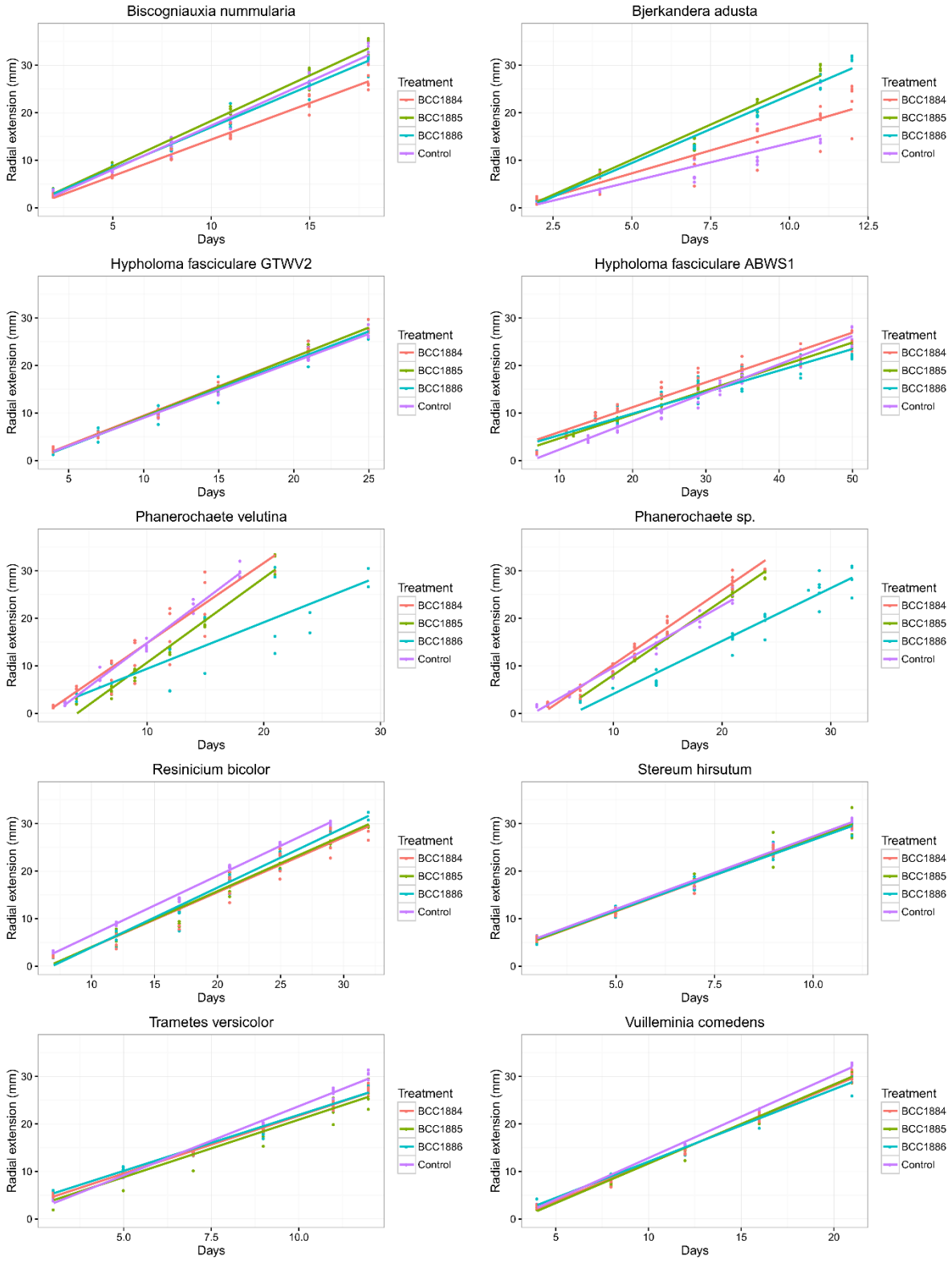

Figure 6.5 Mycelial extension rates of ten wood-decay fungi on 2% malt agar at 10°C in the presence and absence of *Paraburkholderia*. Different colours indicate the three different strains.

6.4.3 Effects of bacteria on inter-fungal competitive interactions

B. adusta had the highest average interaction score overall, followed by *T. versicolor*, *S. hirsutum*, *P. velutina* and *Phanerochaete* sp.; in the controls, *B. adusta* still scored highest, followed by *S. hirsutum*, *T. versicolor*, *Phanerochaete* sp. and *P. velutina*. The presence of bacteria significantly affected fungal performance in a species-specific fashion (Table 6.3). *Phanerochaete* sp. PW271 had significantly reduced performance scores when inoculated with any of the three bacteria. *Paraburkholderia* sp. BCC1885 also significantly reduced the performance of *B. adusta* and *P. velutina*, although to a lesser extent. In some cases the addition of bacteria could reverse the outcome of the interaction (*e.g. T. versicolor vs. Phanerochaete* sp. and *P. velutina vs. Phanerochaete* sp. at 20°C; Fig 6.6), and there were also instances where bacteria could make an otherwise-consistent outcome unpredictable (*e.g. B. adusta vs. P. velutina* at 10°C, *P. velutina vs. T. versicolor* and *P. velutina vs. S. hirsutum* at 20°C; Figs 6.6 & 6.7). All the fungal self-pairings met and merged as expected, regardless of bacterial treatment.

6.4.4 Observations on fungal morphology and bacterial behaviour

The presence of bacteria induced highly localised pigment production in *S. hirsutum* (Fig 6.8); the progress of bacterial migration was marked by bright orange colouration. Bacterial establishment on mycelium was often asymmetric, resulting in parts of the mycelium being colonised and others remaining (visually) bacteria-free. Bacteria were sometimes observed to cross the interaction front and become established on the other fungus. This indicates that whilst migration preferentially occurred towards the hyphal tips, there was at least some capacity to move in the opposite direction. Confocal microscopy revealed bacteria massing around hyphae, including at the point where several hyphae were starting to aggregate into a cord (Fig 6.9).

6.5 Discussion

This is the first report of migratory bacteria isolated from wood-decay fungi, and indicates that they co-occur naturally both in wood and on mycelial cords. Whilst most fungi appeared unaffected by hosting bacteria, active fungal-bacterial interaction was revealed by growth and competitive inhibition of two strains of *Phanerochaete*, and by morphological responses in *S. hirsutum*. This is the first assessment of how bacteria influence fungal inter-specific interactions, and reveals that they consistently impede the performance of some strains. The addition of bacteria can also change or destabilise the outcome of interactions in a pairing-specific fashion.

Table 6.3 Model output for outcomes of competitive interactions between wood-decay fungi and the effect of inoculating each competitor with fungal-migratory *Paraburkholderia*.

Term	**Coefficient**	**Std error**	***t***	***Pr(>|t|)***
(Intercept)	**-0.275**	**0.027**	**-10.3**	**<0.001**
Ba	**0.448**	**0.031**	**14.6**	**<0.001**
Sh	-0.021	0.024	-0.905	0.366
Psp	**-0.278**	**0.031**	**-9.07**	**<0.001**
Pv	-0.040	0.026	-1.57	0.115
Ba-BCC1884	-0.041	0.048	-0.842	0.400
Sh-BCC1884	0.018	0.048	0.382	0.703
Psp-BCC1884	**-0.205**	**0.048**	**-4.25**	**<0.001**
Pv-BCC1884	0.033	0.048	0.691	0.490
Tv-BCC1884	0.014	0.048	0.292	0.771
Ba-BCC1886	-0.025	0.048	-0.525	0.600
Ba-BCC1885	**-0.122**	**0.048**	**-2.54**	**0.011**
Sh-BCC1885	-0.053	0.048	-1.09	0.275
Psp-BCC1885	**-0.296**	**0.049**	**-6.04**	**<0.001**
Pv-BCC1885	**-0.111**	**0.048**	**-2.31**	**0.021**
Tv-BCC1885	0.073	0.049	1.49	0.137
Sh-BCC1886	0.001	0.048	0.012	0.990
Psp-BCC1886	**-0.602**	**0.048**	**-12.5**	**<0.001**
Pv-BCC1886	-0.068	0.048	-1.41	0.159
Tv-BCC1886	0.046	0.049	0.942	0.346
Temp20	**0.157**	**0.022**	**7.22**	**<0.001**

The fungal identity matrix is calculated with zero-sum contrasts; the estimate for Tv may be obtained by subtracting the coefficients for the other fungi. Null deviance = 213.96 on 986 degrees of freedom; residual deviance = 112.33 on 966 degrees of freedom; R^2 *= 0.47. Significant terms are shown in bold. All numbers are given to three significant figures. Fungal abbreviations are given in Table 6.1.*

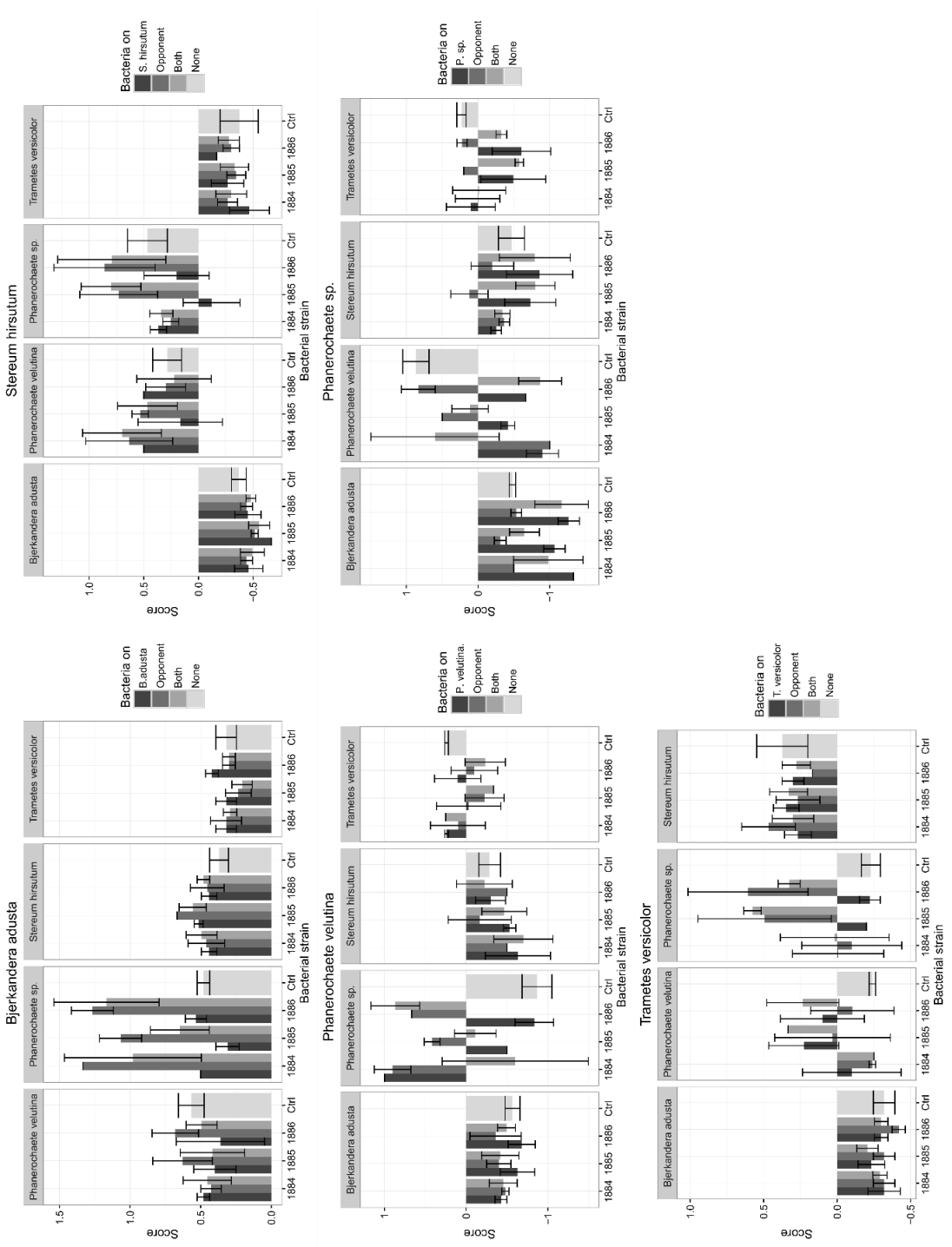

Figure 6.6 Outcomes of competitive interactions between wood decay basidiomycetes on 2% malt agar at 20°C in the presence and absence of *Paraburkholderia*. Interactions are scored on a metric that combines outcome of interaction and the time taken to reach a conclusion. Error bars represent standard deviation.

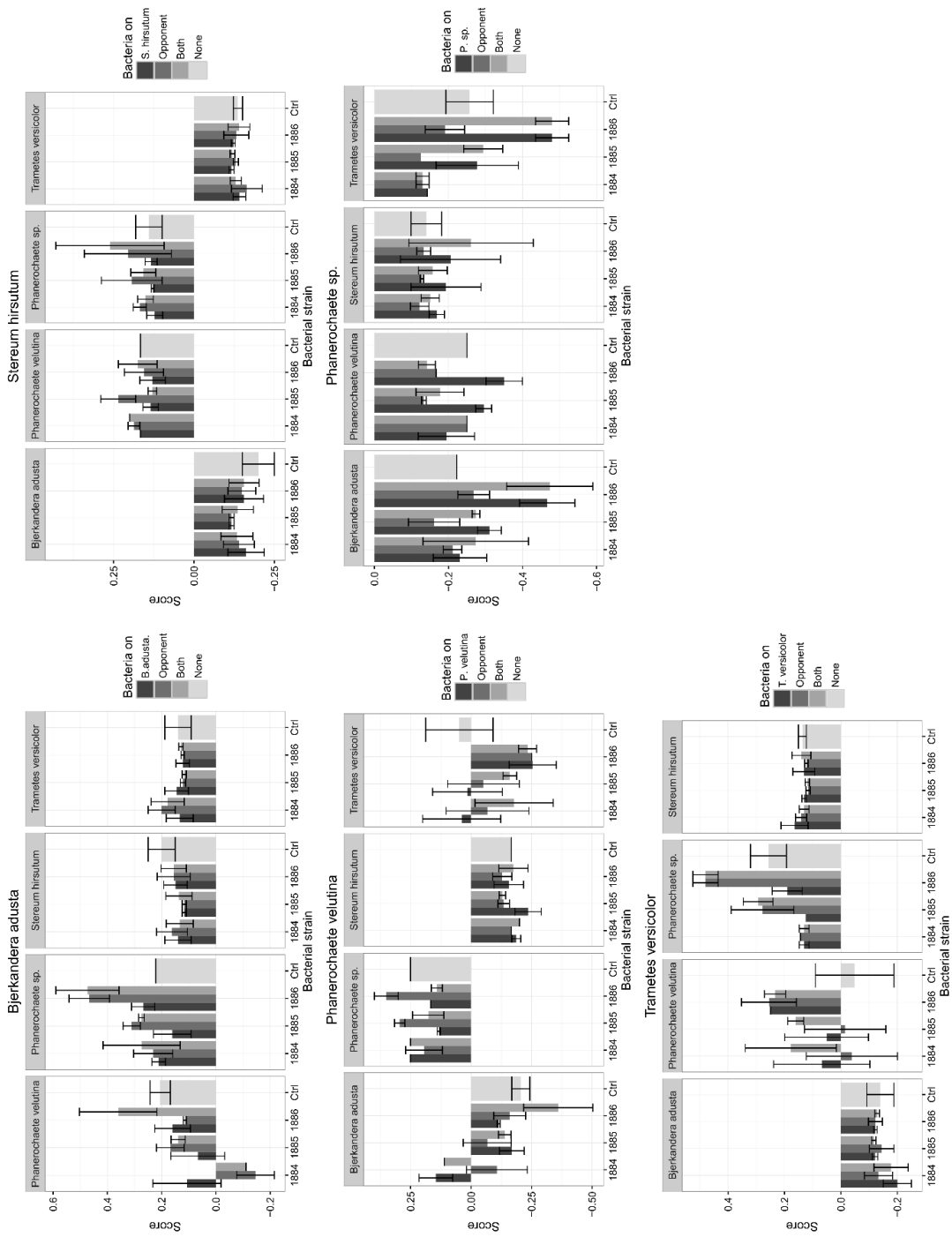

Figure 6.7 Outcomes of competitive interactions between wood decay basidiomycetes on 2% malt agar at 10°C in the presence and absence of *Paraburkholderia*. Interactions are scored on a metric that combines outcome of interaction and the time taken to reach a conclusion. Error bars represent standard deviation.

All three of the bacterial strains isolated were from a single clade of *Paraburkholderia*, even though no attempt was made to discriminate taxa in the isolation. This lends further support that *Burkholderiaceae* are an important fungal-associated family, and *Paraburkholderia* in particular are a major genus among fungal-migratory bacteria (Nazir *et al.* 2012; Stopnisek *et al.* 2015; Johnston *et al.* 2016; Simon *et al.* 2017; Chapters 2 & 4). None of the three present strains formed a cluster with each other, suggesting that all three may be different species. All three fell within the *P. xenovorans* group, close to *P. fungorum, P. terricola, P. xenovorans* and *P. terrae*. This clade encompasses many migratory or otherwise fungal-associated species. *P. fungorum* was originally isolated from a wood-decay fungus, *Phanerochaete chrysosporium*, with which it formed close hyphal associations (although whether *P. fungorum* migrated along the host was not reported) (Seigle-Murandi *et al.* 1996; Coenye *et al.* 2001). *P. terricola* and *P. xenovorans* are both capable of single-strain migration on *Lyophyllum* sp. strain Karsten (Nazir *et al.* 2012). *P. terrae* is the best characterised of all migratory bacteria, with multiple strains known to migrate on a range of fungi (Nazir *et al.* 2012; Haq *et al.* 2014; Nazir *et al.* 2014). The *P. terrae* genome has been sequenced (Haq *et al.* 2014), opening the door for future comparison between *P. terrae* and the present strains that may reveal a genomic signature of migration. Previous work has pointed to type III secretion systems, flagella and pili as likely enablers of migratory behaviour (Yang *et al.* 2016; Yang *et al.* 2017; Nazir *et al.* 2017).

6.5.1 Migratory bacteria occur naturally with wood-decay fungi and have a broad host range

Migratory bacteria could be isolated from mycelial cords, but the retrieval rate was nonetheless low. The strains isolated from wood were discovered serendipitously, so detection rates cannot be compared. Nazir *et al.* (2014) reported low rates of migration over cords compared to hyphae, suggesting that cords may represent a more challenging venue for bacteria. Mycelial cords are toughened dispersal organs, aggregations of hyphae organised within a protective rind (Boddy 1993). This may provide the opportunity for migration of bacteria over the surface of the cord, and/or along hyphae inside the rind. Living within the rind would afford the bacteria direct access to the hyphae, and protection from the environment. This idea is entirely speculative at present, but a small measure of support is provided by the microscopic evidence of bacteria adhering to hyphae which were in the process of forming a cord. Note that this is not evidence of endosymbiosis, as at no point were bacteria observed within hyphae.

The three strains of *Paraburkholderia* obtained were generalist migrators, capable of becoming established on multiple different fungi and thus supporting hypothesis 1. Each

of the bacterial strains was able to migrate on a range of fungal hosts, representative of the species that would be encountered within their habitat. Two of the three bacteria (BCC1884 and BCC1886) migrated over all ten fungi (although with varying success), irrespective of fungal taxonomic position or ecological strategy.

6.5.2 Wood-decay fungi show inter- and intra-specific variation in their responses to migratory bacteria

Most of the fungi tested were neither inhibited nor stimulated due to the presence of migratory *Paraburkholderia*, in accord with previous work (Nazir *et al.* 2014). Sometimes, this was because bacteria were largely unable to establish on the mycelium (*e.g. H. fasciculare* GtWV2). The difference in bacterial establishment between the two *H. fasciculare* strains was both unexpected and unexplained, but it indicates that the factors required for colonisation by migratory bacteria can vary at the intra-specific level. *R. bicolor* supported very limited bacterial movement, perhaps related to the calcium oxalate crystals which cover the hyphae (Connolly & Jellison 2011). *S. hirsutum* had very high rates of bacterial establishment and movement, yet with no effect on its mycelial extension rate – although its abundant pigmentation shows that it actively responded to bacterial presence.

The two *Phanerochaete* strains were notable exceptions to this general lack of growth response. Both provided a very successful platform for bacterial migration, and both were markedly inhibited in the process, thus offering partial support for the second hypothesis. Curiously, Nazir *et al.* (2014) used the same strain of *P. velutina* as the present study, and found it was unaffected when growing across soil with *Paraburkholderia terrae* BS001. This may reflect the difference in bacterial identity, venue of interaction (soil *vs.* agar) or fungal morphology (cords *vs.* hyphae). Although the present study contrasts with *P. terrae*'s lack of effect on host growth rate during co-migration, *P. terrae* has been observed to reduce fungal growth prior to physical contact (Haq *et al.* 2016).

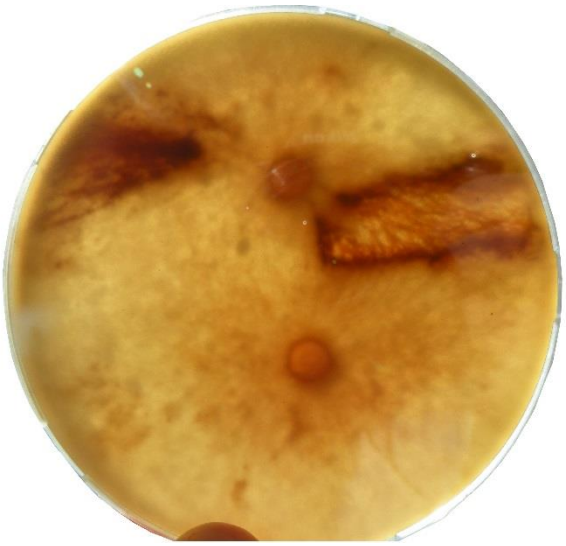

Figure 6.8 *Paraburkholderia* sp. BCC1885 inducing localised pigment production in a self-pairing of *S. hirsutum*. The two inoculation plugs are visible in the centre of the plate. The top plug was inoculated with *Paraburkholderia*.

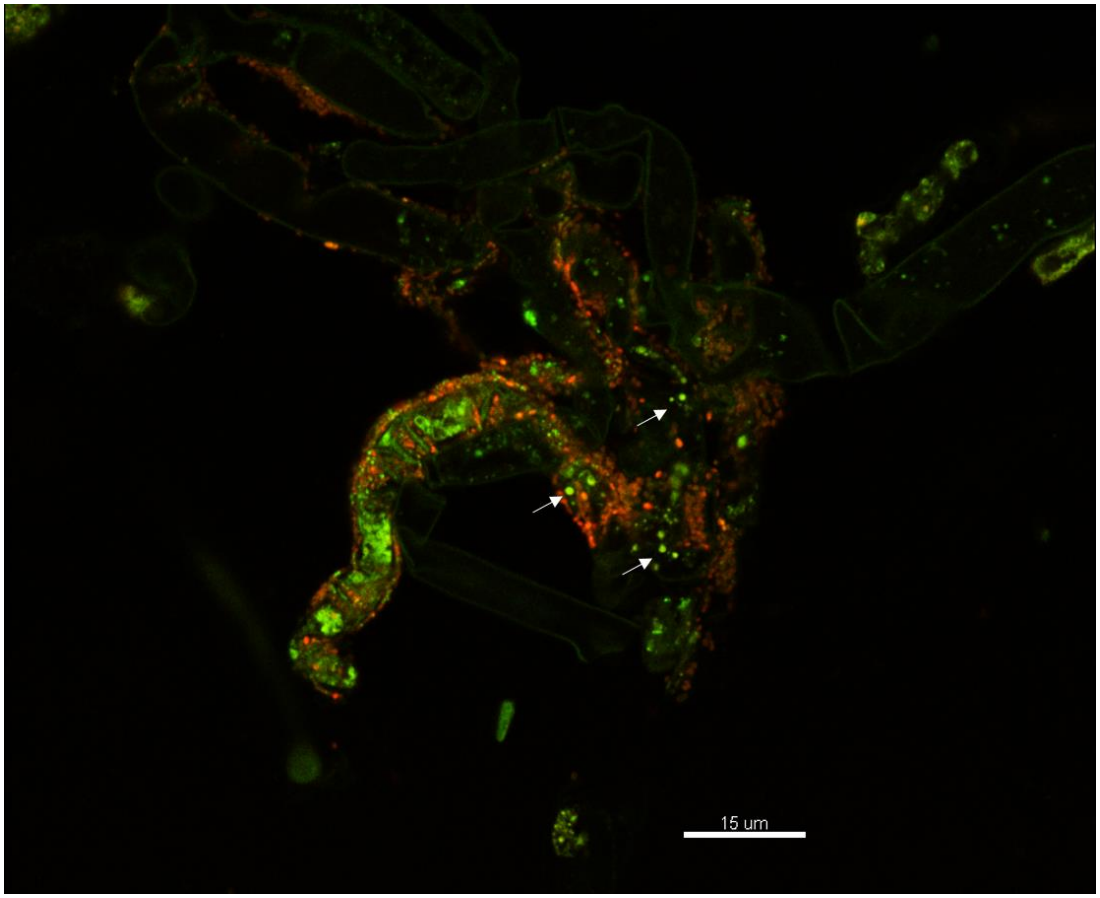

Figure 6.9 Confocal microscope image of *Paraburkholderia* BCC1884 adhering to the hyphae of *Phanerochaete* sp. PW271, stained with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. The hyphae appear to be aggregating into a cord at the centre of the image. Arrows indicate clusters of bacteria. Some autofluorescence is also visible from the hyphae.

6.5.3 Migratory bacteria can reduce the competitive ability of wood-decay fungi

As with extension rate, bacterial effects on competitive ability were fungus-specific, and so the third hypothesis was likewise partially supported. Once again, *Phanerochaete* sp. was the most affected, with a reduction in competitive ability when inoculated with any of the three bacteria. This fungus was isolated alongside *Paraburkholderia* sp. BCC1886, and it is noteworthy that the fungus most affected by bacteria was known to naturally host migratory bacteria. It is possible that this strain is particularly prone to exploitation by bacteria. Migratory bacteria are able to partner with a range of fungal strains and species, yet with a varying strength of interaction, mediated by different mechanisms (Nazir *et al.* 2014; Haq *et al.* 2016). The negative effects on both growth and competition suggest that these *Paraburkholderia* may be parasites, dispersing along fungal hyphae whilst disadvantaging the host. Conversely, *P. terrae* BS001 can protect its host from harmful bacteria and anti-fungal compounds (Nazir *et al.* 2014). Future work on the strains from the current study should investigate whether they also exhibit this ability, in which case there may be a fitness trade-off for fungi between the cost of hosting migratory bacteria and the protection that they afford. Wood-decay fungi exert active selection over the bacteria in their resource (Chapters 4 & 5), so the persistence of *Paraburkholderia* actually on mycelium indicates that it must be either tolerated by the fungus or resistant to its anti-bacterial mechanisms.

Wood-decay fungi form competitive hierarchies, whereby one species will outcompete another in a predictable (though not deterministic) manner (Boddy 2000). The competitive hierarchy observed in the present study was reversed compared to that normally expected (*e.g.* Hiscox *et al.* 2017), with the early coloniser *B. adusta* scoring highest and the late-stage *Phanerochaete* strains scoring lowest. On a rich agar medium, fast-growing earlier-successional species are at an advantage, which probably explains why they were able to outcompete the slower-growing secondary colonisers. This may also help to explain the reduced performance of *Phanerochaete* sp., as the bacteria slow its growth rate even further.

In some combinations, the addition of the bacteria reversed the outcome of the interaction, or destabilised an otherwise predictable relationship. The effect is reminiscent of how grazing invertebrates can reverse interaction outcomes by preferentially consuming the stronger competitor (Crowther *et al.* 2011). Likewise, the addition of a third fungal competitor into a pairwise interaction can shift the outcome from consistent to unstable (Hiscox *et al.* 2017). These examples underline the importance of considering competition

not only in terms of the competitors' own traits, but also as a process under cross-kingdom, top-down influences.

The addition of bacteria did not impede fungal self-recognition mechanisms, as all self-pairings showed normal recognition and mycelial fusion. Altering the bacterial community associated with the zygomycete *Mucor hiemalis* interfered with this process, producing antagonism between the cured and uncured mycelium (Schulz-Bohm *et al.* 2017). *Pseudomonas syringae* activates programmed cell death (and thus nutrient release) in *Neurospora crassa* by tapping into a somatic incompatibility pathway used in self-recognition (Wichmann *et al.* 2008).

6.5.4 Conclusions

In conclusion, migratory bacteria have been isolated directly from fungal mycelium, in a different habitat and geographically distant location to previous strains. These *Paraburkholderia* are competent to migrate with a taxonomically and ecologically diverse range of wood-decay fungi, which in turn showed species- and strain-specific responses to bacterial presence. Most fungi were largely unaffected in both growth and competitive ability, but the two *Phanerochaete* strains were consistently inhibited by the bacteria. These results show that wood-decay fungi naturally host migratory bacteria, and that these bacteria may influence the results of fungus-fungus interactions.

Chapter 7. General discussion

7.1 Synthesis

The work presented here furthers current understanding of fungus-bacteria interactions during wood decomposition by establishing causative relationships in a natural environment: wood-decay fungi drive bacterial community composition. This is the first time that this relationship has been demonstrated in the field. The work also extends knowledge of fungal interactions with migratory bacteria from a soil context to include wood-inhabiting taxa, and reveals for the first time that bacteria can influence the outcomes of inter-specific fungal interactions.

The major finding of Chapter 4 was that fungi can control bacterial communities in the field. Radical changes in bacterial community composition have been seen when fungi were introduced to wood microcosms (Folman *et al.* 2008; Hervé *et al.* 2014), but the present study provided the first evidence of a causative association in the field. Additionally, it provided support for the idea that pH modification is a mechanism for fungal control of the wood environment (de Boer *et al.* 2010). Unexpectedly, the best predictor of bacterial community composition was whether or not the dominant fungus was a cord-former, adding a new aspect to the distinctive ecology of cord-forming basidiomycete fungi in woodland ecosystems (Boddy 1993). This degree of control over the bacterial community may be due directly to the highly competitive nature of cord-formers; additionally, this competitive dominance allows cord-formers to occupy larger territories for longer periods of time compared to less competitive fungi, giving them more opportunity to make a mark on the bacterial community.

Chapter 5 extended this causative association to less-competitive fungi at the early stages of community development. Wood-decay fungi actively repel other fungi from entering their territory (Hiscox *et al.* 2017), but this was the first time that they have been shown to exclude bacteria. Canopy decay has not previously been considered as a driver of bacterial succession, but the differing bacterial communities between fresh and pre-colonised wood highlight the importance of this decay stage.

The complexity of individual fungus-bacteria interactions was demonstrated in Chapter 6. Bacterial migration on wood-decay fungi has been observed in the lab (Nazir *et al.* 2014), but had not previously been demonstrated to occur naturally. The competitive inhibition of *Phanerochaete* sp. by migrating bacteria adds a new entry to the list of biotic controls on fungal competition (Crowther *et al.* 2011; Hiscox *et al.* 2017). None of the fungi showed a significant improvement in performance when inoculated with *Paraburkholderia*,

which was surprising given that other strains convey considerable benefits to the host (Nazir *et al.* 2014). It may be that the scenarios tested revealed the cost to the host, but concealed benefits that would become apparent under different conditions.

Across all the above chapters, the *Burkholderiaeae* repeatedly emerged as major bacterial players: they were positively associated with fungi in Chapters 4 & 5, and all three migratory bacteria isolated in Chapter 6 belonged to the *Paraburkholderia*. This adds further evidence for their importance among fungal-associated bacteria (Stopnisek *et al.* 2015; Chapter 2). *Acidobacteria* were notable for their fungal associations in Chapter 4, yet their scarcity in Chapter 5 suggests that they are slow to colonise wood. This is consistent with previous reports of *Acidobacteria* as a major bacterial taxon in wood (Valásková *et al.* 2009; Sun *et al.* 2013; Sun *et al.* 2014; Hoppe *et al.* 2015; Kielak *et al.* 2016a), and their general association with acidic, low-nutrient environments (Kielak *et al.* 2016b). More surprising was the consistently low proportion of *Actinobacteria* (Chapters 4 & 5), in contrast to previous studies (Sun *et al.* 2013; Sun *et al.* 2014; Hoppe *et al.* 2015; Kielak *et al.* 2016b; Rinta-Kanto *et al.* 2016). One possible explanation is that most of the above studies examined coniferous wood, whereas the present work used deciduous wood – although Hoppe *et al.* (2015) recorded little difference in relative abundance of *Actinobacteria* between deciduous and coniferous wood. *Actinobacteria* may become abundant at later stages of decay (Rinta-Kanto *et al.* 2016), although conversely they have been postulated to be early colonisers (Hoppe *et al.* 2015). Overall, the ecology and determinants of *Actinobacteria* in wood remain unclear.

Most wood-decay fungi used in these experiments were basidiomycetes, although an ascomycete was included in Chapter 6; fungi were primarily selected based on their ecological strategy, rather than their taxonomic classification. The exploratory aspect of Chapter 4 found no correlation between bacterial community composition and the phylum of the dominant fungus. However, it remains an area for future work to explore specific associations between bacteria and ascomycete wood-decay fungi.

7.2 Issues and innovation in methodology

One recurring theme that emerged during the course of the work is the need for new and improved methods to tackle the increasing complex questions in microbial ecology. Attempts to quantify bacteria in wood by quantitative PCR revealed the unexpected but substantial co-amplification of fungal DNA. The diagnosis and exploration of this problem form Chapter 3, which found that universal bacterial qPCR primers are non-specific when fungal DNA is abundant, and particularly where fungal DNA is equal or greater in

concentration than the target. Amplification of host DNA is a known problem in microbiome studies (Galkiewicz & Kellogg 2008) but this is the first time it has been reported for a fungal host, despite having been predicted in fungus-rich environments (Huws *et al.* 2007).

The robust analysis of OTU data is an outstanding challenge in microbial ecology, in light of extremely challenging data properties and the number of decisions that arise in the course of data processing. Chapters 4 & 5 attempt to make progress in this area by replacing the controversial technique of rarefying (McMurdie & Holmes 2014; Weiss *et al.* 2017) with methods that account explicitly for uneven sample sizes and overdispersion in the data. Whilst analysing interaction outcomes is a less common problem, Chapter 6 also required statistical innovation, such as the creation of a combined outcome-and-time metric and the collaborative development of an advanced matrix-based linear model.

The combination of laboratory and field approaches provides a powerful insight into community processes. Field studies are the gold standard for realism, yet their complexity and unpredictability can obscure genuine patterns amidst large amounts of noise. Until now, field assessments of fungus-bacteria interactions in wood have relied on correlation (Hoppe *et al.* 2014; Kielak *et al.* 2016b). Using wood that had been pre-colonised by fungi in the lab gives insight into real-world communities whilst employing the increased inferential power of manipulative experiments (Chapters 4 & 5). A potential drawback of this method is that it introduces the pre-coloniser fungi into the field, where they may grow out from the experimental wood resources and colonise the site. By the end of the experiment in Chapter 5, *Hypholoma fasciculare* mycelium could be seen proceeding from the blocks and colonising adjacent leaf litter. To mitigate for this, all the pre-coloniser fungi used in field experiments were common species, typical of the habitat at the sites. All strains were originally collected within the UK (apart from *Trametes versicolor* TvAW-HxFP, which is a laboratory cross between two homokaryon strains), and in some cases were local to the field sites.

Culture-based methods are useless for surveying bacterial communities in wood (Chapter 2), but remain valuable tools for investigating specific processes in detail (Fierer 2017). The isolation of fungal-migratory *Paraburkholderia* allowed behavioural and physiological study of their interactions with fungi (Chapter 6), which could not have been observed with gross DNA-based techniques. Simple techniques such as measurement and microscopy still make an important contribution to modern microbiology when used in an appropriate setting. The downside to culture-based approaches is that they are by nature artificial, with limited applicability to a field situation. This was reflected in the atypical fungal

hierarchy seen in Chapter 6, which was probably caused by interaction on nutrient-rich medium.

7.3 Fungi as holobionts

Evidence is mounting that fungi naturally host a bacterial community that intimately interacts with the mycelium (Chapter 2). From this, it has been recently argued that fungi should be considered holobionts: not isolated organisms, but unified combinations of host and microbiome (Partida-Martínez 2017). This presents a unique situation in which the host is itself a microorganism, albeit one that reaches macroscopic size. Perturbing the microbiome of *Mucor hiemalis* affected host growth, morphology, volatile production and self-recognition (Schulz-Bohm *et al.* 2017). The present work contributes to understanding fungi within this framework. Firstly, it demonstrates that wood-decay fungi naturally host migratory bacteria, both within wood and on/in mycelial cords (Chapter 6). These migratory bacteria alter the fitness of the host, but the bacteria-host interaction is species- and genotype-specific. Secondly, it indicates that wood-decay fungi influence and alter the bacterial community within their resource (Chapters 4 & 5). For a wood-decay fungus, the immediate environment may be considered an extension of the organism because fungi acquire nutrients by extracellular enzyme production. The bacterial community within wood under single-species fungal dominance is thus broadly analogous to its gut microbiome.

7.4 The usefulness of a trait-based framework in studying fungus-bacteria interactions

A picture has emerged of the importance of traits when considering fungus-bacteria interactions. The concept of describing species in terms of traits (individual-level properties that influence performance) is gaining popularity in microbial ecology, but remains challenging when a large proportion of microbial diversity is poorly described and cannot even be cultured (Crowther *et al.* 2014; Fierer 2017). The present work indicates the usefulness of trait-based classification. The ability to form mycelial cords is a trait associated with particular wood-decay fungi, indicative of a foraging-based ecology that is correlated with high competitive ability, powerful enzyme systems and a late-secondary successional position (Boddy 1993). The possession of this trait turned out to be a major predictor of associated bacterial communities (Chapter 4). The same experiment indicated that certain traits, notably acid-tolerance, are likely to be common among bacterial symbionts of wood-decay fungi and convey an advantage in a fungus-dominated environment. Bacterial migration on fungi represents a 'trait complex' (*sensu* Crowther *et al.* 2014), and Chapter 6

indicates that it may have far-reaching effects in the wood environment, interacting with fungal traits such as growth rate and competitive ability. The 'true traits' underlying this trait complex are not fully understood, but there is evidence that they include flagella-linked motility, expression of type III secretion systems, and cellular recognition and binding ability (Haq *et al.* 2016; Yang *et al.* 2016; Yang *et al.* 2017; Nazir *et al.* 2017).

7.5 Future directions

With the discovery that wood-decay fungi can control bacterial communities, an obvious next step would be to probe the mechanisms of how this occurs. Chapter 4 indicates the importance of fungal pH modification, and Chapter 5 suggests that the first few months of colonisation would be an appropriate time point to focus on, as fungi are likely to be responding actively to a developing bacterial community. Metatranscriptomics could provide a useful way of simultaneously recording fungal and bacterial responses, and would be particularly powerful if combined with experimental manipulation of potential drivers such as wood pH.

Further to this, the role of traits in predicting fungus-bacteria co-existence merits future consideration. From a fungal perspective, the topic could be explored by selecting a range of pre-colonisers specifically chosen to encompass variation in specific traits, and seeing how well those traits predicted the associated bacterial communities. Examining bacterial traits is more challenging, as many taxa are poorly characterised, if they are described at all. Ascribing and describing traits for a particular organism still rely heavily on the ability to culture it (Crowther *et al.* 2014; Evans *et al.* 2017). Metagenomics could provide a way forward, allowing community-level correlation of genomic potential with environmental conditions. Although this approach would have limited ability to combine traits and identities, it could provide a starting point for identifying important traits by looking for those over-represented within the community. Metatranscriptomics and metaproteomics could also provide valuable insights, albeit with their own caveats: fungal gene expression does not necessarily correlate well with enzyme activity, and there is limited capacity to predict protein function and origin in an environment where most organisms are poorly characterised (Keiblinger *et al.* 2016).

Nutritional interactions are likely to play an important role in fungus-bacteria interactions in wood, and would be a fascinating area for future research. This is a particularly important aspect of the relationship, as the need to access nutrition frames all other aspects of life and interactions within wood. Anything that influences which organisms are best able

to exploit the available nutrients will be a key driver of the communities. Specifically, reciprocal carbon and nitrogen exchange between wood-decay fungi and diazotrophic bacteria is an intriguing possibility that could be tested using stable isotope analysis in microcosms. The same methods could be used to gain insight into how many bacteria in the wood are obtaining nutrition directly from the fungus, rather than the wood itself. Quantifying the relative contributions of fungi and bacteria to direct wood breakdown remains extremely difficult, but a judicious combination of stable isotope analysis and metaproteomics could start to give insight into this relationship.

The fungal-migratory *Paraburkholderia* isolated in the course of this research have shown interesting properties and are worthy of further investigation. In particular, it would be valuable to know if they can protect the host from ambient anti-fungal compounds or harmful bacteria, in the manner *Paraburkholderia terrae* BS001 does (Nazir *et al.* 2014). The genomes for these strains have been sequenced recently, and it will be interesting to learn more of their genomic make-up and capabilities. The number and types of secretion system present are of particular interest, as their role in migration interactions is important but not entirely clear (Haq *et al.* 2016; Yang *et al.* 2016; Nazir *et al.* 2017).

7.6 Conclusions

Fungi profoundly influence the establishment and development of bacterial communities. The presence and identity of the fungus within a woody resource can alter the timescale of bacterial colonisation, as well as which bacteria manage to establish successfully within the resource. Conversely, bacteria have the potential to act as controls on fungi, affecting the competitive interactions which are the major determinant of fungal community development in wood. Together, these findings indicated that fungal and bacterial community succession are tied into one another, with reciprocal influence on the dynamics of dead-wood communities.

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Appendix 1: Chapter 3 supplementary material

Protocol S3.1 R script used to query the NCBI database

Available online at https://github.com/drevansa/qPCR_primer_paper/

Appendix 2: Chapter 4 supplementary material

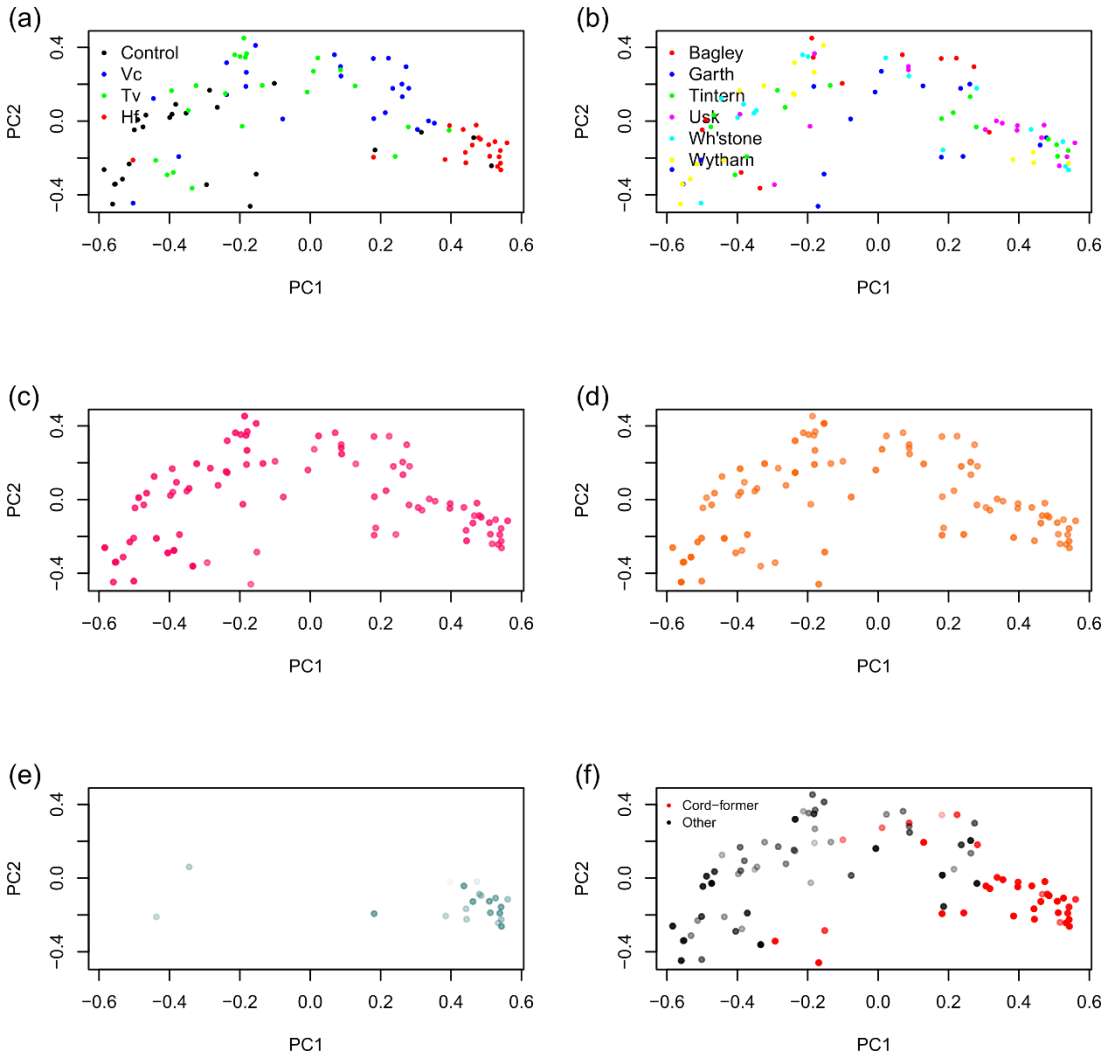

Fig S4.1. Hellinger-transformed principal components analysis of the bacterial community in fungus-colonised wood disks. Points are coloured by (a) pre-coloniser identity; (b) site of origin; (c) gradient of wood pH; (d) gradient of soil pH; (e) pre-coloniser persistence; and (f) whether the dominant fungal OTU belonged to a cord-forming genus (transparency dictated by % of disk held by the dominant OTU).

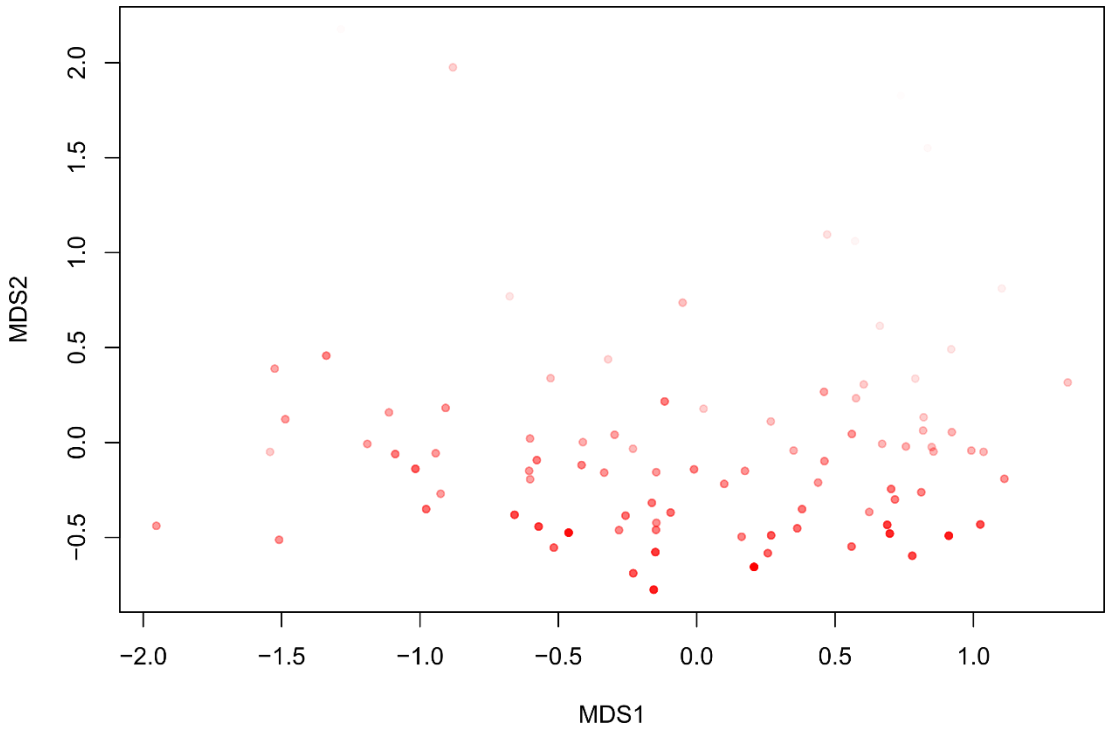

Fig S4.2 NMDS ordination of the bacterial community in fungus-colonised wood disks, with points coloured on a gradient by sequencing depth.

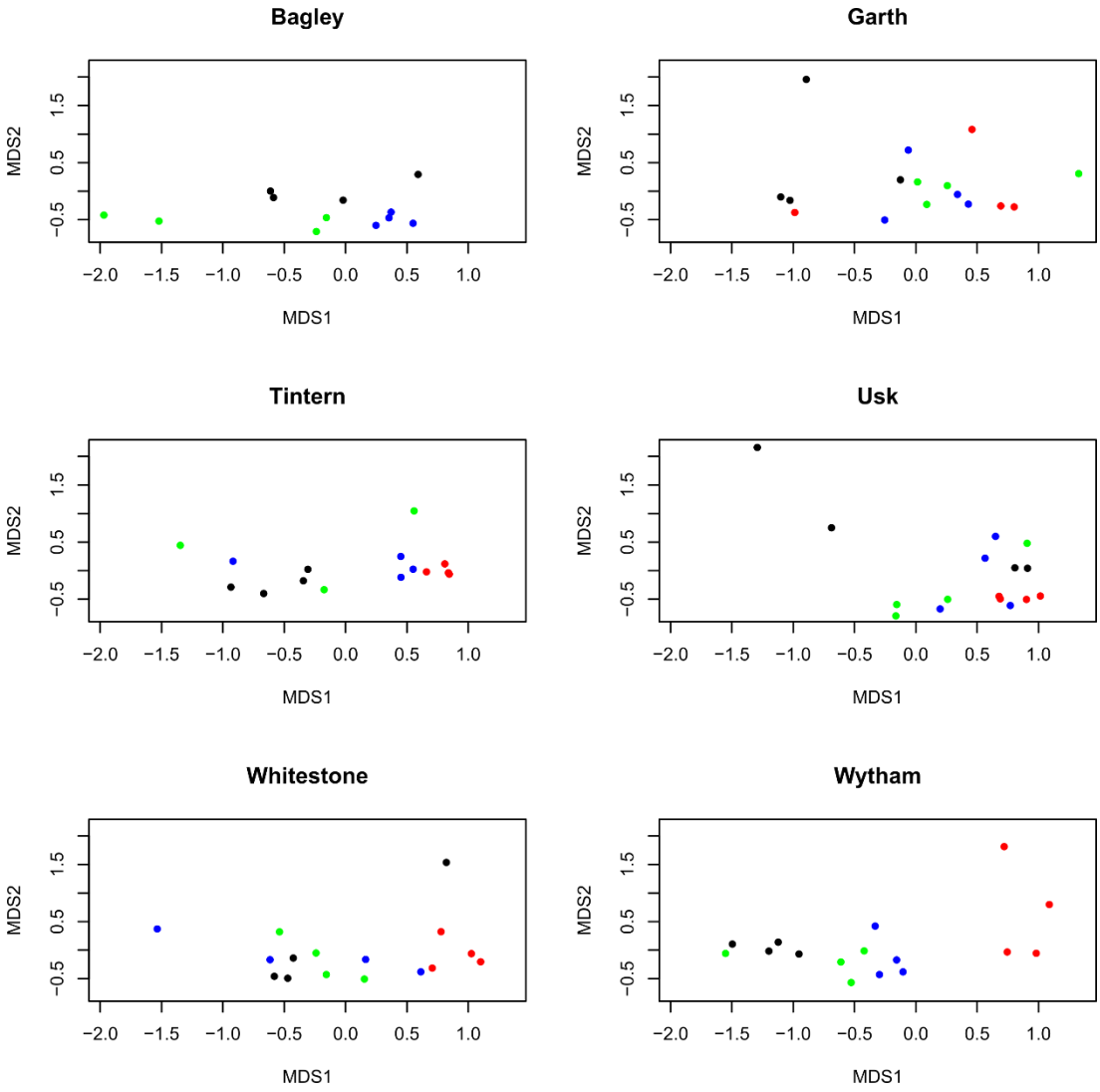

Fig S4.3 NMDS ordination of the bacterial community in fungus-colonised wood disks, coloured by pre-coloniser and panelled by site. Black points are control disks, blue are pre-colonised by *V. comedens*, green are pre-colonised by *T. versicolor*, and red are pre-colonised by *H. fasciculare*. Note that the greatest separation by pre-coloniser occurs on the Wytham site, in keeping with the results of Hiscox *et al.* (2016).

Protocol S4.1. R markdown of the code used to produce the analysis in Chapter 4

Available online at https://github.com/ecologysarah/SR-Johnston-thesis

Appendix 3: Chapter 5 supplementary material

Table S5.1 Number of replicates per treatment from which bacteria could be amplified successfully.

Season	Pre-coloniser	Days	Bacteria present (no. samples)	% samples with bacteria present
Autumn	C	14	10	83
Autumn	C	84	11	100
Autumn	Vc	14	0	0
Autumn	Vc	84	12	100
Autumn	Tv	14	0	0
Autumn	Tv	84	2	17
Autumn	Hf	14	0	0
Autumn	Hf	84	5	42
Spring	C	14	8	67
Spring	C	84	10	100
Spring	Vc	14	1	8
Spring	Vc	84	4	33
Spring	Tv	14	2	20
Spring	Tv	84	3	23
Spring	Hf	14	0	0
Spring	Hf	84	7	64

Abbreviations: C stands for control samples, Vc pre-colonised with V. comedens, Tv pre-colonised with T. versicolor, Hf pre-colonised with H. fasciculare.

Fig S5.1 Pair of blocks wired together and pegged to the forest floor. A metal forestry tag identifies the pair with a unique number. Symbols on the blocks are burnt on with a pyrography iron, identifying to which precoloniser treatment they belong.

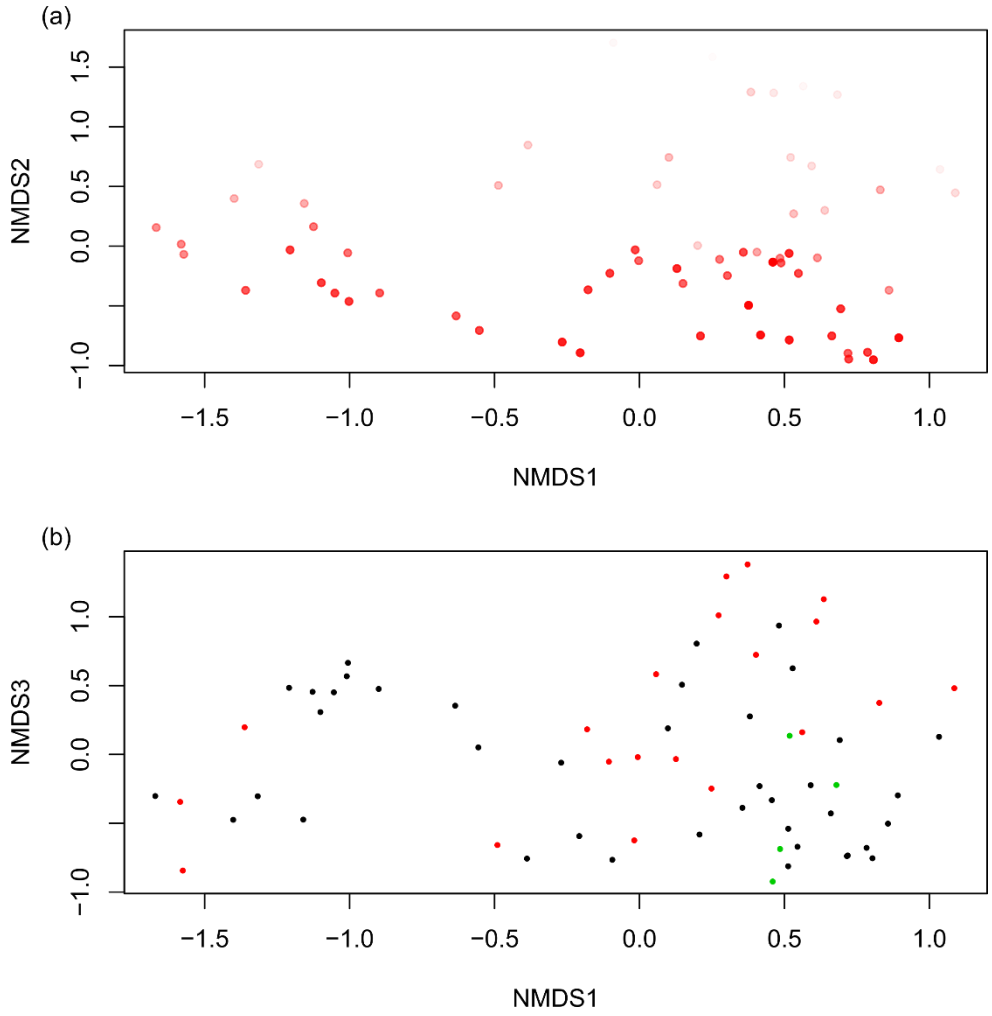

Fig S5.2 Hellinger-transformed principal components analysis of the bacterial community in fungus-colonised wood disks. Points are coloured by: (a) sequencing depth or (b) extraction kit lot number. Note that (a) and (b) are on different axes.

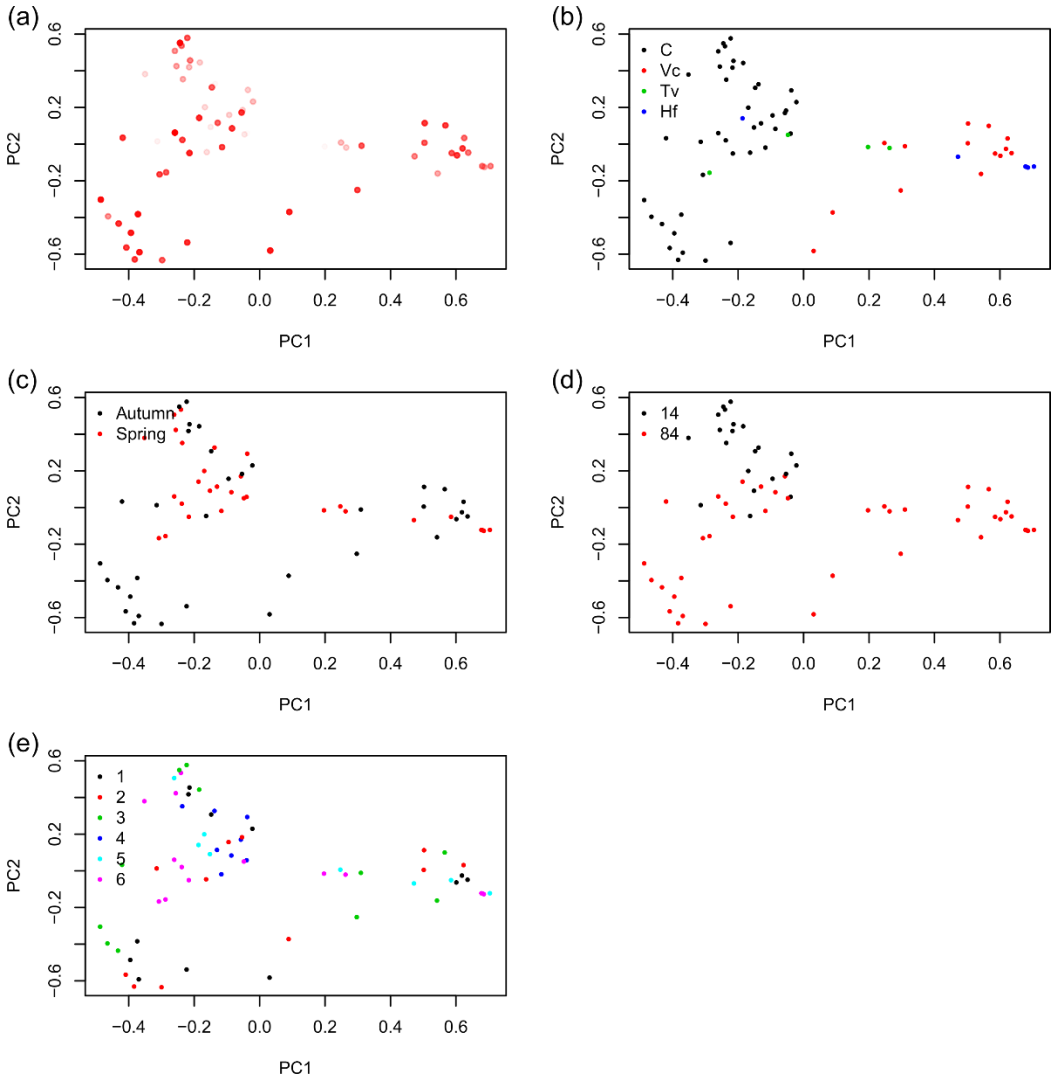

Fig S5.3 NMDS ordination of the bacterial community in fungus-colonised wood blocks, coloured by sequencing depth and extraction kit. Points are coloured by (a) sequencing depth; (b) pre-coloniser identity; (c) season of field incubation; (d) length of time in field; (e) set-out date (1-3 represent autumn set-outs, 4-6 are spring).

Protocol S5.1. An R markdown file of R code used to create the analysis in Chapter 5

Available online at https://github.com/ecologysarah/SR-Johnston-thesis

Appendix 4: Chapter 6 supplementary material

Protocol S6.1 An R markdown file of R code used to create the analysis in Chapter 6

Available online at https://github.com/ecologysarah/SR-Johnston-thesis