

Mycorrhizal fungal biomass and scavenging declines in phosphorus-impooverished soils during ecosystem retrogression



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ABSTRACT

Mycorrhizal fungi enhance plant phosphorus (P) acquisition via their extraradical hyphae (ERH) that scavenge nutrients outside root depletion zones. While soil P availability declines during ecosystem retrogression, how ERH biomass and scavenging vary during ecosystem retrogression remains unknown; it is expected to increase if plants allocate more carbon (C) to mycorrhizal fungi as P availability declines. We measured fungal and bacterial biomass using in-growth cores and lipid biomarkers along a 2-million-year dune chronosequence in an Australian biodiversity hotspot showing a ~60-fold decline in total soil P concentration with increasing soil age. We compared the levels of key fungal biomarkers (ergosterol, NLFA 16:1 ω 5, and PLFA 18:2 ω 6,9) between closed, mesh, and open cores during five months (four sampling dates including the wet winter months), thus allowing us to also determine the dynamics of mycorrhizal fungal scavenging. We found strikingly low and declining biomass of ERH with declining P availability, with minimal long-distance scavenging by ERH. Biomass of ERH was highest in the younger (c. 1 ka) soils that were comparatively rich in P and other nutrients. By contrast, the oldest, most P-impooverished soils had the lowest biomass of ERH, despite high mycorrhizal root colonisation, and high abundance and diversity of potential plant hosts. We show that extremely low P availability constrains ERH biomass. Such low mycorrhizal fungal biomass highlights the need for a more 'myco-centric' view of plant–mycorrhizal relationships in old, severely P-impooverished ecosystems.

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1. Introduction

Mycorrhizal fungi form mutualistic associations with plant roots and contribute significantly to plant nutrition by efficiently scavenging poorly-available soil nutrients, especially phosphorus (P) (Smith and Read, 2008). Arbuscular mycorrhizal (AM) fungi colonise roots of the vast majority of plant species (~80%) in most ecosystems (Brundrett, 2009). They improve P uptake (van der Heijden et al., 2006; Smith et al., 2015), and under certain conditions they can also contribute to nitrogen (N) uptake (Hodge et al., 2010; Whiteside et al., 2012). By contrast, ectomycorrhizal (ECM) fungi colonise fewer plant species (mostly woody), but enhance plant acquisition of inorganic and organic forms of N and P and can

release carboxylates, explaining their greater carbon (C) costs for the plant compared with that of AM (Smith and Read, 2008; Cairney, 2011). The benefits conferred by mycorrhizal fungi for plant nutrient acquisition are partly attributable to their ability to forage well beyond root depletion zones (Owusu-Bennoah and Wild, 1979; Li et al., 1991). Hence, the most-important component of the mycorrhizal fungi, from a functional perspective, is extraradical hyphae (ERH) exploring the soil matrix (Smith and Read, 2008). Although the ecology of ERH has received some attention (Koide and Kabir, 2000; Johnson et al., 2010; Cairney, 2012), current knowledge is biased towards younger forested ecosystems with relatively high levels of soil P (Vitousek et al., 2010), and where plant growth is generally limited by N rather than P availability (Elser et al., 2007).

Important changes in soil N and P availability occur during long-term (i.e. tens to hundreds of thousands of years) soil and ecosystem development. Plant productivity is initially N-limited on

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young soils, because most rocks contain negligible amounts of N, and N enters young ecosystems primarily through biological N₂ fixation. By contrast, P becomes increasingly limiting in older soils as its amount and availability decline through time (Walker and Syers, 1976; Vitousek and Farrington, 1997; Laliberté et al., 2012). Eventually, severe P limitation in old, strongly-weathered soils leads to ecosystem retrogression, whereby rates of ecosystem processes such as primary productivity decline (Wardle et al., 2004; Peltzer et al., 2010; Turner and Condon, 2013). These important changes in soil nutrient availability during ecosystem development may also impact the productivity of soil microbiota, but no studies have yet explored how productivity of ERH varies during ecosystem retrogression, despite the great functional importance of ERH for plant nutrition, especially with regard to P.

Long-term soil chronosequences (i.e. soil age gradients) offer great potential to better understand how soil nutrient availability influences the productivity of plant and associated microbiota during ecosystem retrogression (Peltzer et al., 2010; Walker et al., 2010; Laliberté et al., 2013b). This is because soil chronosequences maximise variation in one factor (i.e. soil age and associated changes in nutrient availability) while minimising variation in other important factors such as parent material, climate and topography (Walker et al., 2010). Yet despite their potential as natural soil nutrient availability gradients, well-studied long-term soil chronosequences have rarely been used in studies of mycorrhizal associations (Dickie et al., 2013; Martínez-García et al., 2014; Clemmensen et al., 2015), particularly with regard to functional aspects such as fungal productivity. The production of ERH by mycorrhizal fungi and scavenging is expected to be more prominent in nutrient-poor soils, if plants allocate more carbon (C) to mycorrhizal symbionts under P limitation (Smith and Read, 2008). Yet, these predictions are mostly based on studies conducted in the ecosystems with orders of magnitude higher soil P concentrations than those found in older, strongly-weathered soils, such as those in south-western Australia (Turner and Laliberté, 2015) and some lowland tropical rainforests (Fyllas et al., 2009). Consequently, there is a need for studies of functional aspects of mycorrhizal associations during ecosystem retrogression and associated declines in soil P.

Production of mycorrhizal ERH has been investigated *in situ* using fungal in-growth cores (Wallander et al., 2001; Hendricks et al., 2006; Wallander, 2006). This approach estimates the production of ERH per volume of soil, by including a series of different-size mesh-bags filled with root-free substrate (Wallander et al., 2013). The method relies on the use of fungal-specific biomarkers such as ergosterol and certain phospholipid and neutral-fatty acids (PLFAs and NLFAs) to estimate ECM and AM fungal biomass (Frostegård et al., 2011; Wallander et al., 2013; Kandeler, 2015). Specifically, ergosterol concentrations in soil can represent all soil-borne fungi, whereas the NLFA 16:1 ω 5 and PLFA 18:2 ω 6,9 can be used as a proxy to quantify AM and ECM fungal biomass in soils (Lekberg et al., 2012; Wallander et al., 2013; Kandeler, 2015). However, while this method has been successfully used in younger, N-limited ecosystems in the northern hemisphere, we know little about the ecology and functioning of mycorrhizal ERH in old, strongly-weathered soils where P strongly limits plant productivity. Interestingly, the plant communities found on these P-impooverished soils can be very species-rich (Laliberté et al., 2014; Zemunik et al., 2015), such as the biodiverse tropical or Mediterranean ecosystems that contain much of the Earth's plant diversity (Myers et al., 2000; Laliberté et al., 2013a). Therefore, estimates of the production of ERH are not only needed to better understand ecosystem-level C balance, but could help us better understand the belowground mechanisms maintaining plant diversity in these ecosystems (Laliberté et al., 2013a, 2015). For example, only plants

hosting mycorrhizal fungi with efficient ERH scavenging strategies may sustain their growth and fitness to remain competitive, since there is a tendency towards giving-up the 'services' of mycorrhizal fungi as exemplified by a plant community more dominated by non-mycorrhizal plants (Lambers et al., 2008, 2014) and less diverse AM fungal communities in the most impoverished soils (Krüger et al., 2015).

Besides ERH productivity and scavenging, we know little about changes in mycorrhiza-associated soil bacteria during ecosystem retrogression. Soil fungal and bacterial communities interact intensively in a fungal–soil interface, called the mycorrhizosphere, where sharp gradients of nutrients occur (Johannsson et al., 2004). Movements of C from plant roots or litter into the mycorrhizosphere represent the main energy source of mycorrhiza-associated bacterial communities. Thus, it is expected that productivity in plant and belowground communities are correlated, unless the productivity of belowground communities is limited more strongly by soil nutrient availability outside the mycorrhizosphere than by C availability. Bacteria–fungi interactions in the mycorrhizosphere can modify P dynamics in soils (Warmink and van Elsas, 2008; Richardson et al., 2011); therefore, these interactions could have important implications for plant nutrient acquisition. There remain some fundamental questions concerning the role of these bacteria in relation to ERH productivity in P-impooverished soils that need to be addressed. For example, are ERH and bacterial biomass correlated or are bacterial communities more responsive to the presence of roots regardless of scavenging ERH?

The overall aim of this study was to better understand the functional ecology of mycorrhizal fungi (e.g., ERH biomass and scavenging) and associated bacteria during ecosystem retrogression. To achieve this, we set-up a field experiment with fungal in-growth cores in a hyperdiverse Mediterranean region in south-western Australia (Hopper and Gioia, 2004) along a well-studied, retrogressive 2 million-year old coastal dune chronosequence (Hayes et al., 2014; Laliberté et al., 2014; Turner and Laliberté, 2015) showing strong and clear shifts from N to P limitation of plant growth with increasing soil age. Specifically, we aimed to: i) quantify mycorrhizal root colonisation and ERH biomass; ii) quantify the extent of mycorrhizal fungal scavenging ability via fungal in-growth cores; iii) relate the soil nutrient availabilities to the observed mycorrhizal fungal and bacterial biomass; and iv) determine the impact of soil nutrient availability on fungal and bacterial community composition, using lipid biomarkers. Our main hypothesis was that there would be the greatest ERH biomass and scavenging in the oldest, most-severely P-impooverished soils, if plants allocate more C to mycorrhizal fungi under increasingly severe P limitation.

2. Materials and methods

2.1. Study system

We conducted the study along the Jurien Bay >2-million year dune chronosequence (Laliberté et al., 2012, 2013b; Turner and Laliberté, 2015). The Jurien Bay dune chronosequence is located in south-western Australia (~200 km north of Perth), one of the world's 25 biodiversity hotspots (Myers et al., 2000; Hopper and Gioia, 2004). The chronosequence runs roughly parallel to the coast, is approximately 15 km wide, and comprises a series of three main dune systems of marine origin (McArthur and Bettenay, 1974; Wyrwoll and King, 1984; Turgay and Nonaka, 2002). This chronosequence forms an exceptionally strong soil nutrient availability gradient (Laliberté et al., 2012; Turner and Laliberté, 2015), and shows shifts from N to P limitation of plant growth (Laliberté et al., 2012; Hayes et al., 2014) that are expected to occur during long-

term pedogenesis (Vitousek and Farrington, 1997; Wardle et al., 2004; Peltzer et al., 2010). The climate is Mediterranean (hot, dry summers and wet, cool winters) with annual precipitation of ~570 mm, of which about 80% falls between May and September. There is no significant precipitation gradient over the entire 15-km chronosequence. Annual average temperature is 25 °C (Australian Bureau of Meteorology, www.bom.gov.au/climate/data). Vegetation is characterised by shrublands with high levels of woody plant diversity at all spatial scales (Laliberté et al., 2014; Zemunik et al., 2015). Fire is the main disturbance to vegetation, with fire-return intervals <30 years (Griffin and Burbidge, 1990; Enright et al., 2005).

2.2. Selection of chronosequence stages

For quantification of ERH biomass, we selected the three most distinct chronosequence stages, in terms of soil nutrient availability, that have been described and used in previous studies (Laliberté et al., 2014; Turner and Laliberté, 2015; Zemunik et al., 2015), out of a total of six dune stages that were outlined. These three distinct stages differ strongly in age, soil nutrient availability, and the strength and type of nutrient limitation (Table 1), and together represent the retrogressive phase of ecosystem development along this sequence (Laliberté et al., 2012; Turner and Laliberté, 2015). A comprehensive overview of soil characteristics for the entire chronosequence can be found in Turner and Laliberté (2015). Details on plot selection and delineation of the chronosequence stages are given elsewhere (Laliberté et al., 2014; Turner and Laliberté, 2015; Zemunik et al., 2015) and overall rationale for using the Jurien Bay chronosequence, these three stages, and five plots per stage is presented in the Appendix A (Notes S2).

2.3. Flora surveys

Comprehensive flora surveys were conducted on 10 permanent plots per chronosequence stage (each plot = 10 m × 10 m), between August 2011 and March 2012, as described by Laliberté et al. (2014) and Zemunik et al. (2015). In brief, we estimated canopy cover and number of individuals for each plant species within seven randomly-located 2 m × 2 m subplots within each larger 10 m × 10 m plot. Within each subplot, all vascular plants were identified to species level (whenever possible) and counted.

2.4. Root sampling for mycorrhizal colonisation and frequency of cluster roots

All live roots (estimated visually, based on colour and turgescence) were retrieved from the surface soil samples (0–20 cm

depth) collected from each 2 m × 2 m subplot after sieving through a 2-mm screen. We collected and bulked roots at the 10 m × 10 m plot level to determine the overall levels (i.e. community-level) of colonisation by AM and ECM fungi, and the frequency of cluster roots. Mycorrhizal colonisation of the bulked root samples was quantified following a vinegar and ink clearing and staining protocol (Vierheilig et al., 1998) optimised for Australian native plants (Teste et al., 2014). Mycorrhizal colonisation of roots was quantified for all six chronosequence stages. Cluster-root frequency and AM fungi root colonisation were determined using the gridline intersect method (Giovannetti and Mosse, 1980). We considered a cluster root as any live root fragment that had >50 rootlets. For ECM fungi root colonisation, we scored all viable root tips as ECM or non-mycorrhizal (Teste et al., 2006).

2.5. Fungal in-growth cores

In April 2012, we randomly selected five replicate plots in each of the three stages described in Table 1 (out of a total of 10 plots per stage). Between April 19 and 24, 2012, fungal in-growth cores Wallander et al. (2001) were installed on the edge of each 2 m × 2 m subplot. A cardinal direction (N, S, W, E) was randomly assigned to each subplot, and the order of the cores was randomly assigned along the edge of the subplots (Figs. S1 and S2). The cores (depth = 20 cm; diameter = 7.5 cm) were made of either: (i) 50 µm nylon mesh bags (mesh with 100 µm thickness) or (ii) polyvinyl chloride (PVC) tubes (Figs. S1 and S2). Two control treatments were included, the no core with disturbance (No-CoreD) and the no core undisturbed (No-CoreU) and also see the Appendix A (Methods S1 and Notes S2) for details on the installation protocol.

The objective of installing fungal in-growth cores was to estimate fungal ERH biomass of the mycorrhizal fungi capable of exploring the soil at a distance of >100 µm (thickness of the mesh) away from plant host roots (Wallander et al., 2001; Berner et al., 2012; Wallander et al., 2013). The PVC cores only sustain the growth of saprophytic fungi, because mycorrhizal fungi and roots cannot physically access soil inside the PVC core. The mesh bags allow mycorrhizal and saprophytic fungi to enter and explore soil inside the core, but prevent access by plant roots. Finally, the controls allow all fungi to explore, in the presence of roots. To estimate the contribution of the mycorrhizal fungal component, biomass values from the PVC cores were subtracted from the mesh bag values (mesh–PVC) in some analyses (thus removing the contribution by saprophytic fungi), which assumes that the different treatments have additive effects (Wallander et al., 2001; Hendricks et al., 2006; Wallander et al., 2013) and see Fig. S1.

Table 1

Main characteristics of the three contrasting chronosequence stages at Jurien Bay (stage 2 (Young soils), 4 (Middle-aged soils), and 6 (Old soils) in Laliberté et al. (2014)). Data shown here are from the five replicate plots per stage that were used in the present study; a more complete set of characteristics and for all the stages can be found in Turner and Laliberté (2015). Values for soil chemical properties are means with 95% confidence intervals in parentheses.

Chronosequence stage (soil age)	Dune system	Parent material	Estimated soil age (ka)	(Co-) limiting Macro-Nutrient	Total N (mg kg ⁻¹)	Total P (mg kg ⁻¹)	Resin P (mg kg ⁻¹)	Organic C (%)	pH ^a
2 (Young)	Quindalup (medium)	Calcareous sand	1	N, P	35.1 (29.9–40.4)	456 (425–487)	1.90 (0.74–3.06)	1.80 (0.52–3.09)	7.9 (7.7–8.0)
4 (Middle)	Spearwood (west)	Calcareous sand	125	P	13.5 (10.0–17.0)	22 (15–29)	1.11 (0.72–1.50)	0.69 (0.42–0.96)	5.8 (5.5–6.1)
6 (Old)	Bassendean	Siliciclastic sand with smaller carbonate component	>2000	P	13.1 (10.1–16.2)	4 (1–8)	0.48 (0.40–0.56)	0.60 (0.38–0.82)	4.8 (4.4–5.2)

^a Soil pH measured with a glass electrode in a 10 mM solution of CaCl₂ (1:2 soil-to-solution ratio) for the rest of the chemical properties please see Turner and Laliberté (2015) for methodological details. Soil moisture did not vary significantly amongst soil ages (data not shown).

2.6. Soil and fungal sampling

From April 19 to 24, 2012, we sampled ~80 ml (~15 g) of soil with a mini-corer (depth = 20 cm, diameter = 2.25 cm) from the controls on each subplot (total = ~100 g) prior to and after the core installation procedures (i.e. prior-installation sampling had roots and after-installation sampling was of the sieved soil). During the first weeks of June, July and August (i.e. the wet winter months during which fungal ERH biomass is expected to be maximum in this seasonally-dry Mediterranean climate), we sampled all fungal in-growth cores with the mini-corer at different locations inside the cores. Plastic straws of a similar diameter as the mini-corer were left in holes to avoid repeated sampling in the same location. Soils were immediately placed on ice inside a cooler in the field, and then transferred within 4 h to a -20 °C freezer until further processing. On September 2, 2012, soils were promptly weighed and sieved (2 mm), then shipped under dry ice to the Institute of Soil Science and Land Evaluation at the University of Hohenheim for biochemical analyses (see details below).

2.7. Ergosterol, neutral lipid and phospholipid fatty acid analysis

Ergosterol was extracted using a modification of the method described by Djajakirana et al. (1996). Ergosterol served as a general proxy for biomass of all fungi, although excluding AM fungi (Watzinger et al., 2014). Given the good positive relationship with PLFA 18:2 ω 6,9 (Fig. S3) in the sandy nutrient-poor soils along this chronosequence we used ergosterol to better indicate all saprotrophic and ECM fungi. Extraction of lipids and separation into neutral lipid fatty acids (NLFA) and phospholipid fatty acids (PLFA) was done after Frostegård et al. (1991). Data are expressed as nmol g⁻¹ soil dry weight (dw). The NLFA 16:1 ω 5 was used as a biomarker for AM fungi and served as a proxy for AM fungal biomass (Hedlund, 2002; Ruess and Chamberlain, 2010; Ngosong et al., 2012) and it has been widely used in various ecosystems (Lekberg et al., 2012; Birgander et al., 2014; Francini et al., 2014; Mechri et al., 2014). The PLFAs i15:0, a15:0, i16:0, 16:1 ω 7, i17:0, cy17:0 and cy19:0 were considered as bacterial in origin (Frostegård and Bååth, 1996). The branched PLFAs i15:0, a15:0, i16:0 and i17:0 were regarded as gram-positive and cy17:0 and cy19:0 as gram-negative in origin (Zelles, 1999). Lipid 18:2 ω 6,9 has been assessed as a saprotrophic/ECM fungal biomarker (Frostegård and Bååth, 1996; Kaiser et al., 2010). However, there is evidence that it can serve as a good ECM fungal biomarker (Högberg et al., 2007, 2010), particularly in nutrient- and/or organic-poor soils (i.e. sands found at Jurien Bay) and if fungal in-growth cores are deployed (Wallander et al., 2013) to account for saprophytic fungi. We used 18:2 ω 6,9 as a proxy for ECM fungal biomass based on previous studies (Högberg et al., 2007; Yarwood et al., 2009; Högberg et al., 2010) and references therein that demonstrate strong correlations between this biomarker and ECM fungi biomass. Furthermore, we also found many ECM fungal taxa in the mesh cores at the same sites (Table S5), thus we have good evidence to suggest that the use of PLFA 18:2 ω 6,9 as a good proxy for estimating ECM fungal biomass in our study system was justified.

Our on-going mycorrhizal fungal community ecology studies (Krüger et al., 2015; Teste et al., unpublished results) using next generation DNA sequencing support the use of NLFA and PLFA as proxies for the estimation of AM and ECM fungal biomass since the soil in the mesh cores had many relatively common AM and ECM fungal taxa (Tables S4 and S5). It should be noted that if spores represent a substantial component of the fungal biomass in the soils of this Mediterranean ecosystem, the detection of ERH scavenging should not be invalid since the spore biomass signal would

simply be 'additive' to the biomass of the ERH according to our experimental design (see above and Notes S2). Finally, the issue of variable amounts of background spore biomass that can be a concern when aiming to detect ERH biomass (Wallander et al., 2013), was not a concern since this potential variation was removed by our experimental approach given that we pooled and homogenised seven soil subsamples within the same plot (see above and Notes S2).

2.8. Soil analyses

Soil nutrient concentrations were determined by Turner and Laliberté (2015) on the same samples from which roots were extracted for overall mycorrhizal colonisation and cluster-root frequency. All methods are described in Turner and Laliberté (2015) and are summarised in the Appendix A (Methods S1).

2.9. Data analysis

The data were analysed as a partly nested design (i.e. split-plot design) (Quinn and Keough, 2002) with the main factor being soil age (levels: young, middle-aged or old) and the nested factor being the fungal in-growth cores (referred to as 'core' hereafter; levels: PVC, Mesh, No-CoreD, No-CoreU). The 'soil age' and 'core' factors were set as the fixed effects, and sampling plots were set as the random factor. When we included sampling date (month of sampling) in the analyses, data were analysed as a repeated measures partly nested design (Quinn and Keough, 2002). We used a linear mixed-effects model to fit the data to test for differences in fungal biomass with plots set as the random factor, with soil age and core as the fixed effects. Model assumptions (normality and homogeneity of variance of residuals) were assessed graphically in R (R Core Team, 2015) and with ggplot2 (Wickham, 2009). The overall AM or ECM root mycorrhizal colonisation levels and frequency of cluster roots were analysed as proportion data with generalised linear models. Linear and non-linear regressions were used to determine the relationships between fungal biomass and soil chemical properties at the plot level. More details on the data analyses can be found in Appendix A (Methods S1).

3. Results

3.1. Extraradical hyphal biomass

Fungal biomass, expressed as ergosterol concentrations, was significantly greater in the no-core treatments (No-CoreD and No-CoreU) compared with the mesh and PVC (Fig. 1a) across all soil ages. In addition, fungal biomass in the No-CoreU treatment was slightly, but significantly, greater than that in the No-CoreD treatment (Fig. 1a). Differences in AM (NLFA 16:1 ω 5; Fig. 1b) and ECM fungal biomass (PLFA 18:2 ω 6,9; Fig. 1c) among core treatments varied with soil age (NLFA 16:1 ω 5 and PLFA 18:2 ω 6,9 core treatment \times soil age interaction; $P = 0.008$; $P < 0.001$, respectively). There was consistently more AM and ECM fungal biomass in the young soils than in the old soils at all levels of the core treatment except for the PVC cores (Fig. 1b, c). In particular, AM fungal biomass in the old soils, for the two no-core treatments, was consistently lower compared with that in the young and middle-aged soils (Fig. 1b). Finally, ECM fungal biomass in the old soils was the same as that in the middle-aged soils across all core treatments (Fig. 1c). The NLFA 16:1 ω 5 to PLFA 18:2 ω 6,9 biomarker ratio was significantly higher in the PVC and No-CoreU treatments compared with that in the mesh and No-CoreD, although differences were small (Fig. 1d).

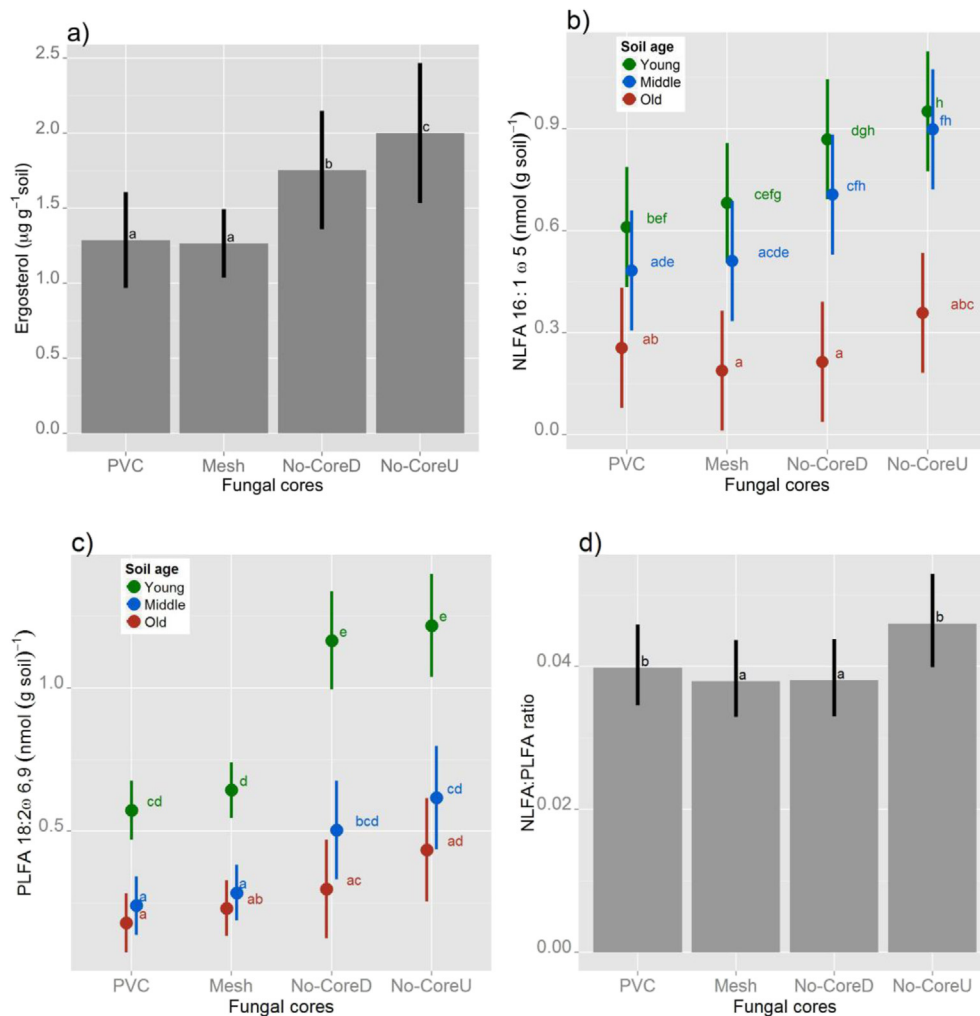


Fig. 1. Soil fungal biomass as measured with the a) ergosterol biomarker, arbuscular mycorrhizal (AM) fungal biomass measured with the b) neutral lipid fatty acid (NLFA) biomarker (16:1 ω 5) and ectomycorrhizal (ECM) fungal biomass measured with the c) phospholipid fatty acid (PLFA) biomarker (18:2 ω 6,9) and d) soil fungal biomass ratio NLFA:PLFA, in the fungal in-growth core treatments across all sampling dates at the Jurien Bay dune chronosequence. Bargraphs show means with 95% confidence intervals (CIs) while graphs with coloured CIs indicate there was a significant interaction between soil age and the fungal in-growth core treatment. Different letters indicate statistically significant differences between means (Tukey HSD tests with $P \leq 0.05$).

3.2. Extraradical hyphal scavenging

We found some evidence for seasonal- and soil-age specific ERH scavenging (mesh-PVC, Fig. 2, Fig. S4). In particular, there was greater AM fungal scavenging in the young soils compared with that in the old soils, but only in August ($t = 3.25$, $P = 0.012$; Fig. 2a, Fig. S4). In addition, there was significant ECM fungal scavenging across all soil ages, but also only in August (Fig. 2b). However, when testing for the main effect of the fungal in-growth cores, we found that there was no overall difference in mycorrhizal fungal biomass between the mesh and PVC treatment (NLFA 16:1 ω 5: Fig. 1b; PLFA 18:2 ω 6,9: Fig. 1c), suggesting that there was no long-distance (>100 μ m) extraradical hyphae (ERH) scavenging (measured as mesh–PVC). Indeed, even in the oldest, most P-impoverished old soils, AM and ECM fungal biomass did not differ among core treatments (Fig. 1b, c).

3.3. Comparison of fungal biomass in soils of other biomes

Overall, fungal biomass was less in the old soils compared with that in the young soils (Fig. 3, Table S3). In particular, AM and ECM fungal biomass showed much lower levels in the old soils

compared with that in the young and middle-aged soils (Fig. 3). In general, AM and ECM fungal biomass levels found in other ecosystem types or biomes, particularly boreal forests, were much greater than those found in our study system (Fig. 3).

3.4. Fungal biomass and soil nutrient concentrations

We found positive curvilinear relationships between the fungal biomarkers and readily-exchangeable soil P across all treatments (Fig. 4, Fig. S5). These positive relationships were more pronounced in the mesh treatment (Fig. 4). Similar relationships were found only between the NLFA and PLFA biomarkers (i.e. not ergosterol) and soil pH (Fig. S6). We also found positive relationships between ECM fungal biomass and soil dissolved organic N for all core treatments and when the mesh treatment was analysed separately (Fig. 5). No other fungal biomarkers showed any significant relationship with soil N (data not shown).

3.5. Seasonality of soil biomarkers

Fungal biomass in soil based on ergosterol was low in April, before the start of the wet winter months and remained

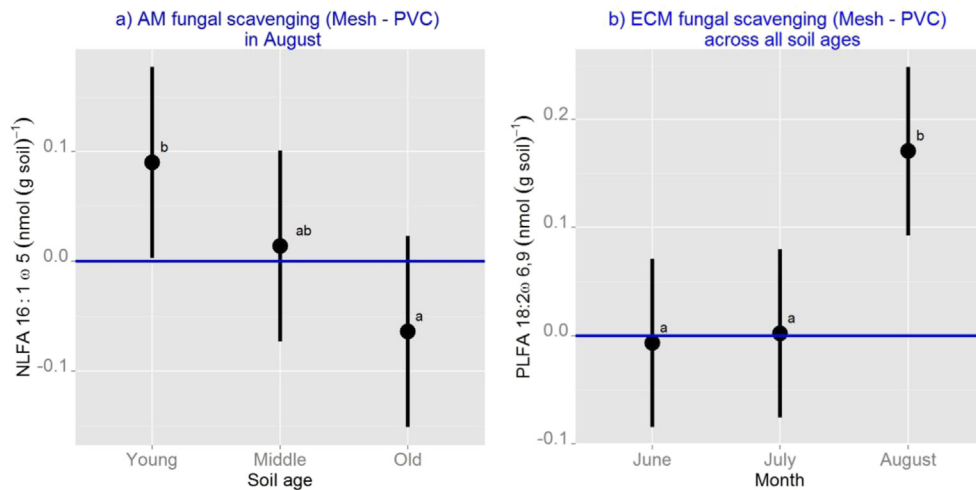


Fig. 2. Fungal biomass differences between the mesh and PVC fungal in-growth core treatments during the rainy winter months in soils at the Jurien Bay dune chronosequence. Error bars are 95% confidence intervals (CIs) from mixed-effect models. If the CIs are greater than, and do not overlap, zero, then this suggests statistically significant scavenging beyond $>100 \mu\text{m}$ occurred. The arbuscular mycorrhizal (AM) fungal biomass (a) was measured as neutral lipid fatty acid NLFA biomarker (16:1 ω 5) while the ectomycorrhizal (ECM) fungal biomass (b) was measured with the phospholipid fatty acid (PLFA) biomarker (18:2 ω 6,9). Different letters indicate statistically significant differences between means (Tukey HSD tests with $P \leq 0.05$).

consistently higher onwards (Fig. 6a). Biomass of AM fungi in soil, estimated with the NLFA 16:1 ω 5 biomarker, was high in April, peaked in June, at the start of the rainy winter season, and then declined in July (Fig. 6b). The relative abundance of AM fungi, estimated from the ratio of NLFA and ergosterol, varied across the season (i.e. peaked in June), but was not influenced by root and hyphal exclusion, since we found no significant effect of the core treatments on this ratio (Fig. S7). Fungal biomass of ECM fungi in soil, across all the fungal in-growth core treatments, did not vary throughout the sampling months (Fig. 6c).

3.6. Effects of plant composition on fungal biomass

We found a positive linear correlation between fungal biomass estimated from ergosterol and percent ECM plant canopy cover across all core treatments (Fig. S8). A stronger correlation was found between the ECM fungal biomarker PLFA 18:2 ω 6,9 and ECM plant cover (Fig. S8). By contrast, arbuscular mycorrhizal host plant richness, diversity, or percent canopy cover showed no relationships with AM fungal biomass across the different soil ages (data not shown).

3.7. Mycorrhizal colonisation and frequency of cluster roots along the chronosequence

The highest levels of AM and ECM fungal colonisation were found on roots from the middle-aged soils (Fig. 7a). Percent root colonisation by AM fungi showed a sharp increase in the middle-aged soils, and was significantly greater than levels found in all other soil ages (Fig. 7a). The young soils had the lowest levels of AM fungal colonisation, although these low levels were only significantly lower than those found in the middle-aged and old soils (Fig. 7a). Colonisation by ECM fungi showed a gradual increase with soil age, reaching the highest levels in the middle-aged soils, although this was not statistically different from the older soils (Fig. 7b). The frequency of cluster roots found in these community root samples also showed a gradually increasing trend with soil age, with the highest absolute values found in the older soils (Fig. 7c).

3.8. Bacterial biomass associated with extraradical hyphal scavenging

The bacteria-specific PLFA biomarkers showed a similar interaction (core treatment \times soil age interaction; $P < 0.001$) and trend seen with the ECM fungal biomass (Figs. 8 and 1c). Bacterial biomass was greater in the young soils compared with that in the other soil ages (Fig. 8). Furthermore, in the young and middle-aged soils, the no-core treatments had greater bacterial biomass than the PVC or mesh treatments (Fig. 8), whereas in the old soils, the bacterial biomass was similarly low across all fungal in-growth core treatments (Fig. 8). We did not find greater bacterial biomass (Fig. S4) associated with greater ERH scavenging (Fig. 2).

4. Discussion

4.1. Extraradical hyphal scavenging by mycorrhizal fungi during ecosystem retrogression

Our main finding was that declining soil P availability in the oldest soils during ecosystem retrogression constrained mycorrhizal fungal extraradical hyphae (ERH) biomass and scavenging. The ERH scavenging that was recorded was likely of very short distance away from roots and occurred only in specific soil ages and only towards the end of the most favourable time of the year in terms of precipitation (August). This key finding is intriguing, given that plant roots were relatively well colonised by AM and ECM fungi and that the most nutrient-impoverished soils harbour the greatest host plant diversity and significant host plant cover (Zemunik et al., 2015). We acknowledge that the declining soil pH along this chronosequence may have also limited the production of ERH. However since: i) we did not find any relationship between soil pH and ergosterol, ii) ECM fungi are not known to be inhibited by low soil pH (Smith and Read, 2008), iii) plants had relatively high levels of mycorrhizal fungal colonisation, iv) the number of mycorrhizal host plant species and relative cover remain high in the old soil with low pH (Zemunik et al., 2015), we surmise that declining soil pH probably inhibited production of ERH of AM fungi via decreased micronutrient or P availability. Our results did not support our initial hypothesis that there would be the greatest

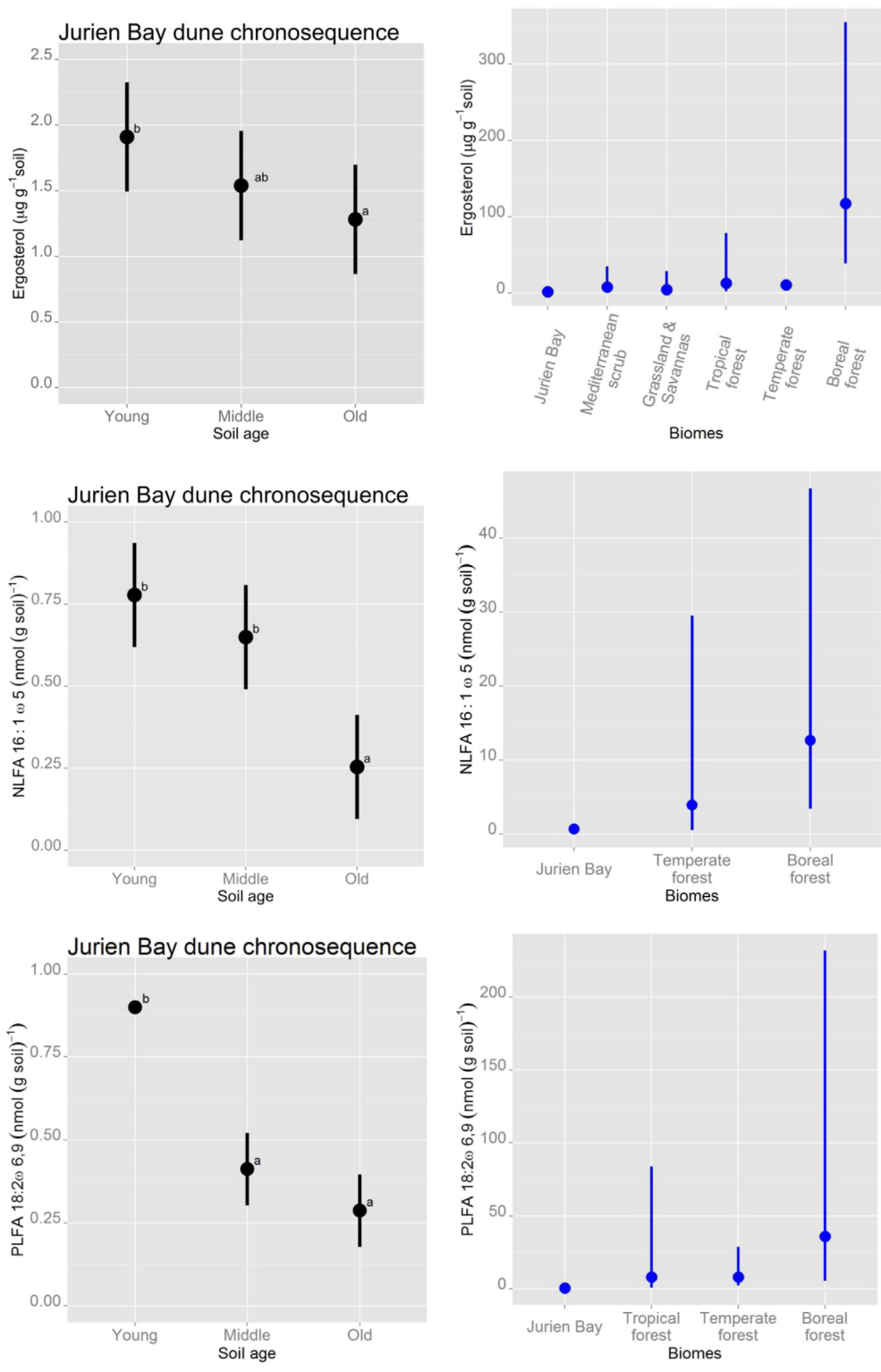


Fig. 3. Fungal biomass along the different soil ages as measured with the ergosterol biomarker, the arbuscular mycorrhizal (AM) fungal biomass measured with the neutral lipid fatty acid (NLFA) biomarker (16:1ω5), and ectomycorrhizal (ECM) fungal biomass measured with the phospholipid fatty acid (PLFA) biomarker (18:2ω6,9) for the core treatments allowing root infiltration (i.e. without PVC and mesh) during the winter months at Jurien Bay. Also shown are the fungal biomass levels found in other biomes for comparison. Values are means with 95% confidence intervals based on mixed-effects models. Different letters indicate statistically significant differences between means (Tukey HSD tests with $P \leq 0.05$).

amount of ERH biomass in the most nutrient-impoverted soils, where we had hypothesised that plants would allocate more C to their mycorrhizal symbionts. Based on previous studies (Koide and Kabir, 2000; Wallander et al., 2010; Smith and Smith, 2011), this

hypothesis seemed plausible, since the prominence of ERH would allow plants to benefit the most from mutualistic mycorrhizal fungi in these soils in terms of nutrient acquisition (Cairney, 2011). The ERH scavenging found only in August, in these seasonally dry

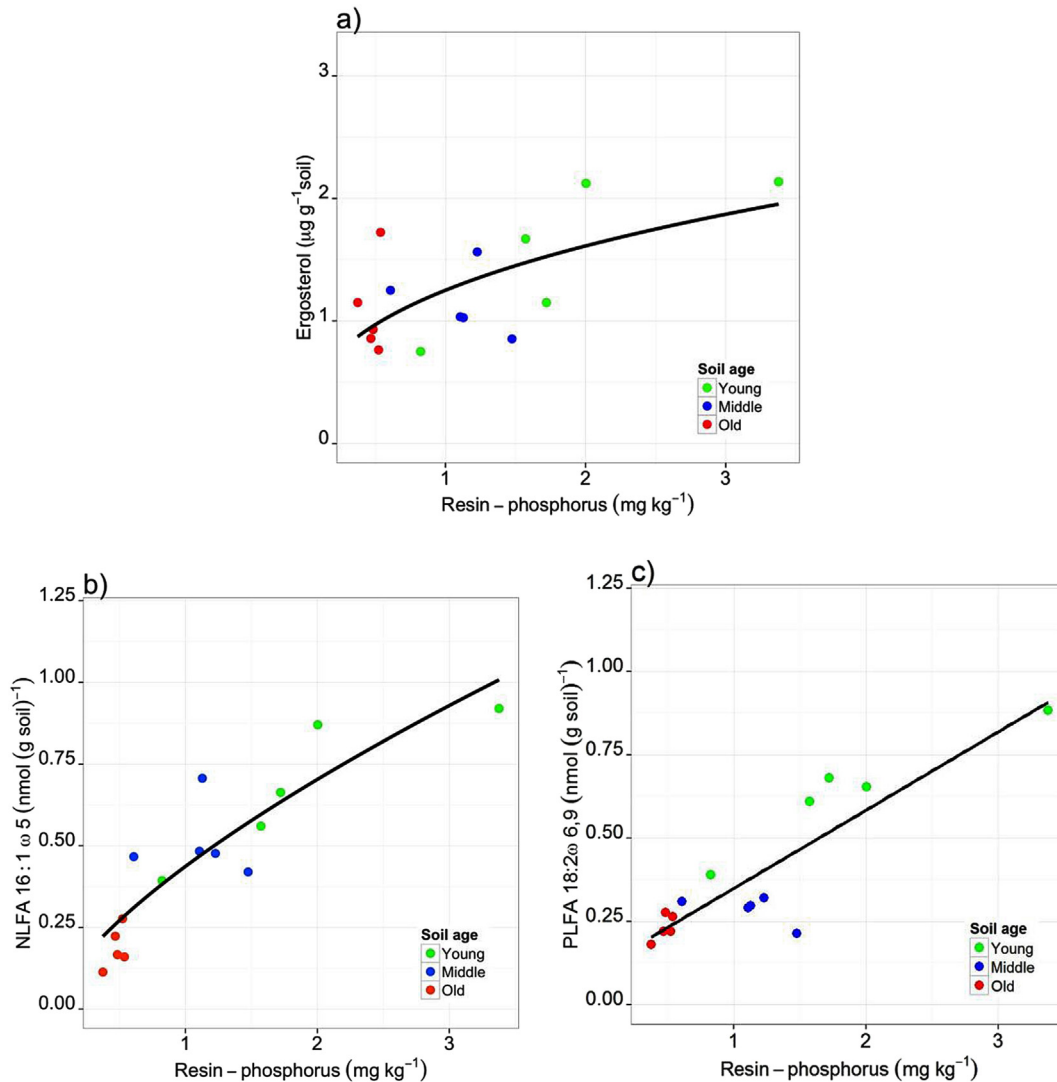


Fig. 4. Regression analyses of fungal biomarkers in soil from the mesh core treatment with readily-exchangeable soil phosphorus (P, resin P) at the Jurien Bay dune chronosequence. An exponential function was used for fungal biomass measured with the a) ergosterol biomarker ($y = 0.91 * \exp(0.26*x)$, quasi- $R^2 = 0.45$, $P < 0.002$, AIC = 15.8). A power function was used for arbuscular mycorrhizal (AM) fungal biomass measured as b) neutral lipid fatty acid (NLFA) biomarker (16:1 ω 5) ($y = 0.44 * x^{0.69}$, quasi- $R^2 = 0.80$, $P < 0.001$, AIC = -17.9). Linear regressions for the c) phospholipid fatty acid (PLFA) biomarker (18:2 ω 6,9) ($y = 0.11 + 0.23x$, $R^2 = 0.78$, $P < 0.001$, AIC = -21.3) was a better fit than non-linear regression models.

Mediterranean plant communities, suggests a modest investment in ERH only after some time of favourable wet conditions and/or root development. In our study, two ‘no-core treatments’ were included and allowed us to measure the ERH normally found in the close presence of roots (i.e. roots were sieved prior to analysis; also see [Appendix A](#) (Notes S2). Furthermore, since ERH biomass was greater in the no-cores compared with that in the mesh cores (only allowed hyphal growth), we suggest that ERH scavenging occurred, but was localised around roots.

4.2. Bacterial biomass associated with extraradical hyphae during ecosystem retrogression

The possibility that associated bacteria may have modified P availabilities in the mycorrhizosphere ([Warmink and van Elsas, 2008](#); [Brooks et al., 2011](#); [Richardson et al., 2011](#)) could be suggested via analyses of total bacterial-specific biomass and differences in community composition along the different soil ages (also see [Appendix A](#) Notes S1). Overall, bacterial biomass followed a

similar interactive pattern found with the mycorrhizal fungal biomass; differences between the fungal in-growth cores (differences generally found between the no core and PVC treatments) was only found in the young soils. Furthermore, biomass of bacterial communities as estimated from bacteria-specific PLFAs was greater in the young soil compared to the other soil ages. We expected greater bacterial biomass would be associated with cases where we found evidence for ERH scavenging, but our results did not support this hypothesis. Rapid bacterial production may occur in synchrony with the ERH burst shown in August, but higher-resolution temporal sampling would be required to detect such changes in bacterial production.

Like fungi, some bacteria are very responsive to increased nutrient availability in soil ([Kandeler, 2015](#)). [Nazir et al. \(2010\)](#) highlighted different key mechanisms that could drive bacterial selection in the mycosphere such as efficient acquisition of specific released nutrients, the capacity for flagellar movement, or the capability of forming biofilms. For example, ECM fungi influence the taxonomic composition of bacterial communities in the

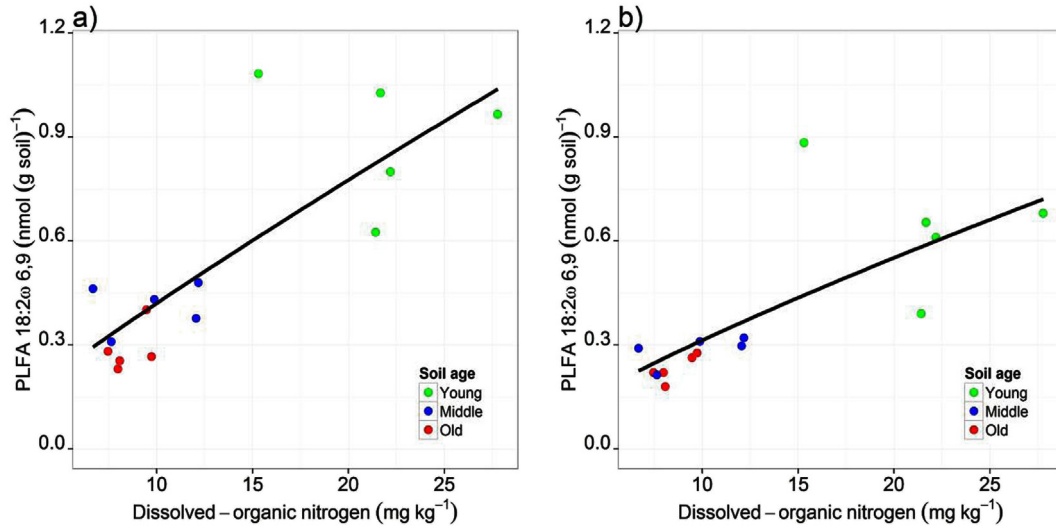


Fig. 5. Regression analyses of the phospholipid fatty acid (PLFA) biomarker (18:2ω6,9) with soil dissolved organic nitrogen (N, DON) at the Jurien Bay dune chronosequence. A power function was used for fungal biomass measured with the PLFA 18:2ω6,9 from a) all core treatments ($y = 0.05 * x^{0.88}$, quasi- $R^2 = 0.68$, $P < 0.001$, AIC = -6.0) and b) only the mesh core treatment ($y = 0.05 * x^{0.81}$, quasi- $R^2 = 0.59$, $P = 0.001$, AIC = -12.2).

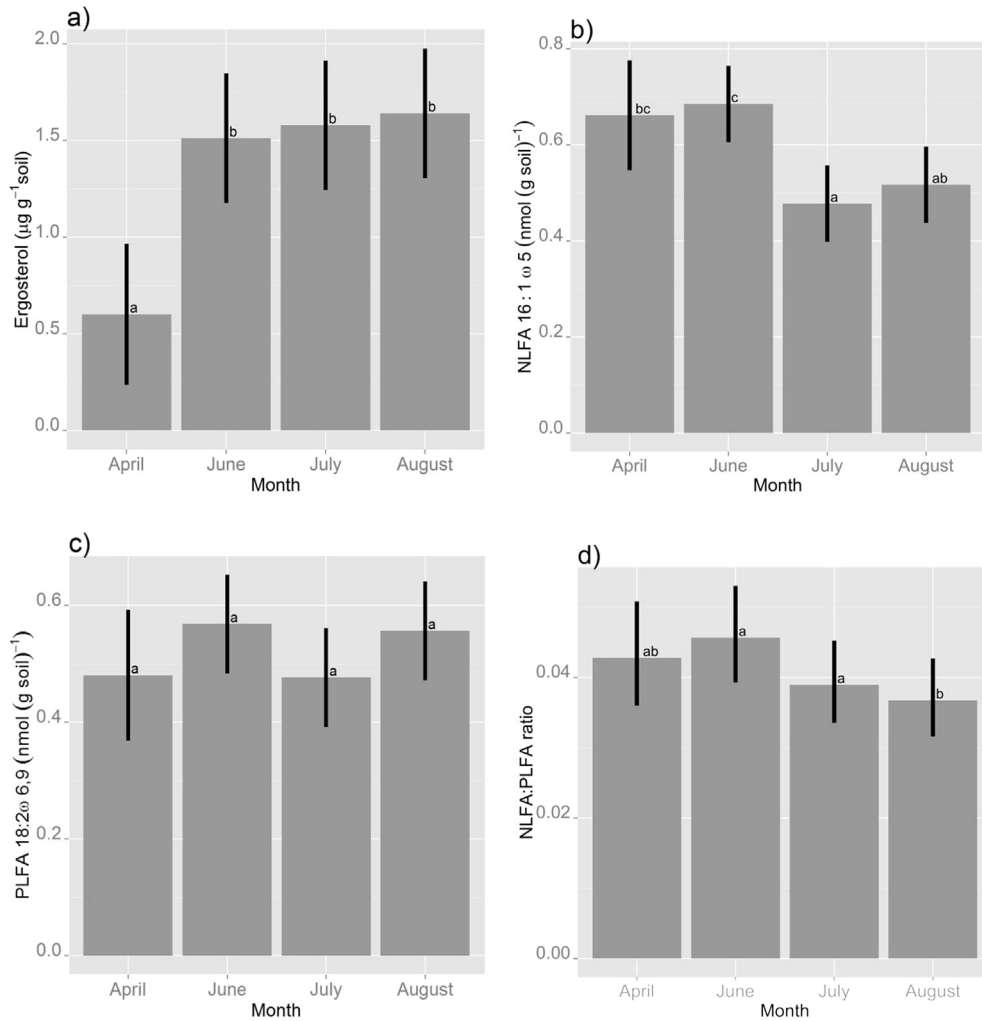


Fig. 6. Soil fungal biomass as measured with the a) ergosterol biomarker, arbuscular mycorrhizal (AM) fungal biomass measured with the b) neutral lipid fatty acid (NLFA) biomarker (16:1ω5) and ectomycorrhizal (ECM) fungal biomass measured with the c) phospholipid fatty acid (PLFA) biomarker (18:2ω6,9) and d) soil fungal biomass ratio NLFA:PLFA at each sampling date across all dunes and fungal in-growth core treatments at the Jurien Bay dune chronosequence. Bar graphs show means with 95% confidence intervals with different letters that indicate statistically significant differences between means (Tukey HSD tests with $P \leq 0.05$).

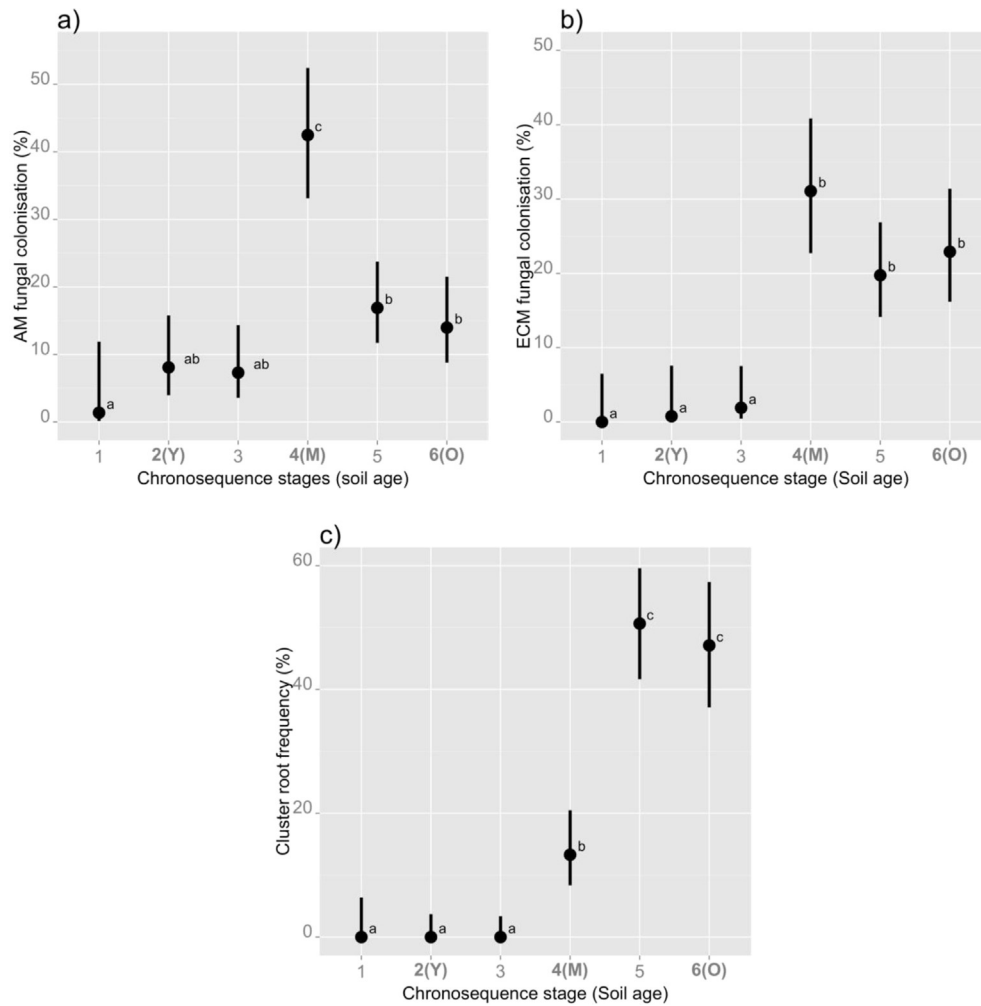


Fig. 7. Colonisation and frequency levels for a) Arbuscular mycorrhizal (AM) fungi, b) ectomycorrhizal fungi (ECM fungi), and c) non-mycorrhizal cluster roots across all six stages of the Jurien Bay dune chronosequence. Values are percent means derived from proportions with binomial 95% confidence intervals generated with the logit parameterisation. Different letters indicate statistically significant differences between means (Tukey HSD tests with $P \leq 0.05$). The six distinct chronosequence stages (1–6 in Laliberté et al. (2014)) are shown here, but for all other results in this study we focused on the three most contrasting chronosequence stages (2, 4 and 6: Young (Y), Middle-aged (M), and Old (O)) soils that are highlighted in bold.

mycorrhizosphere, especially in the close vicinity of ERH (Nurmiaho-Lassila et al., 1997). Our findings suggest that bacterial growth was associated with sites where we found more fungal biomass such as in the no-core treatments and with greater soil nutrient availability. Also, we found that many soil properties such as P availability, total [Mn], organic C, exchangeable Na, Fe and Zn were strongly correlated with the bacterial PLFA concentrations. The three soil ages had distinct bacterial community compositions, where the young soils were most different from the old soils, suggesting that soil nutrient availability is also an important factor shaping bacterial communities. The biomass of these bacteria, like fungi, was likely constrained under extremely nutrient-impooverished conditions.

4.3. Effects of soil nutrient availability on fungal and bacterial biomass

Changes in soil P and N availabilities during ecosystem development (e.g., along dune successional stages) can help explain the occurrence, biomass, and function of mycorrhizal fungi (Read, 1989), and there is good evidence that ECM fungi occur and function across a wider range of soil conditions than previously thought

(van der Heijden and Vosatka, 1999; van der Heijden et al., 1999). Therefore, we also aimed to determine the impacts of soil abiotic conditions on the biomass of mycorrhizal fungi and associated bacterial communities. The positive relationships between the concentrations of available P and N in soil and mycorrhizal fungal biomass suggest that the scavenging ability of mycorrhizal fungi becomes impaired or significantly altered at very low nutrient availability, perhaps because the growth of mycorrhizal fungi themselves becomes P-limited. The optimum curves show that mycorrhizal fungi in these severely nutrient-impooverished soils appear to operate at the far left of typical P supply vs. fungal biomass relationships found in other studies (Smith and Read, 2008; Cairney, 2011). Soil P concentrations in the oldest soils we sampled are among the lowest ever recorded (Turner and Laliberté, 2015), and our findings point to declining—rather than increasing—AM and ECM fungal ERH growth in such severely P-impooverished soils. From relevant glasshouse studies on AM and ECM fungi, the relationship between root colonisation and ERH growth at the extremely low soil P levels tested (Abbott et al., 1984; Jones et al., 1990) support our findings. Our study is the first to demonstrate these relationships in a natural ecosystem, and declining mycorrhizal fungal biomass and scavenging could be a general feature

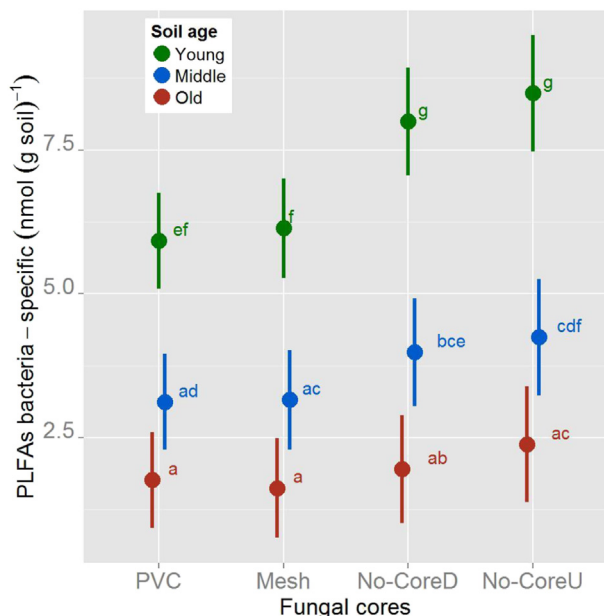


Fig. 8. Soil bacteria biomass as measured with phospholipid fatty acids (PLFAs) biomarkers (i15:0, a15:0, i16:0, 16:1 ω 7, i17:0, cy17:0, cy19:0) in the fungal in-growth core treatments across all sampling dates at the Jurien Bay dune chronosequence. Values are means with 95% confidence intervals. There was statistically significant interaction between the core treatment and soil age ($P < 0.001$). Different letters indicate statistically significant differences between means (Tukey HSD tests with $P \leq 0.05$).

during ecosystem retrogression in very old, strongly weathered soils when P availability declines to extremely low levels.

Plants that produce mycorrhizas in severely P-impoorished soils still need to acquire P and N efficiently for adequate growth; hence, we do not rule out the possibility that mycorrhizal fungi, especially in the oldest and poorest soils, possess efficient physiologies and/or have modified their scavenging strategy to perhaps resemble that of competing and frequently encountered cluster roots from the Proteaceae (Lambers et al., 2014), which are short-lived structures. We expected that the greater relative amounts of organic P (proportional to total P) found in the oldest soils (Turner et al., 2013; Turner and Laliberté, 2015) would increase the relevance of ectomycorrhizas for P acquisition due to their potentially more extensive extramatrical mycelium (Read and Perez-Moreno, 2003; Plassard and Dell, 2010; Cairney, 2011), which could have translated into greater ERH biomass and scavenging; however, our biomarker data suggest greater ERH biomass and scavenging in the younger soils that are characterised by higher N and P availability and lower relative amounts of organic P (Turner and Laliberté, 2015). These findings imply that adequate availability of key nutrients, such as P, appears important for sustained mycorrhizal fungal biomass. Nevertheless, we cannot rule out the possibility that plants may also reduce their carbon allocation to their mycorrhizal symbionts in severely P-impoorished soils, which would also be responsible for the lower fungal biomass that we observed. However, our findings point more towards the fungi themselves becoming P limited, given the relatively high mycorrhizal root colonisation levels found on the poorest soils.

4.4. Seasonality of mycorrhizal fungal scavenging and associated bacteria

Seasonality affected ergosterol concentrations in these nutrient-poor soils, indicating, as expected, that favourable moist conditions generally increased fungal production. However, ECM fungal

biomass was not affected by seasonality, nevertheless we suspect that the small increase in ergosterol after April may have been driven by active ECM fungal hyphae. For future studies, we propose quantifying free and total ergosterol in conjunction with PLFA 18:2 ω 6,9 to tease apart the active from recalcitrant ECM fungal fractions that may be relevant to C sequestration (Clemmensen et al., 2013; Koide et al., 2014). During the wet months, some level of mycorrhizal scavenging probably occurred, but likely only close to the roots, functioning much like root hairs or rootlets of cluster roots. Therefore, based on the evidence gathered in this study, we propose that a 'boom-and-bust' ERH scavenging tactic may be favoured in these seasonally-dry ecosystems, following rain events. However, some ECM fungal sporocarps appeared between June and August which provides evidence that ECM fungi were nevertheless active throughout the winter.

4.5. Ectomycorrhizal fungal biomass estimation and effects of N availability

The fungal biomass estimated by ergosterol is mostly represented by Basidiomycota and Ascomycota fungi found in soil (Kandeler, 2015). The fungal-specific PLFA 18:2 ω 6,9 biomarker is mainly represented by ECM fungi, and has a particularly strong relationship with ergosterol (Frostegård et al., 2011) which was also the case in our system. Ergosterol concentrations gradually declined from the young to the old soils, while PLFA 18:2 ω 6,9 corroborated the ergosterol pattern with soil age. The positive relationship found between ECM fungal biomass and ECM plant host cover is not associated with the greater ECM fungal colonisation levels and highest number of ECM host plant species found in the old soils (Zemunik et al., 2015). Indeed, ECM fungal biomass was greatest in the young soils where the greatest ECM cover was found, despite lower ECM host plant richness, indicating that ECM fungi were more active and perhaps scavenged more readily in the younger, more nutrient-rich soils. This evidence in conjunction with the relationship observed between PLFA 18:2 ω 6,9 and dissolved organic N for the mesh cores suggests that the biomass of ECM fungi in severely nutrient-impoorished soils is also partly driven by readily-available N. By contrast, AM fungal biomass showed no relationship with soil N variables, thus suggesting that reduced AM fungal biomass in this system was more driven by soil P availability.

4.6. Spores of mycorrhizal fungi in Mediterranean ecosystems

Since our PVC cores had detectable amounts of AM and ECM fungal biomass, viable spores likely represented a considerable component of the mycorrhizal fungal biomass in these severely nutrient-impoorished soils. Indeed, detailed lipid analyses suggests that NLFA-rich spores can represent up to 90% of the external biomass in AM fungi (Horton et al., 1998). The possibility that saprophytic fungi in the PVC cores may have been released from potential mycorrhizal-fungal competition was not supported since ergosterol (biomass of all fungi) showed similar trends to that of the mycorrhizal fungal biomass in the PVC. However, facultative saprotrophy does not appear common for ECM fungi (Lindahl and Tunlid, 2015), therefore the relatively high levels of ECM fungal biomass in the PVC cores suggest that resting bodies such as sclerotia are also important components in these soils. Furthermore, AM fungi were slightly more abundant than ECM fungi in the PVC (Fig. 1d) and the NLFA:ergosterol ratio was considerably greater in April (when roots (Pate and Bell, 1999) and fungi (Fig. 6a) are mostly inactive), thus supporting the hypothesis that a high density of mycorrhizal fungal resting spores are found in these soils. Finally, across all soil ages, the AM and ECM fungi in the PVC core that was

likely represented by spores was surprisingly high in April, the driest sampling month before the onset of winter rains; we regard this as evidence for the importance of mycorrhizal spores and dormant structures in these seasonally-dry Mediterranean ecosystems.

The possibility that the biomass due to spores may have 'overwhelmed' the detection of any ERH scavenging was not supported by our data since we found evidence for ERH scavenging but it was context dependent (i.e. only at a given date and soil age). Furthermore, our on-going mycorrhizal fungal community ecology studies suggest there were some common well-known AM and ECM fungal taxa in the mesh cores thus the ERH scavenging detected via the fungal biomarkers and our experimental setup was validated (Tables S4 and S5). Nevertheless if spores did 'overwhelm' the detection of the ERH biomass at the other sampling dates and soil ages, this simply reinforces the evidence for relatively low ERH biomass in this system.

4.7. Mycorrhizal colonisation levels at Jurien Bay and biome comparisons of fungal biomass

Arbuscular mycorrhizal fungal colonisation increased gradually from the youngest to older soils, and peaked in the middle-aged soils. It declined sharply in the old soils during the later stage of ecosystem retrogression where soil P concentrations become extremely low. These data suggest that some AM fungal species may fail to acquire sufficient P for their host plants and are themselves under extreme P limitation. The ECM fungal colonisation also peaked in the middle-aged soils, but it remained high in the old soils. This trend was likely due to the greater host plant species richness or suggesting that ECM fungi may function more reliably as mutualists in the poorest soils. Also, organic P proportionally increases with increasing soil age along this sequence (Turner et al., 2013; Turner and Laliberté, 2015), thus perhaps giving ECM fungi a competitive advantage over AM fungi since ECM fungi possess more diverse foraging strategies (Cairney, 2011). The Mediterranean Jurien Bay soil chronosequence had, compared with other biomes, very low levels of mycorrhizal fungal biomass in soil. This is particularly intriguing, since this chronosequence harbours an impressive diversity of host plant species (Hayes et al., 2014; Zemunik et al., 2015), including the two major mycorrhizal types (AM and ECM).

5. Conclusion

Our results suggest that the productivity of mycorrhizal fungal ERH and associated bacteria is limited by declining soil P availability, and possibly soil pH (co-limiting AM only) during ecosystem retrogression. Our study highlights the need for a more 'myco-centric' view of plant–mycorrhizal relationships (Treseder and Allen, 2002), whereby mycorrhizal fungal production can be more strongly influenced by soil nutrient availability than by C supply from plant hosts. Seasonal water deficit is likely an additional major interacting factor contributing to the performance of the mycorrhizal symbioses in these severely nutrient-impoorished soils. Our study supports the model of Lambers et al. (2008) that suggests mycorrhizal nutrient-acquiring strategies become less dominant in severely P-impoorished soils. Specifically, our results suggest that mycorrhizal fungi may remain dormant in roots during most of the year, mostly scavenge at relatively short distances from host roots, and scavenge at long-distances from roots only when soil moisture and C allocation to roots are both favourable. This ephemeral 'boom-and-bust' functioning, perhaps in conjunction with a large proportion of biomass allocated to spores, may be characteristic of mycorrhizal communities in dry severely nutrient-impoorished

ecosystems entering the retrogressive phase of ecosystem development.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2015.09.021>.

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