Using soil microbial inoculations to enhance substrate performance on extensive green roofs

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Abstract

Green roofs are increasing in popularity in the urban environment for their contribution to green infrastructure; but their role for biodiversity is not often a design priority. Maximising biodiversity will impact positively on ecosystem services and is therefore fundamental for achieving the greatest benefits from green roofs. Extensive green roofs are lightweight systems generally constructed with a specialised growing medium that tends to be biologically limited and as such can be a harsh habitat for plants to thrive in. Thus, this investigation aimed to enhance the soil functioning with inoculations of soil microbes to increase plant diversity, improve vegetation health/performance and maximise access to soil nutrients. Manipulations included the addition of mycorrhizal fungi and a microbial mixture ('compost tea') to green roof rootzones, composed mainly of crushed brick or crushed concrete. The study revealed that growing media type and depth play a vital role in the microbial ecology of green roofs, with complex relationships between depth and type of substrate and the type of microbial inoculant applied, with no clear pattern being observed. For bait plant measurements (heights, leaf numbers, root/shoot biomass, leaf nutrients), a compost tea may have positive effects on plant performance when grown in substrates of shallower depths (5.5 cm), even one year after inoculums are applied. Results from the species richness surveys show that diversity was significantly increased with the application of an AM fungal treatment and that overall, results suggest that brick-based substrate blends are most effective for vegetation performance as are deeper depths (although this varied with time). Microbial inoculations of green roof habitats appeared to be sustainable; they need only be done once for benefits to still been seen in subsequent years where treatments are added independently (not in combination). They seem to be a novel and viable method of enhancing rooftop conditions.

Keywords: Microbial Communities; Resilience; Substrates; Nutrients; Species Richness; Sustainability.

1. Introduction

Extensive green roofs are those with a shallow rootzone – generally between 5 – 15 cm in depth, and often fall into three main types: *Sedum* systems, wildflower systems and biodiverse roofs. From an ecological perspective, biodiverse roofs that mimic brownfield habitat are of great interest and importance in our urban landscapes (Schadek *et al.,* 2009). With increasing construction in our cities it is vital to create wildlife spaces to mitigate associated negative effects. Biodiverse green roofs therefore offer great potential, if designed appropriately (Lundholm, 2015), to offer regional biodiversity at roof level (Connop *et al.,* 2016). The issue is that many green roofs are constructed with a lack of knowledge about how to maximise biodiversity (Kadas 2002). *Sedum* systems are selected by architects for their proven hardiness to rooftop conditions (Monterusso *et al.,* 2005) and the aesthetic value of instant greening (Molineux *et al.,* 2009). Biodiverse roofs are becoming more popular in cities like London, however these are often extremely homogenous – with the same substrate type and depth (Heim & Lundholm, 2014) over the roofs' entirety. Substrate type is particularly important (Molineux *et al.,* 2009; Graceson *et al.,* 2014b; Bates *et al.,* 2015; Molineux *et al*., 2015; Eksi & Rowe 2016), as it is the main green roof component that will support the vegetation. Previous studies suggest that engineered substrates may be biologically limited but that microbial inoculants could be used to enhance the functioning below-ground (Molineux *et al.,* 2014; Ondoño *et al.,* 2014; Young *et al.,* 2015). Thus a physically engineered substrate, that has considered biological functionality, will underpin the success of a specified planting scheme.

Soil microbial communities at ground level have been well studied in many habitats. These microscopic organisms, including bacteria and fungi, are vital for colonization of a substrate by plants (Lavelle *et al.,* 2006). They offer favourable conditions for plants to extract limited nutrients, either by breaking down and recycling dead and decaying matter, or by providing access to nutrient pools that can be unexploitable (Smith and Read, 2010). One group in particular, the arbuscular mycorrhizal fungi (AMF), facilitate this via hyphal networks in plant root cells (Van der Heijden *et al.,* 1998) and in doing so also increase root hair surface area allowing access to water films on soil particles in times of extreme drought stress (Allen, 2009). AMF comprise of about 150 known fungal species and are said to be associated with around 80% of all plant species root systems (Hodge, 2000).

The microbial ecology of green roof habitats is beginning to receive attention McGuire *et al.,* 2013, Rumble & Gange, 2013, John, 2014, Buffam *et al.,* 2015, however little of this research links the effects of microbial communities to plant growth on green roofs (Young *et al.,* 2015) or their effects on substrate nutrient levels. Green roofs can be extreme environments for many plant species; thus microbial groups such as AMF could potentially provide vegetation with a better chance of survival at roof level (Molineux *et al.,* 2014). This in turn would help maintain ecosystem services, like building cooling, evapotranspiration and reduction in the urban heat island effect (Oberndorfer et al., 2007; Lundholm *et al.,* 2010); as well as increased storm water retention (Connop *et al.,* 2016), carbon sequestration (Parras-Alcántara *et al.,* 2015) and urban soil security (Anaya-Romero *et al.,* 2015).

The aim of the research was to determine how substrate type and depth effected plant species richness and plant 'health' determined by performance measurements such as heights, leaf numbers, root and shoot biomass. It also explores the additions of microbial inoculants to green roof substrates and the effect this had, not only on the microbial communities themselves (as described in Molineux *et al.,* 2014), but also on the substrate nutrients and bait plant leaf nutrients. The main research questions regarding the addition of microbial inoculations to various substrate types and depths (described in methods section) were, did they (i) produce larger plants (heights, leaf numbers, root and shoot biomass), (ii) increase root colonisation by beneficial arbuscular mycorrhizal fungi, (iii) effect leaf nutrient levels, (iv) increase species diversity and (v) increase available soil nutrients?

2. Methods

2.1 Field Site

To study the effects of substrate type and depth, an existing experimental set-up on the gift shop at London Zoo (Regents Park, London) was utilised and microbial inoculation treatments were applied. The experimental green roof is approximately 180 m^2 and split into 2m × 2m plots which contain various substrates at five different depths (further details in Kadas, 2007). Molineux *et al.* (2014) fully describes the additions of the microbial treatments, but in short: two substrate types (brick-based

and concrete-based) at two of the depths (5.5cm and 8cm) were chosen for the investigation, each replicated 3 times. Substrate properties data can be found in Appendix I. The existing plots were further divided into quarters, which were then used for the microbial manipulation experiments. The inoculations were applied three times over the summer of 2007. The treatments were a commercial arbuscular mycorrhizal fungal mix (hereafter referred to as 'Fungi'), a live compost tea containing bacteria and fungi (Tea), a combination of both treatments (Fungi + Tea), and finally control plots where no inoculants were added (Control). Information on product content is available at: http://www.symbio.co.uk/horticulture_datasheets.aspx.

2.2 Bait Plants

Before microbial manipulation could begin, bait plants – to be used as indicators for any changes in plant growth due to the addition of microorganisms – were planted into the experimental plots. The bait plant species chosen was *Plantago lanceolata*; as a perennial it retains some leaves over winter and re-sprouts each spring from the rootstock, making the recording of growth from one year to the next possible. It is strongly mycorrhizal and is often used as a model plant in field studies (e.g. Walter *et al*. 2016). By growing the *P. lanceolata* in pumice, in a controlled temperature room, the bait plant roots remained mycorrhizal-free until added to the green roof plots. Colonisation of the roots could then be analysed in the different treatments, by removing one bait plant from each treatment plot annually. This also allowed for the collection of dry shoot and root biomass data whilst leaving the established green roof *P. lanceolata* population undisturbed by the experiment. Four bait plants of *P. lanceolata* were planted into each of the designated experimental plots in May 2007, after three months of growth in a control temperature room at Royal Holloway University. This was to ensure that at least two plants would survive for removal after treatments were applied. Plants were selected for similarity in size in order for height comparisons to be made, and to reduce plant phenotypic variability.

2.2.1 Plant Heights & Leaf Numbers

Plant heights and leaf numbers for the bait plants of *P. lanceolata* were recorded in November 2007, following treatments and November 2008, a year after treatments were first applied. Means taken from three replicates were used to determine any differences between the underlying substrate types (including depth) and the microbial treatments.

All samples were taken in November, so that seasonal variation in microbial biomass (Blume *et al.* 2002) was reduced as much as possible, many studies have also shown microbial biomass is increased under cool and wet conditions, thus November represented an ideal soil sampling time (Van Gestel *et al.* 1992; Arnold *et al.* 1999; Papatheodorou *et al.* 2004). November also represented the end of the growing season on our zoo green roof and therefore the plants were at their largest before the frost began to restrict their growth.

2.2.2 Dry Biomass

In November 2007, the first batch of bait plants were removed from the green roof plots. One plant was taken from each sub-plot and taken back to the laboratory where they were washed, roots stored in 70 % ethanol and leaves transferred to large paper envelopes. This was then repeated with the last batch of *P. lanceolata* bait plants, which were removed from the London Zoo green roof plots in November 2008. Plant leaves were placed into labelled envelopes and dried in an oven at 60 °C for 48-72 h. Once dried, each sample was placed in a weighing boat and weighed to determine total dry shoot biomass for each treatment plot. Means taken from three replicates were used to observe differences between the underlying substrate types (including depth) and the microbial treatments.

2.2.3 AMF root colonisation

The plant roots stored in 70 % ethanol, were washed in distilled water and put into 5% potassium hydroxide and then rinsed again with distilled water. They were transferred to 1% HCl for 15mins, then placed in a simple ink stain comprising of Quink ink, 1% HCl and water in a 0.2:1:50 ratio for 1hr. The samples were then cleaned by soaking in Destain solution (glycerol:water:1%HCl in the ratio 70:24:1) for 24hrs before temporary slides could be made for mycorrhizal analysis. This method was

modified from (Vierheilig *et al.,* 2005). Mycorrhizal occurrence could be calculated by slide scanning under the microscope at a magnification x200 as described by McGonigle et al. (1990). Means taken from three replicates were used to determine any differences between the underlying substrate types (including depth) and the microbial treatments. Once AMF analysis completed, all the roots (including those on temporary slides) were collected and subjected to the same drying technique used for shoot biomass data collection (described in 2.2.2) in order to determine dry root biomass.

2.2.4 Leaf Nutrient Analysis

Following the collection of dry shoot biomass data (as described in 2.2.2), the dried bait plant leaves were ground into a fine powder using a pestle and mortar for leaf nutrient analysis. Approximately 2 µg of leaf material was used for total carbon and total nitrogen analysis using a Nitrogen and Carbon Soil Analyser (Flash EA1112 Series) equipped with a Carbon, Hydrogen, Nitrogen and Sulphur configuration. The leaf material was placed into individual tin containers and dropped by an autosampler into the furnace, where total N and total C could be calculated for each plant collected. Means were found for plants in each microbial treatment, with respect to underlying substrate type and depth.

2.3 Species Diversity

The London Zoo gift shop green roof plots were monitored for plant species diversity where both species type and individual numbers were recorded, using Blamey *et al.* (2003). Surveys took place in July 2007, after microbial treatments were added and May 2008, one year after treatments applied**.**

2.4 Substrate Analysis

Substrate/soil samples were also taken from each treatment plot on London Zoo gift shop green roof to determine the quantity of available nitrates from nitrogen and ammonia, potassium and phosphorus in the sub-plots. These nutrients are essential

196 for effective plant growth, so it was important to assess if the microbial treatments
197 had altered these properties of the substrate. Approximately 100 g of soil was
198 removed in November 2006 (before manipulations), November 2007 (after
199 manipulations) and November 2008 (one year following manipulations) and stored at
200 -20 °C until needed. A segmented flow analyser – Skalar Ltd, UK – comprised of SA1050
201 random access autosampler, chemistry unit SA4000, SA 853 SFA interface with a
202 digital photometer head and Flowaccess software was used to analyze all but
203 potassium nutrients. For all nutrients analysed each sample was replicated three times
204 to give a representative mean.
205
206 2.4.1 Nitrates
207 Substrate nitrogen was determined using a hydrazine reduction method (modified
208 from Henriksen & Selmer-Olsen, 1970) for nitrates and nitrites; and a Berthelot
209 method (modified from Rhine *et al.* 1998) for ammonia.
210 For nitrates and nitrites, 1 g substrate samples were added to 1M potassium
211 chloride in 100 ml conical flasks and placed on a shaker rack for 30 minutes. Three
212 samples of just the KCL reagent were used as control blanks. After this time each
213 sample was filtered through Whatman 25 mm GF/C paper directly into acid washed
214 tubes. These were then capped and stored at 5 °C in a fridge until needed. Reagents
215 for the Skalar SFA were also prepared ready for analysis. These included a buffer
216 solution containing potassium sodium tartrate, tri-sodium citrate and Briji 35, sodium
217 hydroxide, hydrazinium sulphate and a colour reagent containing sulphanilamide and
218 -naphthylethylenediamine dihydrochloride. Standards were also produced to give 1,
219 2, 3, 4 and 5 ppm of sodium nitrate solution. For analysis, each sample was transferred
220 to Skalar vials and placed into an autosampler. The determination of nitrate and nitrite
221 is based on the hydrazine reduction method; which forms a highly coloured azo dye
222 measured at 540 nm.
223 Ammonia was also extracted from substrate samples as above, however
224 different Skalar reagents were used for analysis. These included sodium salicylate,
225 sodium nitroprusside, sodium dichloroisocyanurate and the same buffer solution as
226 above. The standards were 0.4, 0.8, 1.2, 1.6 and 2 ppm of ammonium chloride solution.
227 For analysis, each sample was transferred to Skalar vials and placed into an

autosampler as with the nitrates. The procedure for the determination of ammonia is based on the modified Berthelot reaction; after oxidation and oxidative coupling a green coloured complex is formed and absorption measured photometrically at 660nm.

2.4.2 Phosphates

For phosphates, Olsen's Extractable Phosphorus in soil_method was followed (modified from Watanabe & Olsen 1965), whereby 2.5 g of soil was added to 50 ml Olsen's reagent in 100 ml conical flasks. The Olsen's extractant is a 0.5 M sodium bicarbonate solution with pH of 8.5. The samples were placed on a shaker rack for 30 minutes along with three blanks of just the Olsen's reagent as control samples. After this time each sample was filtered through Whatman 25 mm GF/C paper directly into acid washed tubes. These were then capped and stored at 5 °C in a fridge until needed. To determine phosphorous content, the following reagents were also prepared: ammonium molybdate (1.2 % m/V), ascorbic acid solution and 1.5 M sulphuric acid along with standards of 0, 1, 2, 4, 6 and 8 ppm potassium dihydrogen orthophosphate. Before analysis, 2.5 ml samples were combined with 0.5 ml sulphuric acid, 10 ml ammonium molybdate and 2.5 ml ascorbic acid solutions and allowed to stand for 30 minutes. The automated procedure is based on a reaction that produces an intensely blue coloured complex, with absorbency read at 880 nm.

2.4.3 Potassium

Finally potassium was extracted from substrates based on the Ammonium Acetate (pH 7.0) method (modified from Simard, 1993); whereby 2.5 g of soil was added to 63 ml of ammonium acetate (pH 7) solution. Three blanks to be used as controls containing only the ammonium acetate were also produced. Samples were then placed onto a shaker for 1h then filtered as described above. They were stored at 5 °C in a fridge until needed. Potassium was analysed using a flame photometer with standards of 2, 4, 6, 8 and 10 ppm of the potassium stock solution.

2.5 Statistical Analysis

Plant performance measurements and leaf and soil nutrients analysis were examined using a split-plot multiple analysis of variance (ANOVA) (Zar, 2005) to determine differences between the factors: substrate type, substrate depth and microbial treatment in the years 2007 and 2008. This analysis allowed for interactions between treatments and underlying substrate types and depths to be explored. Data that were not normally distributed were transformed with square roots or logarithms. Means were separated with a Tukey's HSD post hoc test (Fowler et al., 1998). All analyses were conducted using the statistical package UNISTAT®.

3. Results

3.1 Bait Plants

The following data obtained for bait plant performance have been displayed in relation to statistically significant results. Where the microbial treatments did not have an effect on a particular plant measurement, data has been graphed according to underlying variables, such as substrate type and substrate depth irrespective of treatment. Data are displayed in respect to 2007, after microbial treatments applied and 2008, one year after treatments were first added.

3.1.1 Plant Heights

Figure 1a shows the effect of substrate type and depth (irrespective of treatment) on plant heights over the study period. *Plantago lanceolata* bait plants on London Zoo gift shop green roof were considerably taller in 2007 than they were in 2008 ($F_{1,66}$ = 36.98, P <0.01). Substrate depth was also a significant factor affecting how tall plants grew ($F_{1,66}$ = 9.77, P <0.01), and there were interactions between the substrate type and depth ($F_{1,66}$ = 4.56, P <0.05). Plants in concrete-based substrate at 5.5cm depth were similar in height over the two years whilst those in brick-based substrate at 8 cm depth were considerably taller in 2007 and remained so in 2008 ($F_{1,66}$ = 5.66, P <0.05). These interactions mean that the choice of substrate composition for a green roof is vital, as plant performance can change with varying depths.

Figure 1b shows that in 2007 the addition of AM fungi produced the largest increase in heights ($F_{1,66}$ = 4.20, P <0.05). However by 2008, a year after inoculations

292 took place, all heights were reduced to similar levels with no significance found
293 between treatments. Furthermore, the AM fungi treatment and the compost tea
294 treatment were not additive as predicted, instead there was a significant interaction
295 between the two products used in combination ($F_{1,66}$ = 3.82, P <0.05). This is shown
296 by fungi + tea bars being similar in size to all other treatments.
297
298 3.1.2 Leaf Numbers
299 As with the data for plant heights, there were decreased leaf numbers from *P.*
300 *lanceolata* bait plants in 2008 ($F_{1,66}$ = 7.39, P <0.05) following one year without any
301 microbial treatments, Figure 1c & 1d. Figure 1c shows leaf numbers were affected by
302 substrate depth ($F_{1,66}$ = 8.99, P <0.01), where plants in concrete-based substrate at 5.5
303 cm depths had almost twice as many leaves as those in 8 cm plots in 2008.
304 The addition of treatments (Figure 1d) AM fungi and compost tea, appeared
305 to increase leaf numbers compared to controls but this was not statistically significant.
306 Likewise there was no additive benefit when the two treatments were used in
307 combination, instead there was a significant interaction between AM fungi and
308 compost tea products ($F_{1,66}$ = 6.68, P <0.01), suggesting antagonism between the
309 microbial species applied.
310
311 3.1.3 Root & Shoot Biomass
312 Dry shoot biomass (Figure 2a) and dry root biomass (Figure 2b) of *P. lanceolata* plants
313 were both lower in 2008 compared to 2007 ($F_{1,66}$ = 5.71, P = 0.07 and $F_{1,66}$ = 11.09, P
314 <0.05 respectively). Yet, the addition of the AM fungi treatment appeared to increased
315 root biomass slightly ($F_{1,66}$= 3.32, P = 0.07) compared to other treatment plots and
316 control, regardless of year. Root biomass was also affected by underlying substrate
317 depth, where overall 8 cm plots allowed roots to become larger, thus increasing
318 biomass ($F_{1,66}$ = 4.58, P <0.05). In 2007 (Figure 2c) the tea treated plots showed the
319 opposite trend, where substrates that were 5.5 cm deep, contained plants with a
320 larger total plant biomass compared to plots that were 8 cm deep. However by 2008
321 (Figure 2d), there was little difference in biomass between either substrate depths
322 where the tea treatment was applied.
323

3.1.4 AMF root colonisation

Figure 3 shows the levels of colonisation in relation to treatments applied in both years, as well as the percentage of vesicles and arbuscules encountered. From 2007 to 2008 there was a considerable ($F_{1,58}$ = 8.46, P <0.05) increase in arbuscular occurrence in bait plant roots. In 2007, plants from tea treated plots contained approximately four times more AM fungal root colonisation compared to plants from control plots, where both arbuscules ($F_{1,58}$ = 6.69, P <0.01) and vesicles ($F_{1,58}$ = 11.88, P <0.001) were significantly increased. The ratios of arbuscules and vesicles observed also shifted from 2007 to 2008. In 2007 most plots contained more vesicles than arbuscules, except for the tea treated plots, which contained even amounts of each. Yet in 2008 the opposite was true, ratios were more in favour of vesicles where treated with compost tea; for all other treatments, there was an even divide between the vesicle and arbuscular structures.

Furthermore, interactions occurred for arbuscules ($F_{1,58}$ = 6.16, P <0.01) and vesicles ($F_{1,58}$ = 5.14, P <0.05) where compost tea and AM fungi treatments were added together (irrespective of year), resulting in an antagonistic effect rather than the additive one that would have been expected.

3.1.5 Leaf Nutrient Analysis

Figure 4 shows the nutrient content of bait plant shoots after microbial treatments were applied to London Zoo green roof experimental plots in 2007. Data from 2008 have not been displayed as they were very similar to 2007 and year was not a significant factor affecting either leaf nitrogen or leaf carbon.

Figure 4a shows the nitrogen percentage content of shoots. The combination of the fungi and tea treatments increased nitrogen content in the brick-based substrate (additive effect), but reduced nitrogen content in shoots from the concrete-based substrate (antagonistic effect). Therefore there was a significant three-way interaction ($F_{1,63}$ = 6.16, P <0.01) between the substrate type and the fungi and tea treatments; whilst individually the fungi treatment ($F_{1,63}$ = 0.40, P = 0.52) and tea treatment ($F_{1,63}$ = 2.11, P = 0.15) were not significant factors effecting nitrogen in leaves.

Figure 4b shows leaf carbon in bait plants taken from the London Zoo experimental plots in 2007. There was a significant three-way interaction ($F_{1,63}$ = 3.71, P <0.05) between substrate depth and the fungal and tea inoculants; meaning that where treatments were applied to deeper substrate plots (8 cm), plants contained larger quantities of leaf carbon compared to plants grown in shallower plots (5.5 cm).

3.2 Species Diversity

The plant surveys conducted in the summer months of 2007 and 2008 indicated that there was increased plant species diversity ($F_{1,66}$ = 4.91, P <0.05) with the addition of the AM fungi treatment (Figure 5a). Figure 5b shows differences between species richness in the substrate types over the three years where treatments (sub-plots) have been combined to give means for each experimental plot. Results have also shown that the type and depth of substrate play an important role in determining how many plant species are supported on a green roof. Brick-based substrates supported more plant species than the concrete-based substrate ($F_{1,66}$ = 4.91, P <0.05) whilst there was also an interaction between the year and substrate depth ($F_{1,66}$ = 12.66, P <0.001). In 2007, deeper substrates contained more plant species, whilst in 2008 the reverse was true, with shallower substrates (depths of 5.5 cm) becoming more species rich.

3.3 Substrate Analysis

3.3.1 Nitrates

The nitrate and ammonium levels in substrate samples from London Zoo experimental site were combined to give the total amount of nitrogen available in the soil for plant acquisition (Table 1). There was a significant interaction between substrate type and year ($F_{1,51}$ = 4.51, P <0.05); where brick-based substrates contained larger quantities of available nitrogen in 2007 compared to concrete-based substrates in the same year. By 2008, there was little difference in available N levels between the two substrate types. Interestingly however, there were no significant effects observed with the addition of microbial treatments (Appendix II in supplementary material).

3.3.2 Phosphates

The substrate phosphorus levels (Table 1) were higher in 2007 than in 2008, $F_{1,51}$ = 26.08, P <0.01, and this was particularly affected by underlying substrate type, $F_{1,51}$ = 4.90, P <0.05. In 2007, brick-based substrates contained more phosphates than concrete-based substrates, however by 2008, it was these substrate that held more soil phosphorus. The compost tea inoculum increased quantities of available phosphates in 2007, compared to 2008 ($F_{1,51}$ = 5.07, P <0.05). There were also increased levels found in brick-based substrates where this treatment was applied ($F_{1,51}$ = 4.45, P <0.05). Therefore there was a significant three-way interaction between the year, substrate type and compost tea treatment ($F_{1,51}$ = 4.68, P <0.05); implying that in certain substrate types, the addition of compost tea may increase available phosphates to plants in the year of application, but that this is not sustained unless subsequent treatments are carried out.

3.3.3 Potassium

Finally substrate potassium levels (Table 1) were analysed from the green roof experimental plots. Potassium content was significantly increased in 2008 compared to 2007,,$F_{1,51}$ = 54.47, P <0.01 and thus it seems that the addition of microbial treatments had a negative effect on the substrate's potassium. Furthermore, brick-based substrates contained slightly larger quantities of potassium in 2008 compared to 2007 – where both substrate types were similar in levels. The application of compost tea increased potassium in 2008 at the deepest depth of brick-based substrates but decreased this in the 8 cm concrete-based substrate plots. Despite microbial treatments, in 2008, levels of potassium returned to similar levels as those found in the baseline data (around 17-20 mg/kg soil).

4. Discussion

The use of bait plants on London Zoo green roof demonstrated the possible effects of microbial inoculations on general plant performance over time. *Plantago lanceolata* appeared well suited to the green roof environment, with all planted seedlings surviving the course of the study. As a single species, it could not represent every plant response in the green roof community, however it is considered a good model to

measure microbial effect in other field studies (Walter *et al.,* 2016). Results showed inconsistent patterns of microbial treatment benefit, varying with underlying substrate type and depth. Generally, *P. lanceolata* plants increased in height from plots where the AM fungi treatment was applied. As arbuscular mycorrhizal fungi have been shown to significantly increase the survival, establishment and growth of plants with colonised roots (Koske & Gemma, 1997; Bakker *et al.,* 2013; Miransari, 2016) and are said to be key elements in nutrient-unbalanced and xeric environments (Roldan-Fajardo, 1994; Requena *et al.,* 1996; Peña-Becerril *et al.,* 2016); results suggest that the fungal treatment effectively increased AMF root colonisation compared to controls. Despite this, all plants were reduced in size by 2008. Substrates containing 75 % crushed brick at depths of 8 cm, produced plants that were considerably taller than the 5.5 cm plots and any plot containing the concrete-based substrate. This was probably because deeper plots retained more rainwater than shallower ones, providing plants with increased access to water – essential for survival and growth (Kramer & Boyer, 1995). Interestingly, plants grown in 75 % crushed concrete at 5.5 cm depths remained unchanged in height from 2007 to 2008, perhaps due to better water storage capacity or less efficient drainage at shallower depths than the brick-based substrate.

Leaf numbers on *P. lanceolata* plants showed similar patterns to heights, where decreases were seen from 2007 to 2008. Average rainfall (from MetOffice data) in 2006 was 101.2 mm, 86.9 mm in 2007 and 67.0 mm in 2008. This suggests that the application of microbial treatments were successful in increasing plant size and leaf numbers in 2007 but by 2008 - when numbers decreased for all plants - reduced water availability may have been a reason for these changes. Appendix I (in supplementary materials) also shows that mean maximum and minimum temperatures as well as average sunlight hours decreased from 2007 to 2008. Thus weather conditions in 2008 were colder, drier and less sunny which would account for reduced growth rates overall. The interesting findings were where significant interactions between underlying substrate type and depth were observed and often this produced the largest changes in leaf numbers. In 2008, concrete-based substrate contained bait plants with twice as many leaves when grown at 5.5 cm depths compared to those in 8 cm plots. Furthermore, in 2007 *P. lanceolata* plants in 5.5 cm plots had up to six

more leaves when treated with the compost tea, compared to those in 8 cm substrates.

Overall *P. lanceolata* biomass – root and shoot – was decreased in 2008 compared to 2007 (as generally seen with all *P. lanceolata* performance data). In 2007 the total biomass of plants grown in 5.5 cm deep substrates were significant larger where the compost tea treatment was added and in 8 cm deep substrates where the AM fungi treatment was applied. By 2008 however, there was little difference between the total biomass in plants from either substrate depths or with microbial innoculation. The reduction in 2008, as with bait plant heights and leaf numbers, was therefore likely due to abiotic factors as discussed above. The soil nutrients could also have been a contributing factor, which is also addressed further on.

Bait plant root colonisation by arbuscular mycorrhizal fungi increased significantly from 2007 to 2008. After microbial inoculants were applied, experimental plots treated with compost tea increased in mycorrhizal occurrence from 5 % colonisation (in control plots) to approximately 25 % colonisation. However by 2008, colonisation levels in the control plots had increased to over 20 % whilst the fungi and tea treated areas were noticeably higher at over 30 % colonisation. Controls seem to have naturally increased in the substrates at this time, perhaps due to natural processes The structures of AM fungi found within plant roots are important in determining how it is functioning within the substratum (Klironomos *et al.,* 2004). In 2007, vesicles were observed more frequently than arbuscules in control plots and fungi treated plots. Vesicles are storage structures whilst arbuscules are sites of symbiotic nutrient exchange, and as such are thought to be more indicative of active functioning (Klironomos *et al.,* 2004). Therefore these results imply that the mycorrhiza may have been stressed and not that active within the host bait plants (Duckmanton & Widden, 1994; Titus & Leps, 2000; Wearn, 2006) until 2008, where there was an increase in arbuscules.

Even though colonisation increases were recorded, the microbial treatments often had small effects on plant performance measurements, with other parameters such as underlying substrate type and depth being the most significant variables. Therefore it appears that plants are not exploiting the usually beneficial root AMF. Reasons for this could be because nutrients such as phosphorus (Koide, 1991), are so

limiting on a green roof, that the fungi are not helping plants gain any more than they could without the symbionts. It has been said that optimal phosphorus levels, for AMF to produce the greatest benefits to host plants is approximately 50 ppm (Swift *et al.*, 1979; Schubert & Hayman, 1986; Smith & Read, 1997) but the exceedingly low (< 5 ppm) plant phosphates from this study (see Table 1) suggest that green roof substrates are extremely P limited. This probably means that, regardless of increased AM fungi colonisation, mycorrhizae are ineffective in these environments. The use of alternative aggregates in green roof growing media could provide more favourable conditions for both plants and AM fungi. Molineux *et al.,* (2009) found that clay pellet substrates contained five times more phosphorus pentoxide – a common form of P in many fertilizers (Bridger *et al.*, 1953) – compared to red brick (contained in the substrates on London Zoo green roof). This suggests that aggregates produced from recycled waste materials, such as sewage sludge (Debosz *et al.,* 2002), may provide a source of potential phosphates that could be released in rainwater leachates.

An alternative explanation for these results may be that once the mycorrhiza from the inoculation experiments have colonised plant roots, they could be having deleterious effects on their hosts, as shown in more recent microbial studies by Gadhave *et al.* (2016) and L. Jin *et al.* (2016). These studies highlight that AM Fungi can cause growth depressions in plants (Johansen, 1993), particularly when growing conditions are poor (i.e. in low nutrients, during drought periods). L. Jin *et al.* (2016) propose that for AM fungal structures to grow, such as vesicles, the fungus needs to obtain more photosynthetic products from the host plant, resulting in plant growth depression. This would help explain why, in general, all bait plant performance measurements in this investigation were reduced in 2008 compared to 2007 despite the observed increase in AMF colonisation from 2007 to 2008. Furthermore, vesicles were increased due to non-favourable conditions for the fungus, which would account for the negative relationship between plant performance and AMF root colonisation.

Results from bait plant leaf nutrients have shown significant interactions between the substrate type, depth and microbial treatments. For leaf nitrogen, there were significantly higher levels found in plants from substrate composed of 75 % brick compared to those that were 75 % concrete, where both the fungi and tea treatments were added. Leaf carbon was also increased with the combination of AM fungi and

compost tea treatments, but only in the deepest substrate plots (8 cm). Increased root biomass as well as higher nitrogen and carbon content of shoots, points to an increased photosynthetic capacity by plants (Field & Mooney, 1986). This heightened rate of photosynthesis implies that microbial treatments enhanced plant access to soil nutrients, such as nitrogen and phosphorus – vital constituents of the photosynthetic process (Blevins, 1999) – leading to improved plant fitness. Leaf nitrogen analysis indicated that, in brick dominated media, the two microbial treatments were additive, meaning that the fungi and tea treatments together resulted in higher concentrations of leaf nitrogen than those from plots where just AM fungi or just compost tea was applied. Conversely, in the concrete-based substrate, the two treatments were competitive resulting in decreased concentrations of leaf nitrogen compared to plots that were treated with just the AM fungi or just the compost tea. Possible reasons for this could be substrate N and P content. As already seen, soil phosphates can vary considerably with different aggregate types, and this is probably the same for soil nitrogen. In the London Zoo plots, crushed brick dominated substrates may contain higher N and P levels than the predominately crushed concrete ones. The applications of the treatments together may have increased microbial mobilisation of phosphorus and nitrogen for plant availability in the brick dominated substrates, because more nutrients pools were present for symbiotic benefits to be exploited (Koide, 1991). Previous microbial inoculation experiments by Requena *et al.,* (1996) showed that leaf nitrogen was increased with AMF root colonisation, and suggested this was due to an increased exploration of soil nitrogen pools (Ames *et al.,* 1984; Barea *et al.,* 1991; Azcon-Aguilar *et al.,* 1993; Johansen *et al.,* 1993). However, they also showed that interactions between AMF and certain bacteria could lead to decreased shoot nitrogen, indicating that limited P in soils could lead to antagonism between the microbial groups due to resource competition. This may help explain the reduced leaf nitrogen results from the concrete-based substrates.

The London Zoo green roof experimental site was originally seeded with a wildflower mix but since then, natural colonisation of the plots has occurred with the effect of increasing plant coverage and diversity (Kadas, 2007). Results from the species richness surveys showed that in 2007, the 8 cm plots supported the most plant species, correlating with previous research showing that deeper green roof substrates

are far more biodiverse than shallower ones (Brenneisen, 2006; Dunnett *et al*., 2008). However, by 2008 the 5.5 cm plots became more species rich. In addition, the brick-based substrate was also more effective at supporting increased diversity than the concrete dominated media. The applications of compost tea did not affect plant diversity in the green roof plots, however the use of the AM fungi treatment significantly increased species numbers where added. Many studies have shown similar positive effects on plant species diversity with the presence of AMF (Grime *et al.*, 1987; Gange *et al.*, 1993; Klironomos *et al.,* 2000); proposing that AM fungi provide hyphal links between plants allowing a more even distribution of soil nutrients – reducing competition by strong plant species that usually monopolise resources.

Soil nutrient analyses have shown that for both nitrogen and phosphates, levels were higher in brick-based substrates in 2007, whereas potassium levels were not increased in this substrate until 2008. For soil P, further increases were found with the applications of compost tea. This supports the discussion above, where increased substrate nitrogen and phosphates would account for increases in leaf nitrogen content. Overall, the levels of total available nitrogen and phosphates were similar below 5 ppm, and potassium was found at levels of around 20 ppm (Table 1). These levels are extremely low compared to other habitats. Wearn, (2006) found levels of nitrogen and potassium in field soil (grassland area on the Royal Holloway campus) to be approximately 32 ppm and 80 ppm respectively and phosphates to be found on average at 20 ppm. These were considered to be very low levels (Allen, 1989; Edwards *et al.,* 1999); in fact Swift *et al.,* (1979) stated that phosphorus levels can reach above 150 ppm in grasslands/pastures. Phosphates are one of the most limiting nutrients to plants in soils, especially in habitats like brownfield sites (Schadek *et al.,* 2009), shingle beaches (Scott, 1960; Lee *et al.,* 1983) and xeric Mediterranean ecosystems (Azcon-Aguilar *et al.,* 1993; Requena *et al.,* 1996). The extremely low levels found in the London Zoo green roof plots would be a significant factor affecting floral growth (Hinsinger, 2001).

Statistical analysis of data from *Plantago lanceolata* heights, leaf numbers and AMF root colonisation identified significant interactions between the arbuscular mycorrhizal fungi treatment and the compost tea treatment. When combined and applied to the green roof plots, there was not always an additive effect as would be

expected, instead there was frequently competition between the two. Recent work by Gadhave *et al.* (2016) has explored possible reasons for commercial inoculants competing against each other when used in combination and there is evidence of antagonism in other studies looking at the interactions between plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (Bethlenfalvay & Linderman, 1992); as well as specific interactions between AM fungi and other soil microbes (Vosatka *et al.,* 1992; Requena *et al.,* 1996; Saison *et al.*, 2006; Ondoño *et al.,* 2014). These authors suggest that competition arises due to soil nutrient availability – especially the phosphorus content, supporting the nutrient analysis of the London Zoo experimental plots previously discussed.

5. Conclusion

The results indicate that the addition of microbial treatments to London Zoo green roof were variable in terms of having an effect on vegetation compared to controls. The interactions between the AM fungi and compost tea applications and the different substrate types and varying substrate depths produced significant changes in plant heights, leaf numbers, species richness, and leaf/soil nutrient contents. Yet there were inconsistent patterns with regards to the 'best' substrate type and the 'most appropriate' substrate depth; generally speaking brick-based media at 8 cm depths were more favourable but this did vary with time as well as microbial treatment. However, what was clear from most results was that 2007 data were significantly different from post-treatment data from 2008. This seemed to be due to a combination of variables including the microbial inoculations, soil N and P and abiotic factors such as the amount of rainfall (water), mean max. and min. temperatures and sunlight hours. From previously published work, the treatments do seem to have long-lasting effects on the microbial communities themselves, but more research is needed to determine how much benefit they provide to the green roof plants over time. This short-term study shows that enhancement of soil microbial functioning can have positive impacts on some plant health/performance measurements on extensive biodiverse roofs and, with the right substrate, also increase plant species diversity. Green roofs need to be considered as habitats, albeit those with harsh conditions for

their flora and fauna; and should therefore be engineered, not only mechanically, but biologically as well. The introduction of microbial communities through various inoculations can help to improve green roof biodiversity and future research should look at how this then boosts their role in urban green infrastructure; particularly as a provision for ecosystem services and in respect to climate change mitigation.

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7. References

Ames, R.N., C.P.P. Reid, and E.R. Ingham (1984). Rhizosphere bacterial population responses to root colonization by a vesicular-arbuscular mycorrhizal fungus. **New Phytology**, 96:555 – 563.

Allen, S. E. (1989). **Chemical analysis of ecological analysis of ecological materials.** In: Allen, S.E. (Editor), Blackwell Scientific Publication.

Allen, M. F. (2009). **Water relations in the mycorrhizosphere**. In: Luttge, U., Beyschlag,W., Budel, B., Francis, D. (Eds.), Progress in Botany, vol. 70. Springer, Berlin,Germany, 257–276.

Anaya-Romero, M., S. K. Abd-Elmabod, M. Muñoz-Rojas, G. Castellano, C. J. Ceacero, S. Alvarez, M. Méndez, and D. De la Rosa (2015). Evaluating Soil Threats Under Climate Change Scenarios in the Andalusia Region, Southern Spain. **Land Degradation and Development,** 26 (5): 441-449. doi:10.1002/ldr.2363.

643
644 Azcón-Aguilar C., C. Alba, M. Montilla and J. M. Barea (1993). Isotopic (15N) evidence
645 of the use of less available N forms by VA mycorrhizas. **Symbiosis**, 15:39 – 48.
646
647 Bakker, P. A, R. L. Berendsen, R. F. Doornbos, P. C. Wintermans and C. M. Pieterse
648 (2013). The rhizosphere revisited: root microbiomics. **Frontiers in Plant Science**, 4:165.
649 doi:10.3389/fpls.2013.00165.
650
651 Barea, J. M., C. Azcón-Aguilar and R. Azcón, (1991). The role of vesicular-arbuscular
652 mycorrhizae in improving plant N acquisition from soil as assessed with 15N. In: IAEA-
653 SM 313/67 ed. **Stable Isotopes in Plant Nutrition, Soil Fertility and Environmental**
654 **Studies,** Vienna: International Atomic Energy Agency, 209 – 216.
655
656 Bates, A., J. Sadler, R. Greswell, and R. Mackay (2015). Effects of recycled aggregate
657 growth substrate on green roof vegetation development: a six year experiment.
658 **Landscape and Urban Planning**, 135: 22–31.
659
660 Bethlenfalvay, G. J and R. G. Linderman (1992). **Mycorrhizae in sustainable agriculture.**
661 Madison: ASA Special Publication.
662
663 Blamey, M., Fitter, S. R., & Fitter, A. (2003). **Wild Flowers of Britain & Ireland.** A. & C.
664 Black.
665
666 Blume, E., M. Bischoff, J. M. Reichert, T. Moorman, A. Konopka, and R. F. Turco (2002).
667 Surface and subsurface microbial biomass, community structure and metabolic
668 activity as a function of soil depth and season. **Applied Soil Ecology,** 20(3): 171-181.
669
670 Blevins, D. G. (1999). Why plants need phosphorous. **Better crops**, 83(2):29 – 30.
671
672 Buffam, A. P. D. I and M. E. Mitchell (2015) **Nutrient cycling in green roof ecosystems**.
673 In: Sutton R (ed) Green roof ecosystems, 1st edn. Springer International Publishing,
674 New York, pp. 107–137

675

676 Bridger, G. L., D. R. Boylan, and J. W. Markey (1953). Colorimetric determination of
677 phosphorus pentoxide in fertilizers using standard calibration plot. **Analytical**
678 **Chemistry,** 25(2): 336-338.
679
680 Brenneisen S (2006) Space for urban wildlife: designing green roofs as habitats in
681 Switzerland. **Urban Habitats,** 4:10
682
683 Connop, S., Vandergert, P., Eisenberg, B., Collier, M. J., Nash, C., Clough, J., & Newport,
684 D. (2016). Renaturing cities using a regionally-focused biodiversity-led multifunctional
685 benefits approach to urban green infrastructure. **Environmental Science & Policy**, 62,
686 99-111.
687
688 Debosz, Kasia, Søren O. Petersen, Liv K. Kure, and Per Ambus (2002). Evaluating effects
689 of sewage sludge and household compost on soil physical, chemical and
690 microbiological properties. **Applied Soil Ecology,** 19(3): 237-248.
691
692 Duckmanton, L. and P. Widden (1994). Effect of ozone on the development of
693 vesicular-arbuscular mycorrhizae in sugar maple saplings. **Mycologia**, 181-186.
694
695 Dunnett, N., Nagase, A., Booth, R., and Grime, P. (2008). Influence of vegetation
696 composition on runoff in two simulated green roof experiments. **Urban Ecosystems**
697 11:385-398.
698
699 Edwards, G. R, M. J Crawley and M. S. Heard (1999). Factors affecting molehill
700 distribution in grassland: implications for controlling the damage caused by molehills.
701 **Journal of Applied Ecology,** 36:434 – 442.
702
703 Eksi, M. & D. B. Rowe (2016). Green roof substrates: Effect of recycled crushed
704 porcelain and foamed glass on plant growth and water retention. **Urban Forestry &**
705 **Urban Greening**, 20 :81-88.
706

Field, C. and H. A. Mooney (1986). The photosynthesis-nitrogen relationship in wild plants. In: Givnish, T. J. (ed). **On the economy of plant form and function**. Cambridge University Press, Cambridge, 25 – 55.

Fowler, J., Cohen, L., & Jarvis, P. (1998) **Practical Statistics for Field Biology**, second edition. John Wiley & Sons.

Gadhave, K. R., J. E. Hourston & A. C. Gange (2016). Developing Soil Microbial Inoculants for Pest Management: Can One Have Too Much of a Good Thing? **Journal of chemical ecology**, *42*(4): 348-356.

Gange, A. C. (1993) Translocation of mycorrhizal fungi by earthworms during early succession. **Soil Biology and Biochemistry,** 25, 1021 – 1026.

Graceson, A., J. Monaghan, N. Hall, and M. Hare (2014b). Plant growth responses to different growing media for green roofs. **Ecological Engineering**, 69:196–200.

Grime, J. P., J. M. L. Mackey, S. H. Hillier and D. J. Read (1987). Floristic diversity in a model system using experimental microcosms. **Nature,** 328:3.

Heim, A., and J. Lundholm (2014). The effects of substrate depth heterogeneity on plant species coexistence on an extensive green roof. **Ecological Engineering,** 68:184-188.

Henriksen, A. A., and A. R. Selmer-Olsen (1970). Automatic methods for determining nitrate and nitrite in water and soil extracts. **Analyst,** 95(1130): 514-518.

Hinsinger, P. (2001). Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. **Plant and Soil,** 237:173 – 195.

Hodge, A. (2000) Microbial ecology of the arbuscular mycorrhiza. **FEMS Microbiology Ecology,** 32, 91 – 96.

739 Johansen, A., I. Jakobsen and E. S. Jensen (1993). External hyphae of vesicular-
740 arbuscular mycorrhizal fungi associated with Trifolium subterraneum L. 3. Hyphal
741 transport of 32P and 15N. **New Phytologist***,* 124:61 – 68.
742
743 John, J., J. Lundholm and G. Kernaghan (2014). Colonization of green roof plants by
744 mycorrhizal and root endophytic fungi. **Ecological Engineering,** 71:651-659.
745
746 Kadas, G. (2002). **Study of invertebrates on green roofs: How roof design can**
747 **maximise biodiversity in an urban environment***.* Master of science thesis. University
748 College, London.
749
750 Kadas, G. (2007) **Can Green Roofs provide a habitat for invertebrates in an urban**
751 **environment?** Unpublished PhD thesis. Royal Holloway University of London.
752
753 Klironomos, J.N., McCune, J., Hart, M., Neville, J., (2000) The influence of arbuscular
754 mycorrhizae on the relationship between plant diversity and productivity. **Ecology**
755 **Letters,** 3:137–141
756
757 Klironomos, J. N., J. McCune and P. Moutoglis (2004). Species of arbuscular
758 mycorrhizal fungi affect mycorrhizal responses to simulated herbivory. **Applied Soil**
759 **Ecology,** 26(2):133-141.
760
761 Koide, R. T. (1991). Nutrient supply, nutrient demand and plant response to
762 mycorrhizal infection. **New Phytologist,** 117:365 – 386.
763
764 Koske, R. E., and J. N. Gemma, (1997) Mycorrhizae and succession in plantings of
765 beachgrass in sand dunes. **American Journal of Botany,** 84, 118 – 130.
766
767 Kramer, P. J. and J. S. Boyer (1995). **Water Relations of Plants and Soils.** Academic
768 Press, San Diego, USA.
769
770 Lavelle, P., Decaens, T., Aubert, M., Barot, S., Blouin, M., Bureau, F., Margerie, P.,

771 Mora, P. and J. P. Rossi, (2006) Soil invertebrates and ecosystem services. **European**
772 **Journal of Soil Biology**, 42, S3 – S15.
773
774 Lee, J. A., R. Harmer and R. Ignaciuk (1983). Nitrogen as a limiting factor in plant
775 communities. **Nitrogen as an Ecological Factor** (eds J. A. Lee, S. McNeil & I. H. Rorison),
776 Blackwell Scientific Publications, Oxford, 95 – 112.
777
778 Lundholm, J. T. (2015). Green roof plant species diversity improves ecosystem
779 multifunctionality. **Journal of Applied Ecology**, 52(3):726-734.
780
781 Lundholm, J., J. S. MacIvor, Z. MacDougall, M. Ranalli (2010). Plant species and
782 functional group combinations affect green roof ecosystem functions. **PLoS ONE**, 5
783 (3):e9677.
784
785 Jin, L., Q. Wang, Q. Wang, X., Wang, and A. C. Gange (2016). Mycorrhizal-induced
786 growth depression in plants. **Symbiosis,** 1-8.
787
788 McGonigle, T. P., M. H. Miller, D. G. Evans, G. L. Fairchild, and J. A. Swan (1990). A new
789 method which gives an objective measure of colonization of roots by vesicular—
790 arbuscular mycorrhizal fungi. **New phytologist,** 115(3): 495-501.
791
792 McGuire, K.L., S. G. Payne, M. I. Palmer, C. M. Gillikin, D. Keefe, S. J. Kim, S. M.
793 Gedallovich, J. Discenza, R. Rangamannar, J. A. Koshner, and A. L. Massmann (2013).
794 Digging the New York City skyline: soil fungal communities in green roofs and city parks.
795 **PloS one,** 8(3):58020.
796
797 Miransari, M. (2016). Stress and Mycorrhizal Plant. In: **Recent Advances on**
798 **Mycorrhizal Fungi,** 63-79. Springer International Publishing.
799
800 Molineux, C. J., Fentiman, C. H., & Gange, A. C. (2009) Characterising alternative
801 recycled waste materials for use as green roof growing media in the U.K. **Ecological**
802 **Engineering**, 35, 1507 – 1513.

803
804 Molineux, C. J., S. P. Connop, and A. C. Gange. (2014). Manipulating soil microbial
805 communities in extensive green roof substrates. **Science of the Total Environment,**
806 493:632-638.
807
808 Molineux, C. J., A. C. Gange, S. P. Connop and D. J. Newport (2015). Using recycled
809 aggregates in green roof substrates for plant diversity. **Ecological Engineering**, 82:596-
810 604.
811
812 Monterusso, M. A, D. B. Rowe and C. L Rugh (2005). Establishment and persistence of
813 Sedum spp. and native taxa for green roof applications. **HortScience** 40: 391–396.
814
815 Oberndorfer, E., Lundholm, J., Bass, B., Coffman, R., Doshi, H., Dunnett, N., Gaffin, S.,
816 Köhler, M., Rowe, B., (2007) Green Roofs as urban ecosystems: ecological structures,
817 functions and services. **Bioscience***,* 57, 823 – 833.
818
819 Ondoño, S., Bastida, F., and J. L. Moreno (2014). Microbiological and biochemical
820 properties of artificial substrates: A preliminary study of its application as Technosols
821 or as a basis in Green Roof Systems. **Ecological Engineering**, 70: 189-199.
822
823 Papatheodorou, E. M., M. D. Argyropoulou, S. J. Grayston, C. D. Campbell, R. D.
824 Bardgett, J. L. Mawdsley, C. D. Clegg, H. Ferris, R. C. Venette and K. M. Scow (2004).
825 Soil management to enhance bacterivore and fungivore nematode populations and
826 their nitrogen mineralisation function: The effects of large-and small-scale differences
827 in soil temperature and moisture on bacterial functional diversity and the community
828 of bacterivorous nematodes. **Transport,** 25(1).
829
830 Parras-Alcántara, L., B. Lozano-García, E. C. Brevik, and A. Cerdá (2015). Soil Organic
831 Carbon Stocks Assessment in Mediterranean Natural Areas: A Comparison of Entire
832 Soil Profiles and Soil Control Sections. **Journal of Environmental Management,** 155:
833 219-228. doi:10.1016/j.jenvman.2015.03.039.
834

835 Peña-Becerril, J. C., A. Monroy-Ata, M. S. Orozco-Almanza, and E. M García-Amador
836 (2016). Establishment of catclaw plants (Mimosa biuncifera Benth.) inoculated with
837 arbuscular mycorrhizal fungi in greenhouse and field drought conditions. **Revista de**
838 **Biología Tropical/International Journal of Tropical Biology and Conservation,** 64(2):
839 791-803.
840
841 Requena, N., P. Jeffries and J. M. Barea (1996). Assessment of natural mycorrhizal
842 potential in a desertified semiarid ecosystem. **Applied and Environmental**
843 **Microbiology,** 62:842 – 847.
844
845 Rhine, E. D., R. L. Mulvaney, E. J. Pratt, and G. K. Sims (1998). Improving the Berthelot
846 reaction for determining ammonium in soil extracts and water. **Soil Science Society of**
847 **America Journal,** 62(2): 473-480.
848
849 Roldan-Fajardo, B. E. (1994). Effect of indigenous arbuscular mycorrhizal endophytes
850 on the development of six wild plants colonizing a semiarid area in south-east Spain.
851 **New Phytologist,** 127:115 – 121.
852
853 Rumble, H., & A. C. Gange (2013). Soil microarthropod community dynamics in
854 extensive green roofs. **Ecological engineering**, 57, 197-204.
855
856 Saison, C., V. Degrange, R. Oliver, P. Millard, C. Commeaux, D. Montange, and X. Le
857 Roux (2006). Alteration and resilience of the soil microbial community following
858 compost amendment: effects of compost level and compost-borne microbial
859 community. **Environmental Microbiology,** 8(2):247-257.
860
861 Schadek U., B. Strauss, R. Biedermann and M. Kleyer (2009) Plant species richness,
862 vegetation structure and soil resources of urban brownfield sites linked to
863 successional age. **Urban Ecosystems,** 12:115–126
864
865 Schubert, A., and D. S. Hayman (1986). Plant growth responses to vesicular ‐
866 arbuscular mycorrhiza. **New Phytologist,** 103(1): 79-90.

Scott, G. A. M. (1960). **The biology of shingle beach plants with special reference to the ecology of selected species**. PhD thesis, University of Wales.

Simard, R. R. (1993). Ammonium acetate-extractable elements. **Soil sampling and methods of analysis,** 39-42.

Smith, S. E., and D. J. Read (1997). Mycorrhizal symbiosis. **Mycorrhizal symbiosis**. Edition 2.

Swift, M. J., O. W. Heal and J. M. Anderson (1979). **Decomposition in terrestrial ecosystems,** Blackwell Scientific, Oxford.

Titus, J.H. and J. Lepš (2000). The response of arbuscular mycorrhizae to fertilization, mowing, and removal of dominant species in a diverse oligotrophic wet meadow. **American Journal of Botany**, 87(3):392-401.

Van der Heijden M. G. A, J. N. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, and T. Boller (1998). Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. **Nature,** 396:72–75

Van Gestel, M., J. N. Ladd, and M. Amato (1992). Microbial biomass responses to seasonal change and imposed drying regimes at increasing depths of undisturbed topsoil profiles. **Soil Biology and Biochemistry,** 24(2): 103-111.

Vierheilig, H., P. Schweiger, and M. Brundrett (2005). An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. **Physiologia Plantarum** 125:393-404.

Vosatka, M., M. Gryndler and Z. Prikryl (1992). Effect of the rhizosphere bacterium Pseudomonas putida, arbuscular mycorrhizal fungi and substrate composition on the growth of strawberry. **Agronomie,** 12:859–863.

Walter, J., J. Kreyling, B.K. Singh and A. Jentsch (2016). Effects of extreme weather events and legume presence on mycorrhization of Plantago lanceolata and Holcus lanatus in the field. **Plant Biology** 18: 262-270.

Watanabe, F. S., and S. R. Olsen (1965). Test of an ascorbic acid method for determining phosphorus in water and NaHCO3 extracts from soil. **Soil Science Society of America Journal,** 29 (6): 677-678.

Wearn, J. A. (2006). **Effects of above-ground herbivory on rhizosphere community dynamics in lowland grasslands.** PhD Thesis. Royal Holloway University of London.

Young, T., Cameron, D.D., Phoenix, G.K (2015). Using AMF inoculum to improve the nutritional status of Prunella vulgaris plants in green roof substrate during establishment. **Urban Forestry and Urban Greening**, 14 (4): 959-967.

Zar, J. H. (2005) **Biostatistical Analysis**, Pearson Education, Upper Saddle River, NJ.

931
932
933
934
935
936
937
938
939
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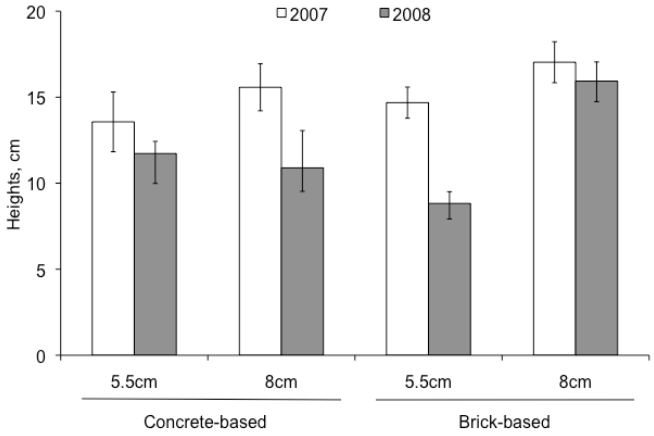

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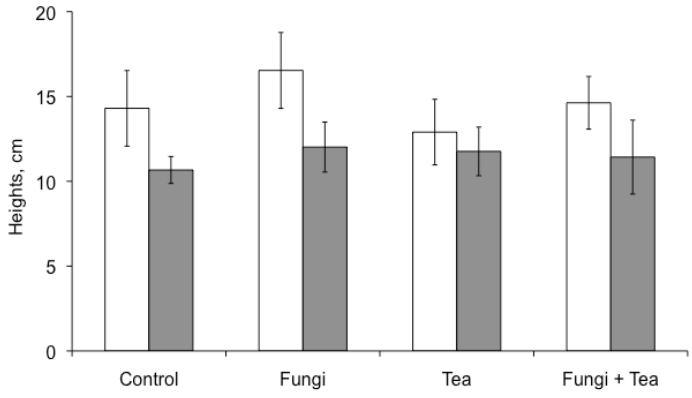

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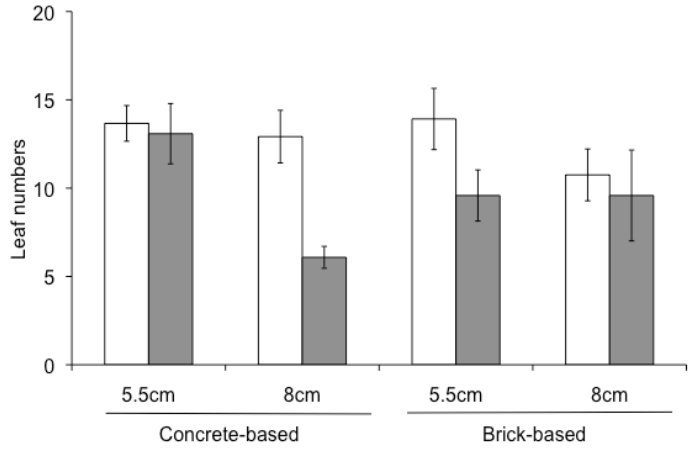

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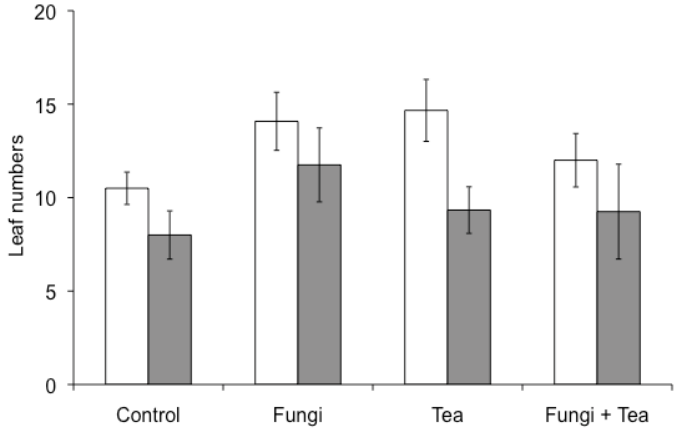

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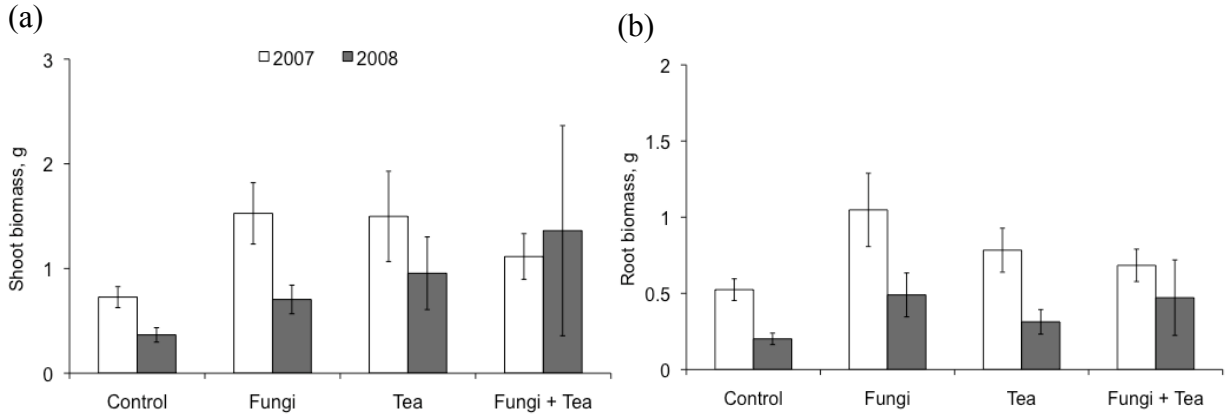
946 **Figure 1.** (a) Bait plant heights and (c) bait plant leaf numbers, with regards to underlying
947 substrate type and depth; and (b) bait plant heights and (d) bait plant leaf numbers, with
948 microbial treatments on London Zoo green roof experimental site, where: 2007 = after
949 treatments and 2008 = one year after treatments applied. Bars represent means ± S.E.



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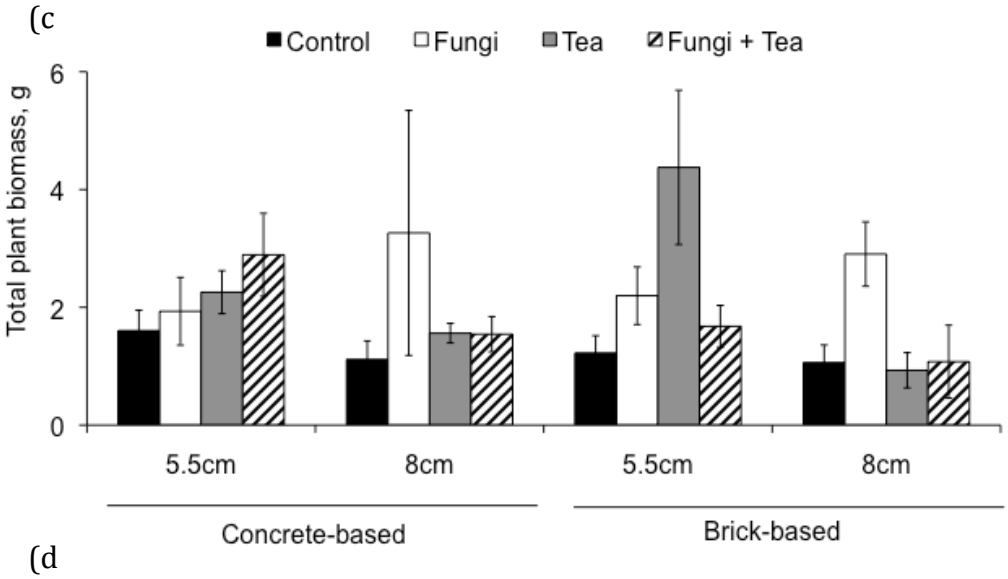

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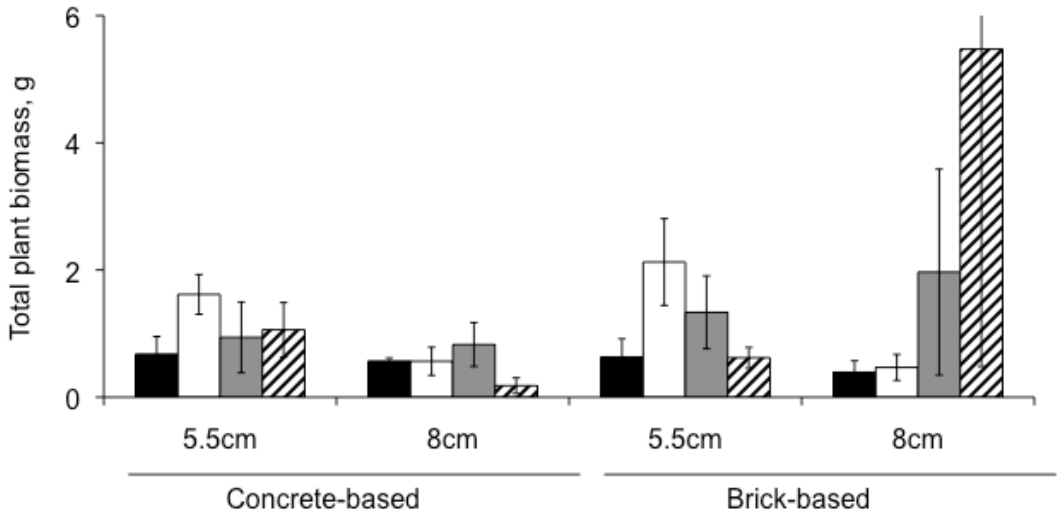

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975 **Figure 2.** Bait plant from the treated plots on London Zoo green roof experimental site,
976 where (a) shoot biomass and (b) root biomass in grams from 2007 = after treatments and
977 2008 = one year after treatments applied, means from 12 replicates per year; and total bait
978 plant biomass with respect to underlying substrate type/depth in (c) 2007 and (d) 2008,
979 means from three replicates. Bars represent means ± S.E.

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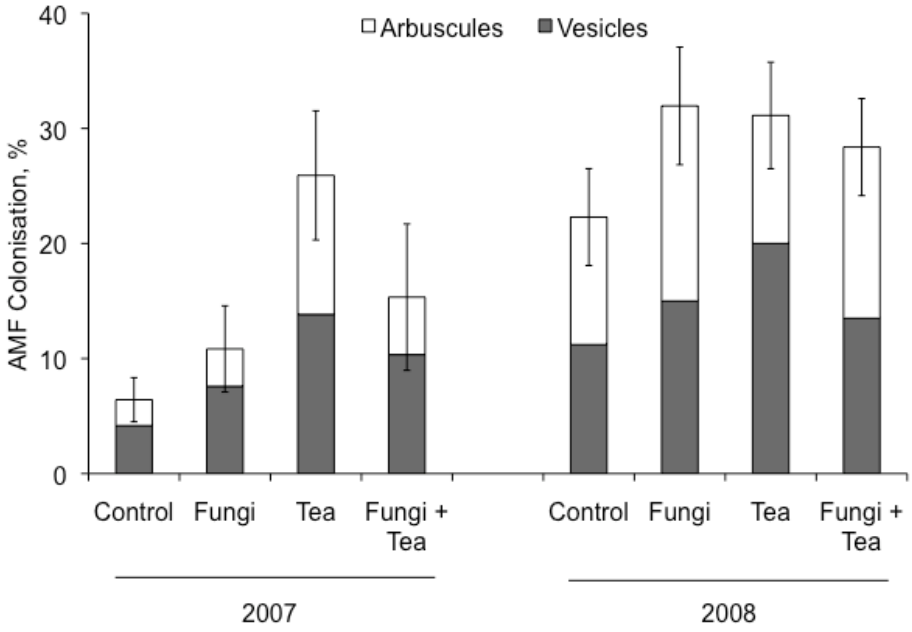

Figure 3. Bait plant root colonisation with AM fungi, from the treated plots on London Zoo experimental site in 2007 and 2008. Bars represent both arbuscule and vesicle colonisation means ± S.E. (of total AMF colonisation).

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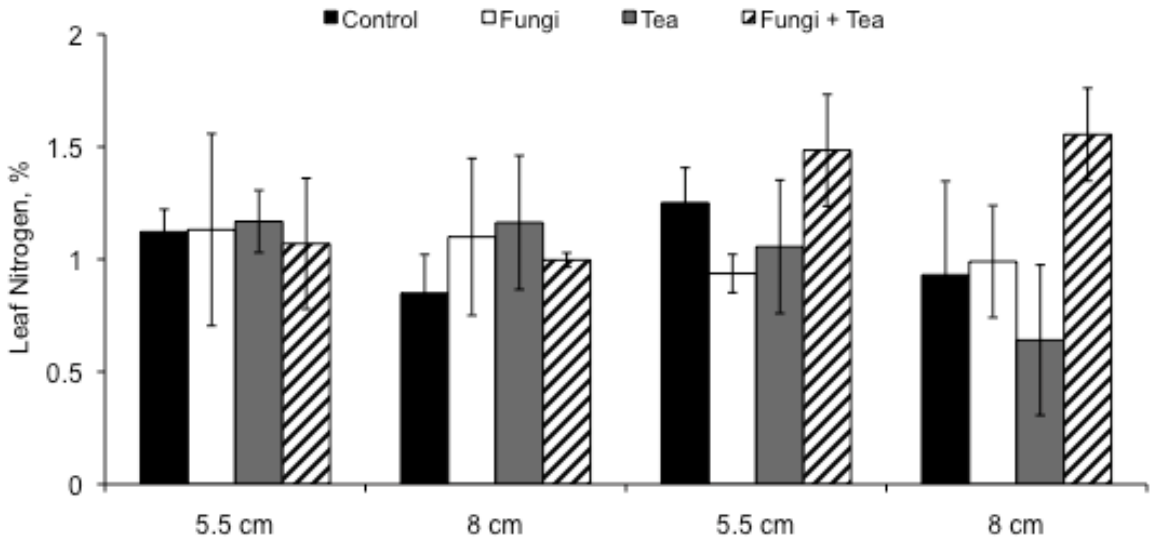

(b

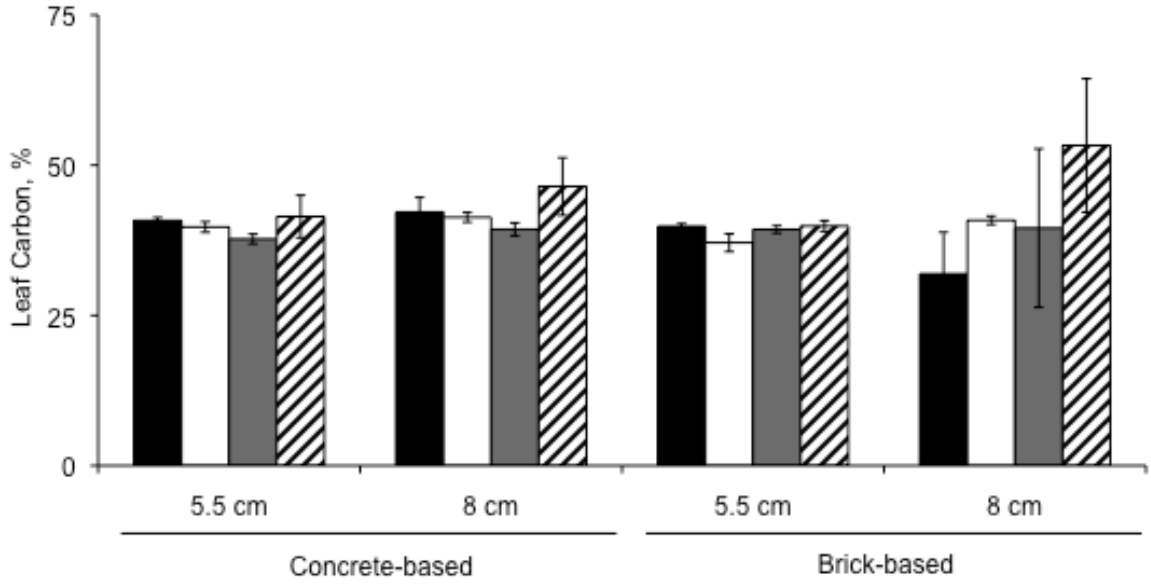

Figure 4. Leaf nitrogen (a) and leaf carbon (b), % content in bait plant shoots from each microbial treatment in 2007. Means from three replicates, bars represent means ± S.E.

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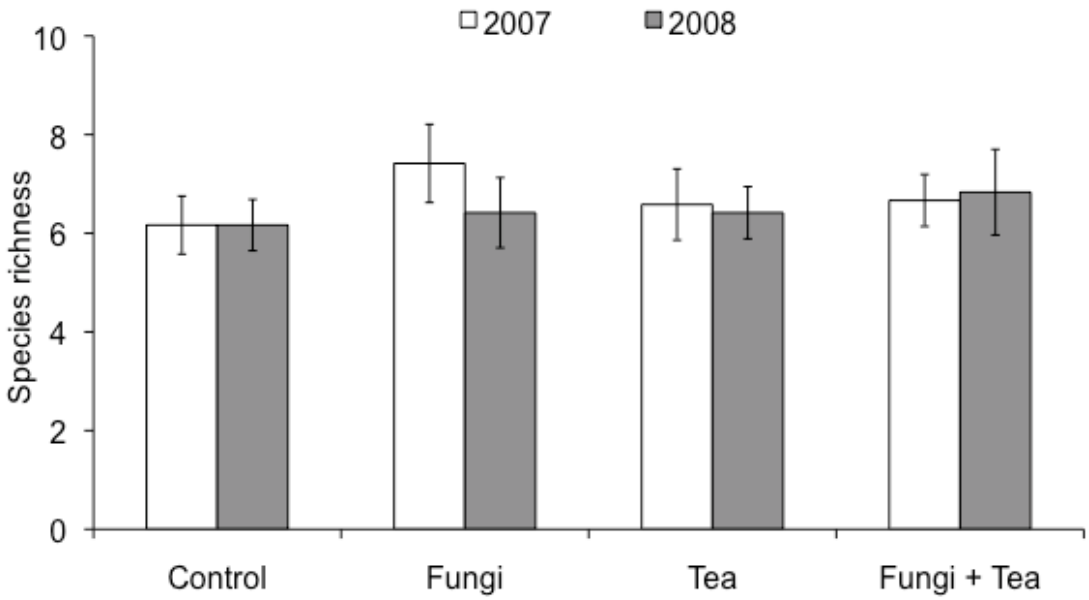
2007
2008
Species richness
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8
6
4
2
0
Control
Fungi
Tea
Fungi + Tea

(b

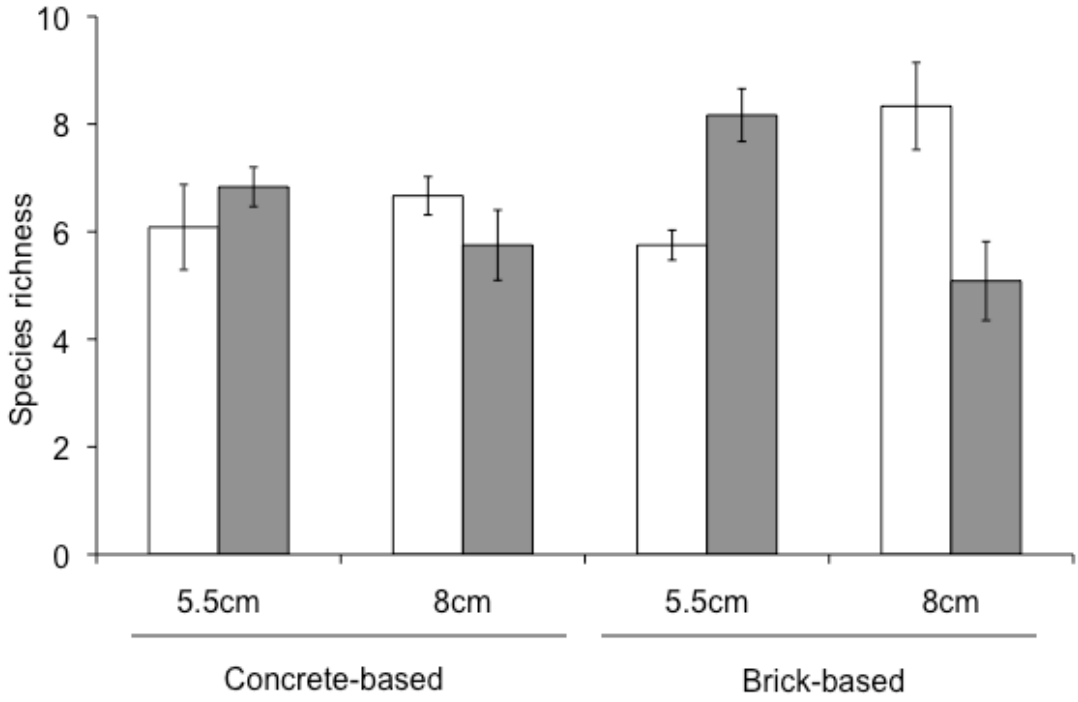
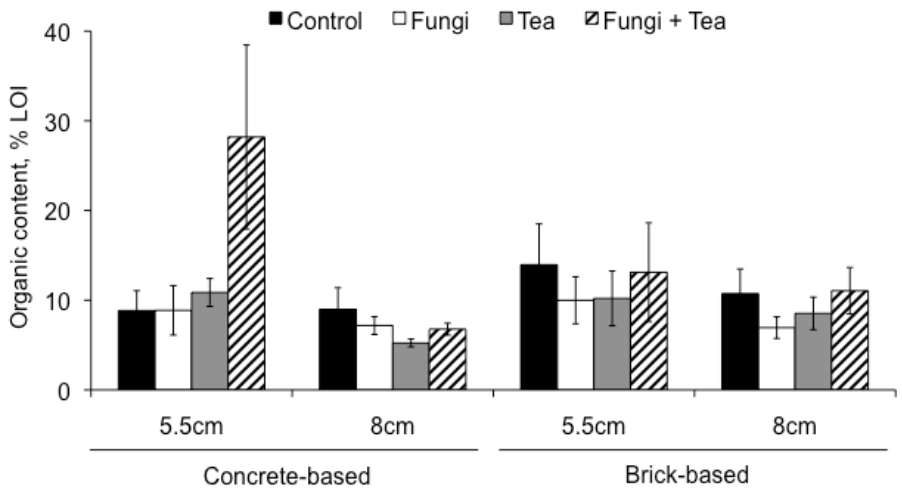


Figure 5. Species richness in (a) the four treatment types irrespective of underlying substrate and (b) in different substrate types, irrespective of treatment where: 2007 = after treatments and 2008 = one year after treatments. Bars represent means ± S.E.

	Baseline				2007				2008		
	Concrete-based		*Brick-based*		*Concrete-based*		*Brick-based*		*Concrete-based*		*Bri*
	5.5 cm	8 cm	5.5 cm	8 cm	5.5 cm	8 cm	5.5 cm	8 cm	5.5 cm	8 cm	5.5 cm
Treatment											
Control	2.14	2.95	0.29	2.21	0.81	0.69	1.45	2.05	0.85	0.86	0.75
Fungi					1.67	0.88	0.93	0.75	0.98	0.85	0.72
Tea					0.41	0.84	4.96	1.00	0.97	1.33	0.73
Fungi + Tea					0.99	1.57	3.40	2.70	0.65	1.01	0.65
Control	1.51	0.63	0.66	1.59	1.14	1.62	1.40	1.79	0.94	1.23	0.76
Fungi					1.18	1.44	0.78	0.78	1.10	1.14	0.84
Tea					1.58	0.82	2.77	2.41	1.15	1.09	0.77
Fungi + Tea					1.53	0.95	1.39	3.13	0.59	0.98	0.57
Control	15.79	16.88	15.15	24.60	5.27	9.08	0.01	0.01	18.42	14.44	18.70
Fungi					7.10	12.23	6.84	12.11	17.16	13.85	18.00
Tea					12.12	4.63	6.65	17.13	14.92	11.13	18.03
Fungi + Tea					10.43	11.00	11.12	4.97	16.78	11.61	17.61

Table 1. Substrate nutrients analysis, with regards to microbial treatment and underlying substrate type and depth on London Zoo green roof experimental site, where: Baseline = before microbial treatments added, 2007 = after treatments and 2008 = one year after treatments applied.

Appendix I
London Zoo Substrate Properties



Appendix Figure 1. Soil organic matter (as % weight loss on ignition) in the different microbial treatments and substrate types in 2007. Means from three replicates and bars represent means ± S.E.

Characteristic	2007	2008
Substrate Water Content (%)	34.8	32.7
Mean rainfall (mm) *	86.9	67.0
Max Temperature (°C)	15.8	15.2
Min Temperature (°C)	8.1	7.6
Sun (hours)	127.4	117.5

Appendix Table 1. London Zoo substrate characteristics. Means taken from 48 experimental plots.
* From Heathrow weather station, 51.479, -0.449, available from Met Office data records.

Appendix II: Statistical Results – ANOVA Table

Main effects & interactions	Nitrates		Phosphates		Potassium	
	F	*P*	*F*	*P*	*F*	*P*
Year	1.31	0.33	**26.08**	**<0.01**	**54.47**	**<0.01**
Substrate type	2.03	0.16				
Substrate depth	0.12	0.72	0.90	0.34	0.02	0.87
Fungi	0.22	0.63	2.16	0.14	0.61	0.43
Tea	1.33	0.25	1.06	0.30	1.01	0.31
			1.31	0.25	0.50	0.48
Year x Substrate type	**4.15**	**<0.05**	**4.90**	**<0.05**	**3.82**	**=0.05**
Year x Substrate depth			0.13		1.30	
Year x Fungi treatment	1.71	0.19	0.70	0.71	3.30	0.25
	0.42	0.52		0.40		0.07
Year x Tea treatment	1.09	0.30	**5.07**	**<0.05**	2.52	0.11
Substrate depth x Substrate type	0.06	0.80	1.44		0.04	0.83
Substrate depth x Fungi treatment	0.19	0.66	0.84	0.23	0.04	0.82
Substrate depth x Tea treatment	0.21	0.64	0.04	0.36	1.26	0.26
Substrate type x Fungi treatment	0.43	0.51	0.12	0.82	0.08	0.77
Substrate type x Tea treatment	1.10	0.29	**4.45**	0.72	1.16	0.28
Fungi treatment x Tea treatment	0.11	0.73	0.01	**<0.05**	0.60	0.43
				0.95		
Year x Substrate type x Substrate depth	1.20	0.27	0.50	0.48	0.01	0.92
	2.17	0.14	1.70	0.19	1.17	0.28
Year x Substrate type x Fungi treatment						
Year x Substrate type x Tea treatment	2.09	0.15	**4.68**	**<0.05**	0.49	0.48
	0.01	0.99	0.18		0.01	0.94
Year x Substrate depth x Fungi treatment	0.19	0.66	0.37	0.66	**4.44**	**<0.05**
	0.94	0.33	0.64	0.54	2.48	
Year x Substrate depth x Tea treatment				0.42		0.12
Year x Fungi treatment x Tea treatment						
Substrate Type x Substrate Depth x Fungi treatment	0.74	0.39	0.54	0.46	1.19	0.28
Substrate type x Substrate depth x Tea treatment	2.18	0.15	1.57	0.21	0.53	0.46
Substrate type x Fungi treatment x Tea treatment	0.60	0.44	0.43	0.51	3.21	0.07
	0.97	0.32	2.84	0.09	0.33	0.56
Substrate depth x Fungi treatment x Tea treatment						

Appendix Table 2. ANOVA results for main effects and interactions with London Zoo substrate nutrients. Showing the *F* statistic and probability value. Degrees of freedom = 1, 51. Significant results highlighted in **bold**.