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Light-dark cycles influence soil bacterial composition *in situ*

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Abstract

Soil bacterial taxa have important functional roles in ecosystems (e.g., nutrient cycling, soil formation, plant health). Many factors influence their assembly and regulation, with land cover type (e.g., remnant vegetation, agriculture, urban parks) and plant-soil feedbacks being two well studied factors. However, changes in soil bacterial communities in situ over light-dark cycles have received little attention, despite plants and some bacteria having endogenous circadian rhythms that could influence soil bacterial communities. We sampled surface soils in situ across 24-hour light-dark cycles (at 00:00, 06:00, 12:00, 18:00) at two land cover types (remnant vegetation vs. cleared, grassy areas) and applied 16S rRNA amplicon sequencing to investigate changes in bacterial communities. We show that land cover type strongly affected soil bacterial diversity, with soils under native vegetation expressing 15.41-16.42% lower alpha diversity but 4.92-10.67% greater heterogeneity than soils under cleared vegetation. In addition, we report time-dependent and site-specific changes in bacterial network complexity and between 598-922 ASVs showing significant changes in relative abundance across times. Native site node degree (bacterial interactions) at phylum level was 16.0% higher in the early morning hours compared to the afternoon/evening. Our results demonstrate for the first time that light-dark cycles have subtle yet important effects on the composition of soil bacterial communities in situ and that land cover influences these dynamics. We provide a new view of soil microbial ecology and suggest that future studies should consider the time of day when sampling soil bacteria.

Original Article

Light-dark cycles influence soil bacterial composition *in situ*

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ABSTRACT

Soil bacterial taxa have important functional roles in ecosystems (e.g., nutrient cycling, soil formation, plant health). Many factors influence their assembly and regulation, with land cover type (e.g., remnant vegetation, agriculture, urban parks) and plant-soil feedbacks being two well studied factors. However, changes in soil bacterial communities*in situ* over light-dark cycles have received little attention, despite plants and some

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bacteria having endogenous circadian rhythms that could influence soil bacterial communities. We sampled surface soils *in situ* across 24-hour light-dark cycles (at 00:00, 06:00, 12:00, 18:00) at two land cover types (remnant vegetation vs. cleared, grassy areas) and applied 16S rRNA amplicon sequencing to investigate changes in bacterial communities. We show that land cover type strongly affected soil bacterial diversity, with soils under native vegetation expressing 15.41-16.42% lower alpha diversity but 4.92-10.67% greater heterogeneity than soils under cleared vegetation. In addition, we report time-dependent and site-specific changes in bacterial network complexity and between 598-922 ASVs showing significant changes in relative abundance across times. Native site node degree (bacterial interactions) at phylum level was 16.0% higher in the early morning hours compared to the afternoon/evening. Our results demonstrate for the first time that light-dark cycles have subtle yet important effects on the composition of soil bacterial communities *in situ* and that land cover influences these dynamics. We provide a new view of soil microbial ecology and suggest that future studies should consider the time of day when sampling soil bacteria.

Keywords : bacterial ecology; biodiversity; circadian rhythm; land cover; soil microbiome

INTRODUCTION

Soil bacterial communities are highly diverse and are involved in many ecosystem processes (e.g., nutrient cycling, soil formation, plant health; Van Der Heijden et al., 2008). Our understanding of soil bacterial communities is increasing due in part to their links with human and ecosystem health (Liddicoat et al., 2019; Roslund et al., 2022; Singh et al., 2023) and advances in genomics (Berg et al., 2020; Liu et al., 2021). While it is understood that plants and soil bacteria have a close relationship, further research is required to fully understand the breadth of connections and mechanisms involved. Filling this knowledge gap will contribute to an improved understanding of soil bacterial ecology, with potential implications for improving ecosystem integrity and soil microbial monitoring approaches.

Land cover type (e.g., forests, lawns) has a strong effect on soil bacterial communities, as demonstrated in many studies where more natural land cover, such as remnant vegetation, has been compared to nearby human-dominated land cover types (e.g., urban parks, agricultural lands; Delgado-Baquerizo et al., 2021; Liddicoat et al., 2019; Wan et al., 2021). While studies have reported higher soil bacterial heterogeneity across samples from remnant vegetation compared to cleared or urban lawns (Delgado-Baquerizo et al., 2021) and the occurrence of notable bacterial functional groups in soils (e.g., butyrate-producers) (Liddicoat et al., 2020; Roslund et al., 2020), further research is needed to improve knowledge on how aboveground biodiversity and land cover types influence soil bacterial communities. Indeed, different land cover and vegetation communities may have subtle impacts on soil bacterial communities that have been largely overlooked.

Plants display diurnal cycles due to endogenous circadian clock genes and *zeitgebers* - external cues that regulate organismal processes to a circadian rhythm or light-dark cycle (from the German terms Zeit "time" + geber "giver"; Hörnlein & Bolhuis, 2021). While many circadian clock genes function independently of light, *zeitgebers*such as light or temperature are crucial for maintaining light-dark cycles (Hörnlein & Bolhuis, 2021; Staley et al., 2017). While some bacteria, such as cyanobacteria, display true circadian rhythms via circadian clock genes, many bacterial taxa do not possess these genes (Kondo et al., 1993). Despite this, all bacteria may still be subject to the influence of *zeitgebers* such as light, temperature, plant processes via plant-soil feedbacks, and bacteria-bacteria interactions (Hörnlein & Bolhuis, 2021; Kelly et al., 2019; Van der Meer et al., 2007). Plants and soils, for example, are intertwined primarily through the *rhizosphere* – the interface between roots and soil (Haichar et al., 2008). In often commensal relationships, plants make exudates available to bacteria (Badri & Vivanco, 2009; Haichar et al., 2008), while bacteria contribute to plants via increased access to soil nutrients and reduced presence of pathogenic bacteria, and these processes can fluctuate over short timeframes (Canarini et al., 2019; Doornbos et al., 2012). Therefore, while not all soil bacteria exhibit*true* circadian rhythms, soil bacterial communities may undergo light-dark cycles in their community composition due to wider ecological processes and zeitgebers.

Light-dark cycles of soil bacterial communities have been studied in greenhouse experiments with model and agricultural plant species*Arabidopsis thaliana* , rice, and barley (Baraniya et al., 2018; Lu et al., 2021; Staley

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et al., 2017). These studies have generally indicated that plant rhizodeposition of carbon and other exudates into the soil affects soil bacterial community growth, composition, and the expression of various genes over light-dark cycles (e.g., rhizodeposition is generally greater during daylight hours), but we found only one study on the effect of light-dark cycles on soil bacterial community composition outside of these model plant systems (Landesman et al., 2019). Improving our understanding of light-dark cycles in natural soil bacterial communities and their interactions has the potential to impact soil bacterial ecology as their composition may change in a time-dependent way.

Accordingly, we aimed to better understand how soil bacterial composition and network complexity change across different land cover types and light-dark cycles. First, we hypothesised that bacterial community composition, diversity (α- and β-diversity), and network complexity would be affected by land cover type. Second, we hypothesised that bacterial community composition (β-diversity and taxa abundances) and networks would be different depending on the time of day because of the influence of plant-soil feedback loops and the strong effect light-dark cycles have on plants (i.e., light and temperature).

METHODS

Study Area

We sampled four plots across two study sites, Mark Oliphant Conservation Park (hereafter called Mark Oliphant CP) and Kenneth Stirling Conservation Park (hereafter called Kenneth Stirling CP) in the Mount Lofty Ranges, Adelaide, South Australia, Australia, on Kaurna and Peramangk Country (Table 1; Figure 1). These sites were selected as they had the required land cover types in close proximity and both had similar pre-European vegetation communities. Each site had adjoining and large (i.e., >1 ha) grassy and native vegetation land cover types. The grassy areas were cleared of all non-grass vegetation (hereafter called 'cleared' plots), and the native woodland areas had remnant vegetation (hereafter called 'native' plots). We established spatially-paired 25 x 25 m (NSEW-oriented) plots in each land cover type at both sites. These paired plots were less than 500 m apart within each site, with native plots situated at least 100 m away from the adjacent cleared land to reduce edge effects (Zhao, Song, et al., 2021). We collected soil moisture and below (-10 cm) and aboveground temperatures (+0 cm, +10 cm) with TMS-4 data loggers (TOMST, Prague, Czech Republic), which were installed at each plot for the duration of the study period (6 weeks), recording at 15-minute intervals (Wild et al., 2019).

Vegetation Surveys

Vegetation surveys at each plot were done between 24 and 26 May, 2022. We used an established vegetation survey approach based on White et al. (2012) with north-south facing point-intercept transect surveys done at 0 m, 5 m, 10 m, 15 m, 20 m, and 25 m of each plot. At each 1 m interval within transects ($n = 156$), we recorded the plant species, growth forms, and ground cover (e.g., leaf litter, bare ground) present to generate plant species richness and growth form/ground cover proportions for each plot.

Soil Sampling

Soil samples were collected to assess bacterial communities (described below) at 6-hour intervals (00:00, 06:00, 12:00, and 18:00). We sampled across a 24-hour sampling period at each plot once per week repeated across 6 consecutive weeks from 14 June to 21 July 2022, resulting in six replicates of each site-land cover-time combination ($n = 6$ replicates x 2 study sites x 2 land cover plots x 4 time intervals = 96 total samples).

Bacterial community soil samples: Soils were collected from the top 10 cm at nine grid points following the Biomes of Australian Soil Environments database protocols (Bissett et al., 2016) using a decontaminated trowel (by using 5% bleach and DECON-90 following protocols in Cando-Dumancela et al., 2021; 2022) and homogenised in a sterile sample bag. From this, subsamples of 50 mL were collected and stored on ice, then frozen at -20@C within one hour of sampling.

Physicochemical soil samples: From the composite soil samples taken for bacterial community analysis, 200 g was subsampled from all 12:00 samples (per plot $n = 6$) and sent for physicochemical analysis. Samples

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were analysed by CSBP Labs (Bibra Lake, WA 6163), quantifying levels of phosphorus, potassium, sulphur, organic carbon, nitrate nitrogen, ammonium nitrogen, electrical conductivity, pH, and texture using protocols described on the service provider's website (https://www.csbplab.com.au/tests/soil).

DNA Extraction, PCR, Sequencing and Bioinformatics

DNA extractions were completed in six batches where soil samples were processed within one week of the sample collection. DNA extractions were done using the Qiagen DNeasy PowerLyzer Soil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and DNA quantification was completed using the QuantiFluor dsDNA System (Promega, Madison, WI, USA) and Quantus Fluorometer (Promega, Madison, WI, USA). The 16S rRNA gene region was targeted using the 27F and 519R primer set (Lane, 1991), PCR-amplified, and sequenced on the Illumina MiSeq platform at the Australian Genome Research Facility (AGRF; Adelaide, SA, Australia). Raw FASTQ files are available at https://figshare.com/s/7cd385ad91893d99ea5a.

FASTQ files were processed through the QIIME 2 bioinformatics pipeline (Bolyen et al., 2019) in a conda environment (Continuum Analytics). Due to poor quality reverse reads, only the forward reads were used. The*Cutadapt* plugin was used to remove primer sequences and quality check the raw sequences (Martin, 2011). The *Figaro* tool was used to identify the optimal trimming lengths (Sasada et al., 2020) for forward reads. These were then trimmed according to the *Figaro*output using *DADA2* (Callahan et al., 2016). Amplicon sequence variants (ASVs) were assigned using the *SILVA* version 138.1 rRNA database (Glöckner et al., 2017; Yilmaz et al., 2014; Quast et al., 2013) via the q2-feature-classifier (Bokulich et al., 2018) and classify-sklearn naïve Bayes taxonomy classifier.

Statistical Analysis

All statistics were done in R v4.1.2 (R Core Team, 2021). Soil temperature, moisture, and physicochemical characteristics were analysed with Kruskal-Wallis tests.

Bacterial diversity

The *phyloseq* package (McMurdie & Holmes, 2013) was used for downstream analyses of bacterial community data. Amplicon sequence variants (ASVs) were discarded if they were not classified as Bacteria, classified as "Mitochondria", "Chloroplast", or were not present in at least two samples. Samples were then rarefied to the sample with the lowest read depth (Hong et al., 2022). Rarefied samples were used to assess beta diversity between site, land cover type, and times of the day. Bray-Curtis distances were visualised in a principal coordinates analysis plot using the *ordinate* function in *phyloseq* and permutational multivariate analysis of variance (PERMANOVA) applied with a Type I sum of squares using the *adonis* function in the*vegan* package (Oksanen et al., 2013). The effective number of ASVs (alpha diversity) was calculated using the exponent of Shannon's diversity index values (Jost, 2006). Kruskal-Wallis tests were used to assess the differences in bacterial alpha diversity between sites, land cover types, and times of the day.

Network analysis

To understand bacterial interactions and community structure (i.e., complexity) across sites, land cover types, and the four sampling times (00:00, 06:00, 12:00, 18:00), we evaluated co-occurrence association networks of bacterial ASVs. We constructed networks at the phylum taxonomic level to examine broad complexity and the genus level to visualise higher resolution complexity, recognising that the accuracy of species-level associations would be low due to the high similarity between the 16S rRNA gene from closely related species. In the evaluated networks, vertices (also known as 'nodes') represent ASVs and edges (also known as 'links') connect a pair of ASVs if their frequencies are significantly associated (absolute abundance >0.75, $p = [?]0.01$ for phylum and >0.95,$p = [?]0.01$ for genus). The type of association, whether positive (representing a mutualistic interaction) or negative (representing an antagonistic interaction), was denoted with blue and red edges, respectively. To account for compositional bias associated with ASV data, we used *SparCC* (Friedman & Alm, 2012) to define associations. Only ASVs with sequence counts >10 were included for phylum and >100 for genus to improve visualisation, selection rigour and computational processing. Randomly permuted

(n = 100) data were used to estimate the statistical significance of associations. We used the R package *Matrix* (Bates et al., 2023) to create a matrix from the given set of values and*igraph* (Csardi et al., 2023) to visualise and evaluate the plots.

Differential abundance analysis

Separately within each of the four plots (2 sites x 2 land cover types), globally differentially abundant ASVs across sampling times were identified using the *ANCOM-BC* algorithm (Lin & Peddada, 2020) by assessing log-fold-changes in ASV abundances at 06:00, 12:00, and 18:00 sampling times, compared to midnight (00:00) as the intercept/baseline sampling time. We used the *ancombc()* function in the R*ANCOMBC* package (https://github.com/FrederickHuangLin/ANCOMBC) with settings including alpha = 0.05 significance level, p-value adjustment for multiple comparison using the method of Holm (1979), taxa with fractional prevalence less than 0.1 (= prv_cut) were excluded in the analysis, structural zeros were detected (struc_zero = TRUE) with taxa classified as structural zeros using asymptotic lower bounds (neg_lb = TRUE), and a conservative variance estimator was used for the test statistic (conserve = TRUE). We used the *ANCOM-BC* global test result which identifies differentially abundant taxa between at least two groups across three or more different groups. To determine whether differentially abundant ASVs were shared across the plots, we constructed Venn diagrams to display the number of overlapping or non-overlapping differentially abundant ASVs across plots and comparison periods (06:00 cf. 00:00, 12:00 cf. 00:00, and 18:00 cf. 00:00).

RESULTS

Vegetation

The vegetation communities at Mark Oliphant CP and Kenneth Stirling CP were native eucalypt woodlands with a shrubby understory. The cleared plots were characterised by graminoid and herb species (Figure S1), with a higher plant species richness in Mark Oliphant CP compared to Kenneth Stirling CP (Mark Oliphant CP: n = 8; Kenneth Stirling CP: n = 6; Figure 1b, d; Table 1). *Eucalyptus obliqua* and *E. baxteri*dominated the canopy at both native plots, with a range of small trees, shrubs, herbs, and graminoids in the understory (Figure 1c, e; Figure S1). Plant species richness was greater in Mark Oliphant CP compared to Kenneth Stirling CP (Mark Oliphant CP: n = 22; Kenneth Stirling CP: n = 18; Table 1).

Soil Temperature, Moisture and Physicochemical Characteristics

Soil temperature and moisture data were collected at each plot at 15-minute intervals for the duration of the study. Within Kenneth Stirling CP, soil temperature 10 cm below ground was higher in the cleared land cover plot (mean +- SD; cleared: 9.01degC +- 1.07degC; native: 8.79degC +- 1.06degC; chi-squared = 77.81, $p < 0.001$; Table 1). The same pattern followed at Mark Oliphant CP with higher soil temperature 10 cm below ground in the cleared land cover plot (cleared: 9.80degC +- 1.54degC; native: 9.57degC +- 1.16degC; chi-squared = 58.70, $p< 0.001$; Table 1). Soil temperatures at 0 cm and 10 cm above ground followed the same patterns in both Kenneth Stirling CP and Mark Oliphant CP (Table 1). Similarly, mean soil moisture was higher in the cleared land cover plot at Kenneth Stirling CP (cleared: 50.07% +- 2.13%; native: 41.53% +- 2.12%; chi-squared = 5924.3, $p< 0.001$; Table 1) and Mark Oliphant CP (cleared: 47.02% +- 1.81%; native: 37.25% +- 3.63%; chi-squared = 5689.1, $p< 0.001$; Table 1).

Soil physicochemical characteristics differed dramatically between plots but without clear patterns. At Mark Oliphant CP, organic carbon was higher in the native land cover plot (mean +- SD; cleared: 2.85% +- 0.14%; native: 4.12% +- 0.47%; P-adj < 0.001, 95% CI [-1.75, -0.784]; Table S2), but was lower in the native land cover plot at Kenneth Stirling CP (cleared: 3.20% +- 0.21%; native: 2.57% +- 0.27%; P-adj = 0.008, 95% CI: [0.139, 1.104]; Table S2). Sulphur was also greater in the native land cover plot at Mark Oliphant CP (cleared: 5.03 mg/kg +- 0.50 mg/kg; native: 9.00 mg/kg +- 3.00 mg/kg; P-adj = 0.004, 95% CI [-6.71, -1.22]; Table S2) but was not different between land cover types at Kenneth Stirling CP (cleared: 6.67 mg/kg +- 1.30 mg/kg; native: 4.60 mg/kg +- 0.76 mg/kg; P-adj = 0.185, 95% CI [-0.679, 4.81]; Table S2). Notably, potassium, phosphorus, and pH were substantially higher in the cleared land cover plot at Kenneth Stirling CP compared to all other plots (Table S2).

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Bacterial Community and Diversity

After cleaning and filtering of sequence data, a total of 12,173 bacterial amplicon sequence variants (ASVs) were detected across the 96 samples (Table S1). Sample reads were rarefied to the lowest read count of 23,073 reads (from #30 sample – KSN 00:00 week 6; Table S1). The effective number of ASVs was calculated to compare alpha diversity between sites, land cover, and times. Alpha diversity at Mark Oliphant CP was higher than Kenneth Stirling CP (mean +- SD, Mark Oliphant CP: 554 +- 132 effective number of ASVs; Kenneth Stirling CP: 479 +- 149 effective number of ASVs; chi-squared = 6.28, $p = 0.012$; Figure 2a). Alpha diversity was higher at the cleared land cover type at Kenneth Stirling CP (cleared: 519 +- 123 effective number of ASVs; native: 439 +- 164 effective number of ASVs; chi-squared = 4.42, $p = 0.035$) and at Mark Oliphant CP (cleared: 603 +- 104 effective number of ASVs; native: 504 +- 139 effective number of ASVs; chi-squared = 8.82, $p = 0.003$; Figure 2a).

Time did not have an effect on alpha diversity in Kenneth Stirling CP at either the cleared (mean +- SD; 00:00: 505 +- 109 effective number of ASVs; 06:00: 513 +- 132 effective number of ASVs; 12:00: 529 +- 161 effective number of ASVs; 18:00: 529 +- 119 effective number of ASVs; chi-squared = 0.313, $p = 0.957$; Figure 2b) or native land cover plot (00:00: 520 +- 140 effective number of ASVs; 06:00: 339 +- 180 effective number of ASVs; 12:00: 462 +- 203 effective number of ASVs; 18:00: 436 +- 99.4 effective number of ASVs; chi-squared = 4.547,$p = 0.208$; Figure 2b). Time also had no effect on alpha diversity at Mark Oliphant CP at the cleared (00:00: 602 +- 134 effective number of ASVs; 06:00: 589 +- 88.3 effective number of ASVs; 12:00: 591 +- 82 effective number of ASVs; 18:00: 631 +- 127 effective number of ASVs; chi-squared = 0.393, $p = 0.942$; Figure 2c) or native land cover type (00:00: 591 +- 188 effective number of ASVs; 06:00: 518 +- 103 effective number of ASVs; 12:00: 450 +- 105 effective number of ASVs; 18:00: 457 +- 129 effective number of ASVs; chi-squared = 2.61, p= 0.456; Figure 2c).

The principal coordinate analysis and PERMANOVA based on Bray-Curtis distance showed that land cover ($p = 0.001$) had a stronger and significant effect on beta diversity than site ($p = 0.001$) and time ($p = 1.0$) (Figure 2d). The site effect was stronger in the cleared land cover types, with Kenneth Stirling CP and Mark Oliphant CP samples separating more distinctly than in the native land cover (Figure 2d). There was also a greater bacterial community heterogeneity measured by distance to the centroid in the native land cover plot compared to the cleared land cover plot in Kenneth Stirling CP (mean +- SD; cleared: 0.477 +- 0.062 distance to centroid; native: 0.534 +- 0.0456 distance to centroid; P-adj = 0.001, 95% CI [-0.096, -0.018]; Figure 3). The same trend was seen in land cover at Mark Oliphant CP though this was not significant (cleared: 0.483 +- 0.046; native: 0.508 +- 0.040; P-adj = 0.351, 95% CI [-0.063, - 0.014]; Figure 3).

Bacterial networks

The association network analyses highlighted differences in community complexity and interactions across sampling times and land cover types, defined by node degree (the average number of edges connecting the vertices), network size, and edge weight (i.e., whether the interactions were positive or negative) (Figure 4). At the phylum level, bacterial ASVs in the cleared plots had a higher node degree (total x node degree = 45.2) than the native plots (total x node degree = 36) (t = -3.18, df = 148, $p = <0.01$).

There were also clear differences in the node degree across sampling times. The Mark Oliphant CP cleared plot had a steadily increasing node degree from 00:00 through to 18:00. Moreover, the mean node degree was typically higher for native plots between 00:00 and 06:00 (combined x node degree =19.9) than between 12:00 and 18:00 (combined x node degree =16.1) (t = 2.22, df = 80, $p = 0.02$) but was generally lower for cleared plots between 00:00 and 06:00 (combined x node degree =18.4) than between 12:00 and 18:00 (combined x node degree =26.8) (t = -4.36, df = 83, $p = <0.01$).

Evaluation of either positive or negative edge types highlighted differences across land cover types with more negative associations among ASVs in the native plots. One striking finding was that the mean edge weight between 00:00 and 06:00 in the native Mark Oliphant CP plot dropped from 0.88 (a high level of positive interactions) to 0.10 (a high level of negative interactions).

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At the genus level (Figure 5), bacterial ASVs in the cleared plots had a higher node degree (total x node degree = 27.6) than the native plots (total x node degree = 20.3), as expected following the phylum-level analysis. There were also clear differences in the node degree across sampling times; however, the patterns were variable. The native Kenneth Stirling CP plot had a steadily decreasing node degree from 00:00 through to 18:00. The cleared Kenneth Stirling CP plot had a steadily increasing node degree from 18:00 to 12:00. There were also differences in edge weights across site types with more negative associations among ASVs in the native sites (combined x edge weight = 4.78) than cleared plots (combined x edge weight = 5.47). The striking difference in mean edge weight between 00:00 and 06:00 in the native Mark Oliphant CP plot was also visible in the genus-level network plots. Conversely, the mean edge weight *increased* between native Kenneth Stirling CP plot at 00:00 and 06:00.

The evaluation of hub taxa (bacterial groups with the highest degree of either positive or negative associations) showed that the keystone bacteria at the genus level were different at each sampling time for both the native and cleared plots, with positive degree ranging from 4 to 19 and negative degree ranging from 0 to 15 (Table 2). At the phylum level, Acidobacteriota were the most common taxa with the highest number of positive associations, occurring in native Kenneth Stirling and Mark Oliphant CP plots at 18:00 and the cleared Mark Oliphant CP plot at 06:00 and 12:00. Bdellovibrionota were the top negative hub taxa, featuring in native Kenneth Stirling CP 00:00, native Mark Oliphant CP 12:00, and cleared Mark Oliphant CP 12:00.

Differentially abundant ASVs

Across all the plots, sample ASV relative abundances (%) and corresponding log-fold-change results from ANCOM-BC differential abundance testing were highlighted for the top 50 ASVs in each plot that displayed the largest absolute magnitudes of log-fold change between comparison groups (see Figures S2-S5; Tables S3-S6). These most fluctuating ASVs were predominantly from the phyla Acidobacteriota, Actinobacteriota and Proteobacteria (Tables S3-S6). Respective total numbers of taxa from each plot with at least one occasion of significant differential abundance at later measurement times (06:00, 12:00, 18:00) compared to the midnight (00:00) baseline/intercept were 853 ASVs at the cleared Kenneth Stirling CP, 598 ASVs at native Kenneth Stirling CP, 922 ASVs at cleared Mark Oliphant CP, and 685 ASVs at the native Mark Oliphant CP plot. Time-series changes in relative abundance (%) of these differentially abundant ASVs over the sampling times were also visualised for each site (Figure S6). Non-trivial cohorts of taxa appeared to be varying temporally over the 24-hour period, and these fluctuating taxa were largely plot-specific (see Venn diagrams, Figure S7). Within a plot, numbers of taxa typically in the range of 200-400 ASVs were either increasing or decreasing when comparing baseline midnight (00:00) samples to later measurement times (06:00, 12:00, 18:00). Very few of the ASVs displaying temporal differential abundance were shared across plots, with approximately 1-4% of all differentially abundant (or in the order of 10-50 ASVs) shared between sites.

DISCUSSION

We examined changes in soil bacterial communities with land cover type and light-dark cycles. We investigated these relationships by repeatedly sampling soil at two spatially-paired plots at two South Australian conservation parks (four plots in total) over six weeks. From these soils, we characterised the bacterial community via 16S rRNA amplicon sequencing and generated edaphic physicochemical data (e.g., organic carbon, pH, sulphur, phosphorus). We showed that land cover type strongly affected soil bacterial diversity with soils under native vegetation expressing lower alpha diversity but greater heterogeneity than soils under cleared vegetation. We report time-dependent and site-specific changes in bacterial network complexity and relative abundance of many taxa. Our results demonstrate for the first time that light-dark cycles have subtle yet important effects on the composition of soil bacterial communities *in situ* .

Effect of Land Cover Type on Soil Bacterial Diversity

We show that soil bacterial alpha diversity was generally higher in the cleared land cover plots but that these plots had less heterogeneous bacterial communities – results that are consistent with previous work on the effects of urban versus natural land cover on soil bacteria. The global study by Delgado-Baquerizo et

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al. (2021) showed that soil bacterial communities from human-impacted locations had consistently higher alpha diversity compared to their paired natural ecosystem locations. Similar results were found by Han et al. (2021) comparing different vegetation communities in more urban and more natural areas. Delgado-Baquerizo et al. (2021) also showed that bacterial communities were more homogenous across urban and/or more human-impacted soils. They suggested this was primarily driven by similarities in urban soil management practices and land cover changes. In our case, both cleared land cover plots have been historically impacted by humans through the clearing of all non-grass vegetation and continued mowing.

Similarly, our network analysis provided supporting evidence that soil bacterial community complexity was affected by land cover type. We saw fewer bacterial interactions and network complexity (lower connectance) in our native plots. Moreover, we found more positive interactions in our cleared plots and more negative interactions in the native plots. The presence of more positive associations in the cleared plots suggests these bacteria may undergo greater cooperation for resources or a lack of competition among the interacting bacteria. The results of our 'hub taxa' analysis showed that the bacteria with the highest node degree at the genus level were different at each land cover type. This suggests that the hub taxa have only a fleeting influence on the network structure across time. However, when examining the hub taxa at the phylum level, we found that Gram-negative Acidobacteriota groups were most prevalent, featuring in the top position (highest node degree for positive interactions) twice for both land cover types. This suggests that Acidobacteriota may have a fundamental mutualistic role to play in the rhizosphere. This is corroborated by the literature, which confirms Acidobacteriota roles in C- and N-cycling and plant health, amongst other functions (Kalam et al., 2020; Huber et al., 2022). These ecological functions could conceivably influence rhizosphere bacterial interactions. Regarding negative hub taxa (bacterial groups with an antagonistic association), Bdellovibrionota had the highest node degree for negative interactions, featuring twice in each land cover class. These bacteria are often obligate aerobic predators (Ortiz et al., 2021), consuming Gram-negative bacteria, which could potentially help explain their negative association in our networks.

Additionally, we found that soil temperatures and moisture were greater in the cleared plots than paired native plots, a pattern that was similarly noted by Delgado-Baquierizo et al. (2021). Soil bacteria are generally sensitive to changes in soil temperature and moisture (Wu et al., 2015), which may influence the soil bacterial communities (Delgado-Baquerizo et al. 2021). The presence of distinct soil bacterial communities in different land cover types highlights the need to not only conserve soil bacterial biodiversity present in our natural areas but also to support the development of ways to restore soil bacteria in modified areas for soil biodiversity conservation.

Effect of Light-Dark Cycles on Soil Bacterial Communities

Here, we detected numerous differentially abundant ASVs that displayed fluctuating patterns in ASV relative abundance and differences in network complexity over the 24-hr sampling period. We found that mean node degree (connectance) was significantly higher between 00:00 and 06:00 than between 12:00 and 18:00 for the native plots but significantly lower for the cleared sites between these times. This indicates a level of time-dependent changes in bacterial network complexity with inter-site variability. In other words, it appears that native site bacterial interactions increased in the early morning hours when it is darker (and colder) and decreased in the afternoon/evening when it is lighter, and vice versa for the cleared plots. Further, the 'hub taxa' analysis results showed that the bacteria with the highest node degree at the genus level were different at different sampling times. These findings support that light-dark cycles mediate changes in bacterial interactions (e.g., driven by time-dependent rhizosphere activity). One possible explanation for this light-dark cycling could be the transmission of biological rhythms from plants to the soil bacteria (Newman et al. 2022), as plants alter the physicochemical properties of the soil. Soil temperature, moisture and respiration also vary diurnally (Hu et al., 2016), which could affect these microbial interactions, as could methane fluxes via plant exudates or other microorganisms (Subke et al., 2018). The time-dependent changes observed in bacterial network complexity and ASV relative abundances suggest that the time of sampling should be considered in soil microbial studies. The differences in bacterial interactions between land cover types (between day and night) are equally as interesting. This inter-site variation suggests that

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vegetation complexity may influence rhizosphere microbial community interactions in combination with light-dark cycles. More research is needed to determine the drivers of this variation. However, we can speculate that vegetation community-mediated differences in soil biogeochemistry between the more complex remnant vegetation (i.e., our native plots) and the less diverse lawn (i.e., our cleared plots) sites—-resulting from factors such as variation in transpiration, shade, exudation, and pH—-may be responsible.

We did not observe a strong effect of time or light-dark cycles on the soil bacterial alpha or beta diversity at the community level in any plot. We are aware of no studies that have focused on characterising the light-dark cycles of soil bacterial communities *in situ* and to our knowledge, very few have used DNA-based approaches to study soil bacterial light-dark cycles. Landesman et al. (2019) was the only study prior to ours that had considered the effect of light-dark cycles on bacterial communities however, their primary focus was on seasonal variation. Light-dark patterns in bacterial communities were observed in the rhizospheres of *Arabidopsis thaliana* and rice in a range of*ex situ* greenhouse studies (Lu et al., 2021; Staley et al., 2017; Zhao, Ma, et al., 2021; 2022). It is possible that our study did not detect a light-dark effect at the sample community level because the soils were pooled at the plot level and did not specifically target root rhizospheres (separate from bulk soils), as per the studies mentioned above. While there is variation in the life cycles of different bacterial taxa, the bacterial turnover rates in bulk soils (further away from the rhizosphere) are expected to be slower and therefore may not vary over such short temporal scales (Joergensen & Wichern, 2019; Sokol et al., 2022). However, as used in some greenhouse experiments (Baraniya et al., 2018; Dai et al., 2022; Staley et al., 2017), transcriptomics may reveal new insights into the light-dark cycles of bacterial community activity and plant exudates in vegetation community-wide studies. Future studies could apply these transcriptomic approaches that generate activity level data of soil bacterial communities rather than focusing on changes in community composition.

Conclusions

Soil bacterial communities can be influenced by many factors (Sokol et al., 2022). We highlight the well-studied impact of land cover type on soil bacterial composition, diversity and network connectedness. These findings highlight the need for further improved ecosystem restoration practices that target the soil microbiome, which is a nascent field of research (Farrell et al., 2020; Mohr et al., 2022). Our study also provides a new look into soil microbial ecology by observing *in situ* changes in soil bacterial communities across light-dark cycles, which is a new lens through which to study soil microbial ecology. Future soil bacteria studies using DNA-based tools should improve the control of sampling time to avoid introducing unwanted noise into their soil bacterial datasets.

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Competing interests

The authors declare no competing interests.

Data accessibility

Raw FASTQ files and sample metadata are available on figshare (https://figshare.com/s/7cd385ad91893d99ea5a) and will be made available on the Sequence Read Archive (SRA) upon publication.

Benefit-generated

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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Author Contributions

Nicole W. Fickling, Catherine A. Abbott, Joel E. Brame, and Martin F. Breed contributed to research design. Nicole W. Fickling, Joel E. Brame, and Christian Cando-Dumancela collected and process samples. Nicole W. Fickling, Craig Liddicoat, and Jake M. Robinson contributed to bioinformatics and data analysis. Nicole W. Fickling wrote the draft manuscript, and all authors contributed to reviewing and editing the manuscript.

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Table 1 . Plot metadata, showing location, land cover type, vegetation metrics, soil temperature (10 cm below ground, 0 cm above ground, and 10 cm above ground, and soil moisture (10 cm below ground).

Plot	Latitude	Longitude	Elevation (m)	Plant species richness	Canopy cover (%)	Leaf litter (%)	Soil temp -10 cm (°C)	Soil temp +0 cm (°C)	Soil temp +10 cm (°C)
Mark Oliphant CP Cleared	-35.21355	138.42472	369	8	0.01	0.100	9.80 ± 1.54	8.73 ± 1.54	8.36 ± 3.85
Mark Oliphant CP Native	-35.21215	138.42189	370	22	27.07	26.03	9.57 ± 1.16	8.56 ± 2.43	8.28 ± 2.96

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Plot	Latitude	Longitude	Elevation (m)	Plant species richness	Canopy cover (%)	Leaf litter (%)	Soil temp -10 cm (°C)	Soil temp +0 cm (°C)	Soil temp +10 cm (°C)
Kenneth Stirling CP Cleared	-34.96686	138.77683	512	6	0.0	39.06	9.01 ± 1.07	8.35 ± 2.78	8.09 ± 2.93
Kenneth Stirling CP Native	-34.96762	138.77817	512	18	29.66	27.82	8.79 ± 1.06	7.97 ± 2.12	7.88 ± 2.49

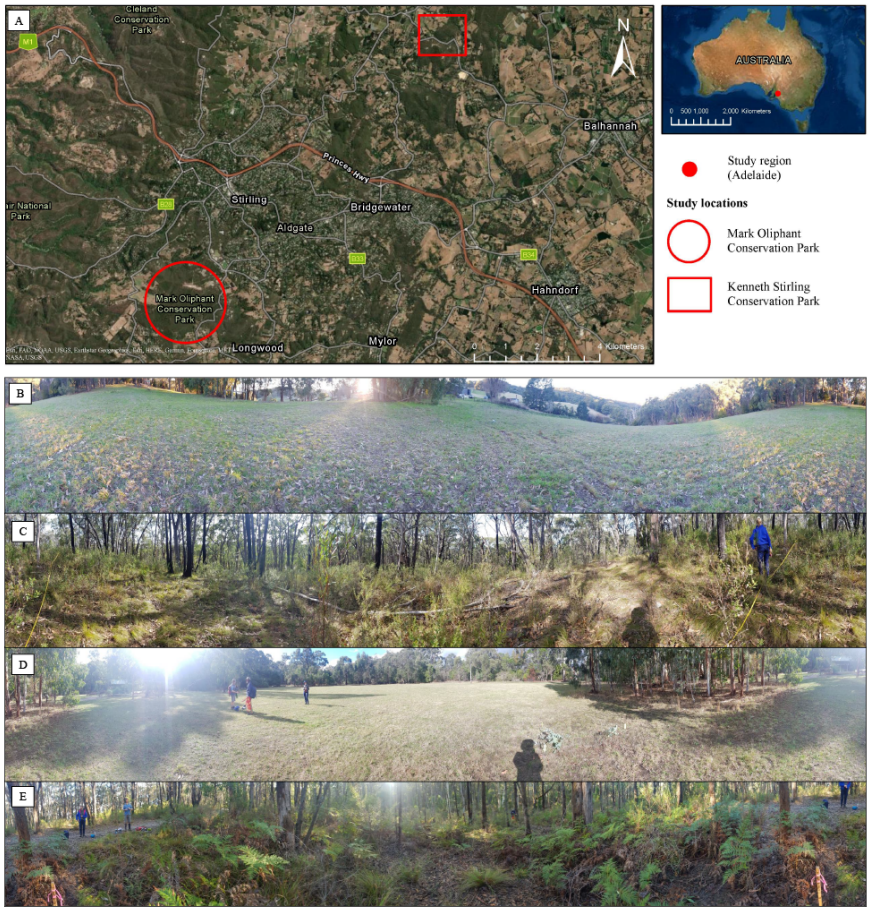

Figure 1. Map of study region and panoramic photographs of the four study plots. (A) Map of study region; (B) Cleared land cover plot at Kenneth Stirling Conservation Park; (C) Native land cover plot at Kenneth Stirling Conservation Park; (D) Cleared land cover plot at Mark Oliphant Conservation Park; (E) Native land cover plot at Mark Oliphant Conservation Park. (Photo credits: Nicole Fickling)

Table 2 . Bacterial taxonomic groups with the most positive and negative edges for each plot/time. The top positive and negative degrees are shown for each taxon, and the table includes the results for the phylum and genus levels.

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Plot/time	**Top positive taxa**	**Top negative taxa**	**Positive degree**	**Negative degree**
Phylum				
Native KS 00:00	Bacteroidota	Bdellovibrionota	9	5
Native KS 06:00	Cyanobacteria	SAR324_clade	6	9
Native KS 12:00	Planctomycetota	N/A	8	N/A
Native KS 18:00	Acidobacteriota	Firmicutes (Bacillota)	7	6
Native MO 00:00	Dependentiae	N/A	12	N/A
Native MO 06:00	SAR324_clade	Proteobacteria	9	8
Native MO 12:00	Armatimonadota	Bdellovibrionota	6	8
Native MO 18:00	Verrucomicrobiota	Firmicutes (Bacillota)	7	7
Grass KS 00:00	Patescibacteria	Elusimicrobiota	11	10
Grass KS 06:00	Acidobacteriota	Myxococcota	9	6
Grass KS 12:00	Gemmatimonadota	Latescibacterota	12	5
Grass KS 18:00	Bdellovibrionota	N/A	10	N/A
Grass MO 00:00	Fibrobacterota	Planctomycetota	5	2
Grass MO 06:00	Acidobacteriota	Latescibacterota	10	4
Grass MO 12:00	Acidobacteriota	Bdellovibrionota	11	1
Grass MO 18:00	Elusimicrobiota	Methylomirabilota	13	3
Genus				
Native KS 00:00	*Armatimonadales*	*Nakamurella*	6	11
Native KS 06:00	*Caulobacter*	*CPla-3 termite group*	6	10
Native KS 12:00	*Tundrisphaera*	*Gaiella*	7	7
Native KS 18:00	*Pseudomonas*	*Actinomycetospora*	7	6
Native MO 00:00	*Vicinamibacteraceae*	*TK10*	11	1
Native MO 06:00	*Jatrophihabitans*	*Inquilinus*	4	7
Native MO 12:00	*Aquisphaera*	*Granulicella*	13	5
Native MO 18:00	*TRA3-20*	*Subgroup_7*	5	4
Grass KS 00:00	*RB41 (Acidobacter)*	*Jatrophihabitans*	7	5
Grass KS 06:00	*CPla-3 termite group*	*Occapllatibacter*	11	6
Grass KS 12:00	*WPS-2*	*Subgroup_7*	12	14
Grass KS 18:00	*Streptomyces*	*Kribbella*	6	3
Grass MO 00:00	*Geodermatophilus*	*Nakamurella*	13	10
Grass MO 06:00	*Anaeromyxobacter*	*Granulicella*	10	15
Grass MO 12:00	*A0839*	*Candidatus*	10	11
Grass MO 18:00	*vadinHA49*	*Obscuribacteraceae*	19	13

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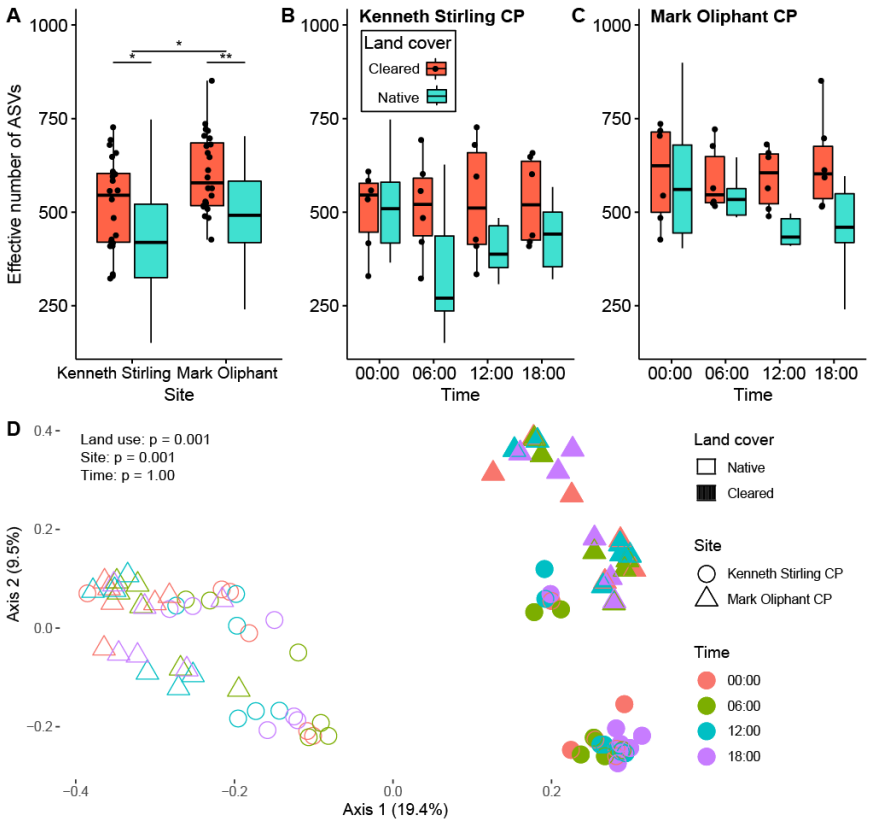

Figure 2. 16S rRNA amplicon sequencing results . Cleared land cover plots are shown in red and native land cover plots are shown in turquoise. Effective number of ASVs between (A) sites/land cover, (B) times at Kenneth Stirling CP, and (C) times at Mark Oliphant CP. Principal coordinates analysis plot using Bray-Curtis distance (D). * $=p < 0.01$, ** $= p < 0.001$.

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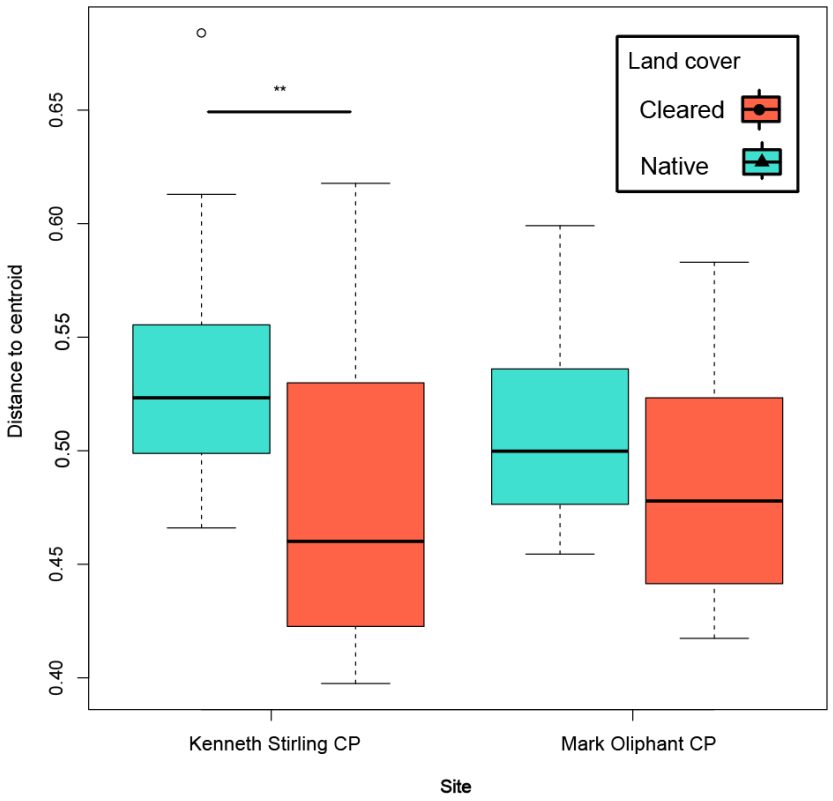

Figure 3. Distance to centroid of samples comparing site/land cover types. Cleared land cover plots are indicated in red and native land cover plots indicated in turquoise. Distance to centroid calculated on the Bray-Curtis distance in a principal coordinates analysis space. ** = $p < 0.001$.

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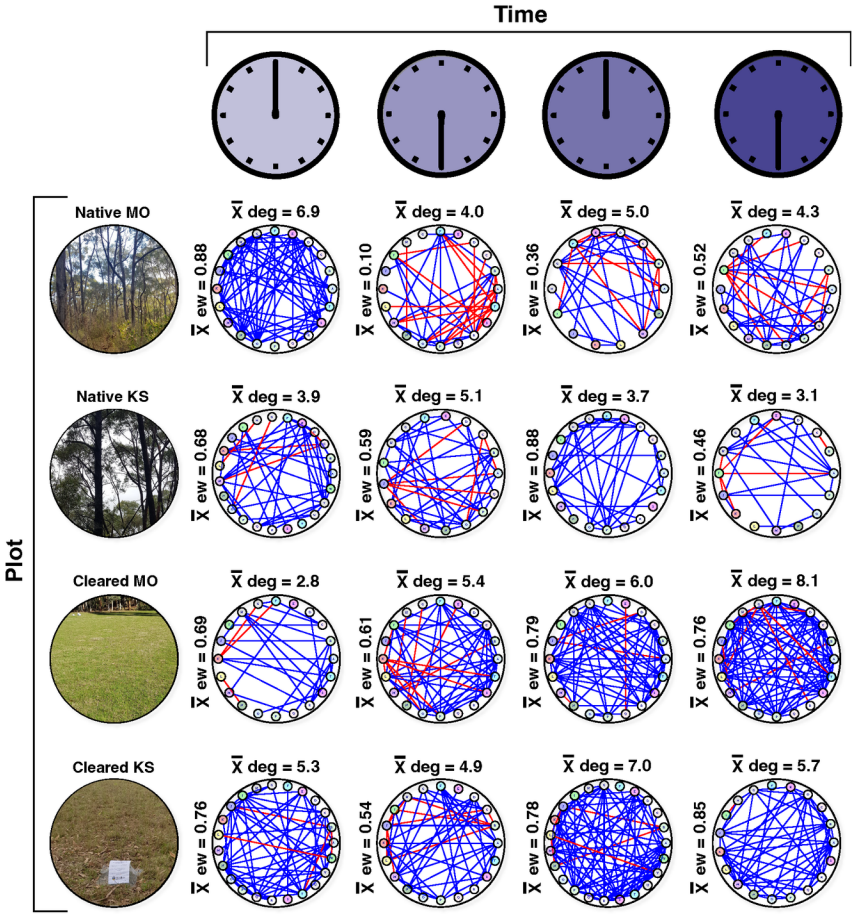

Figure 4. Association network plots of bacterial ASVs for the four plots (Native MO, Native KS, Cleared MO, and Cleared KS) and sampling times (00:00, 06:00, 12:00, and 18:00). Vertex colour represents taxonomic groups at the phylum level (see Supplementary Materials for more information). Blue edges represent positive associations, and red edges represent negative associations. The mean degree is shown above each network, and the mean edge weight (ew) is to the left of each network. MO = Mark Oliphant CP; KS = Kenneth Stirling CP.

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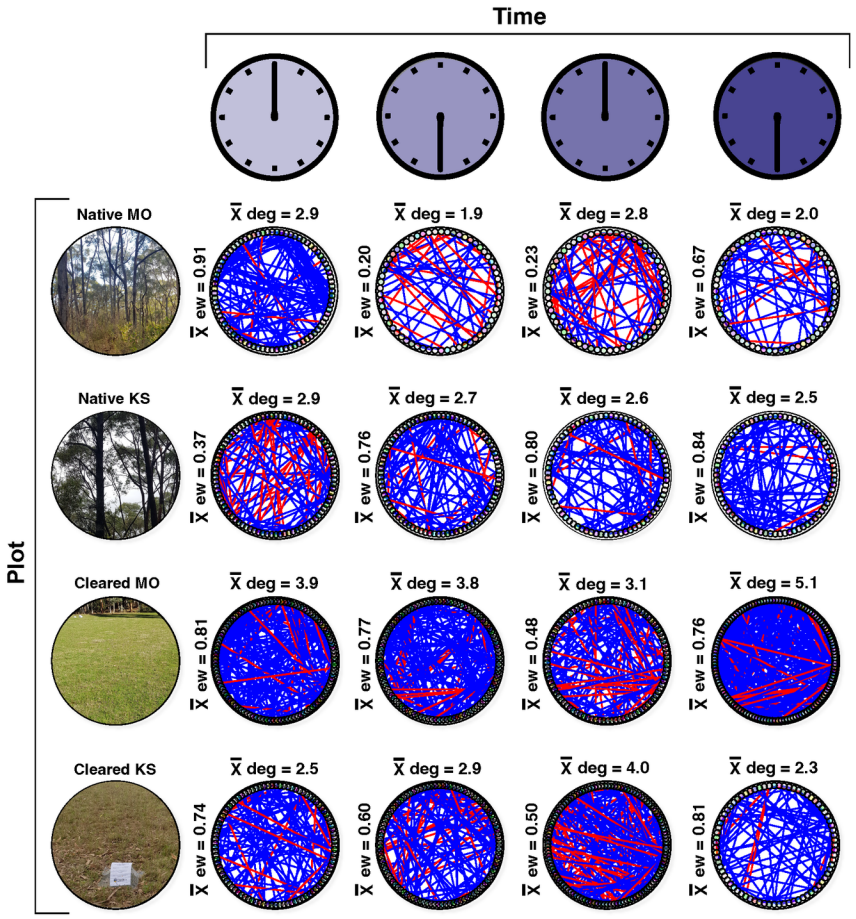

Figure 5. Association network plots of bacterial ASVs for the four plots (Native MO, Native KS, Cleared MO, and Cleared KS) and sampling times (00:00, 06:00, 12:00, and 18:00). Vertex colour represents taxonomic groups at the genus level (see Supplementary Materials for more information). Blue edges represent positive associations, and red edges represent negative associations. The mean degree is shown above each network, and the mean edge weight (ew) is to the left of each network. MO = Mark Oliphant CP; KS = Kenneth Stirling CP.