The Impact of Urbanisation on Microbial Abundances and Diversity: A Comparative Analysis of Urban Environments

Abstract

Urbanisation affects soil health and diversity. DNA sequencing of soil samples can reveal the impact of urbanisation on microbial communities and their nutrient cycles. Using Oxford nanopore amplicon 16S rRNA target gene technology to sequence the DNA found in soil samples collected from urban environments can be processed. Their abundance, diversity and species richness are calculated, which can underpin the microbial communities in abundance or lack of related to specific carbon and nitrogen cycles analysis that can reveal the impact of urbanisation of microorganisms in soil habitats. However, the impact of urbanisation on nitrogen-fixing microorganisms in urban habitats like gardens and university campuses has yet to be discovered. Here, we show a higher abundance in urban universities than in urban gardens. We also found no significant difference between both settings, suggesting that urbanisation around the locations has impacted soil microorganisms and their nutrient cycles similarly due to human activity. Our results demonstrate how urbanisation in these areas affects microbial communities in the soil, yet not all results had a negative impact, like the high abundance of Epsilonproteobacteria. Furthermore, our assay has uncovered the potential for further research into more niche areas, for example, fertiliser application rates and functional genes involved in the nutrient cycles, as well as testing other significance tests in more depth.

Introduction

Amplicon sequencing is an affordable and practical Next-generation technology that enables efficient microbial ecology exploration (Grant K.R., 2022). This technique can effectively identify and quantify microorganisms in soil, including bacteria, fungi, archaea, and viruses, revealing their diversity and abundance (Zimmerman N. *et al.,* 2014). In addition, amplicon sequencing can reveal novel biochemicals that soil microbes acquire, such as antibiotics and enzymes. The use of this technology by Borčinová M. *et al.* (2020) proceeded with the discovery of new pharmaceuticals.

The data produced by amplicon sequencing can help understand the ecosystem processes, such as nitrogen fixation (Graham E.B. *et al.,* 2014), the decomposition of organic matter, and the carbon cycle (Raza T. *et al.,* 2023). Moreover, soil microbes serve as indicators of environmental quality (Karimi B. *et al.,* 2017), and their identification can aid in predicting the impacts of climate change and the effects of human activities, such as pollution and urbanisation like Huang, L. *et al.* (2020) explored on the microbial community. Therefore, amplicon sequencing in exploring these concepts can provide valuable insights to improve soil health and reduce the negative impact that human actions have on the natural world.

Urban gardens can alter microbial populations by adding elements such as compost or fertilisers, which can shift microbial diversity and activity (Tresch S. *et al.,* 2019). However, Wang H. *et al.* (2017) discovered that the excessive use of turfgrass can lead to environmental issues like over-fertilisation and higher emissions of N_2O_3, a potent greenhouse gas.

Although limited research has explored the impact of urbanisation on nitrogen-fixing microorganisms in gardens, the impact of urbanisation on microorganisms in university campuses is more complex. (King G.M., 2014) The higher foot traffic and various university recreational activities can negatively impact microbial habitats, and varying water management, like opposite extremes of irrigation practices, can affect microbial abundance (Teurlincx S. *et al.,* 2019). The study of human activity, which causes microbial shifts and environmental impacts in universities, needs further exploration. Analysing the impact of human activity using amplicon sequencing for soil microbial populations is critical to alter the negative consequences of urbanisation on microbial ecology and improve soil health (Li M. *et al.,* 2023).

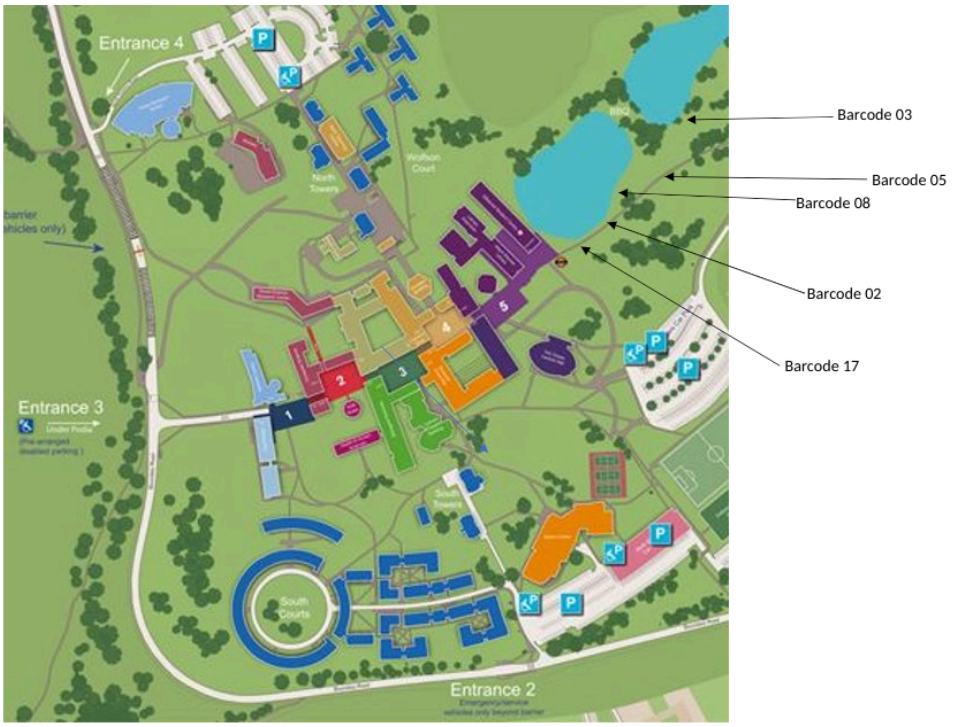
This study examines the impact of urbanisation on which was once rural and agricultural areas of Essex. Which are now constantly being transformed to meet the growing demand for living spaces (Markey S. *et al.,* 2008). Specifically, the study aims to investigate how urban settings affect microbial abundances and activity in nitrogen and carbon cycles. Our study predicts that urban universities will have a higher abundance of more diverse microbes. But we also anticipate that both urbanisation groups will show no significant difference since human activity has impacted microbial communities in both settings.

Methods

Collection of Soil Samples

This study involved two groups of soil samples. Group 1 included samples from the University of Essex campus and a contaminated industrial site at Hythe, noted for heavy metal presence (barcode 10). Specific samples were taken from a lake on the Wivenhoe trail (barcodes 02, 03, 05, 08, and 17). Group 2 consisted of samples from gardens in Colchester, Essex, including the Professor's (barcode 01), students' (barcode 04), and researchers' gardens (barcode 09). Each 2-gram sample was collected using gloves and stored at room temperature for four days pre-DNA extraction.

A map where University samples were collected on campus



(*How to get to our Colchester Campus | University of Essex*, no date) https://www.essex.ac.uk/visit-us/plan-your-visit/travel-to-colchester.

Figure 1. Illustrates a map of the University of Essex campus, where samples were collected. Labels with the barcode numbers related to specifically where the sample was collected from the lake on the Wivenhoe trail.

Isolation and Extraction of DNA

DNA was extracted from 0.25g of each soil sample using the DNeasy® PowerSoil® Pro kit (cat. no. 47014) from Qiagen, UK, following manufacturer instructions. This process helped in dispersing soil particles and dissolving humic acids. The DNA was then frozen between -30 to -15 degrees Celsius for PCR.

PCR

The 16S Barcoding Kit 1-24 (cat.no. SQK-16S024) from Oxford Nanopore, UK, was used for PCR amplification to identify target genes. The PCR mixture consisted of 2 µL genomic DNA, nuclease-free water, and 25 µl LongAmp Hot Start Taq 2X Master Mix, totalling 40 µl. Pre-mixed primers targeting 16S rRNA gene from gDNA were added, and the samples underwent 25 PCR cycles in a Gene Amp® PCR system 9700 Thermocycler. The process included a 1-minute initialisation at 95°C and a 20-second denaturation at the same temperature. Subsequently, the machine cooled down for 30 seconds at 55°C for annealing, then the temperature was increased to 65 degrees Celsius, and the extension lasted an additional two minutes. And a final 5-minute extension, both at the same temperature.

Purifying DNA and Quantification

Barcode	DNA concentrations ug/um
1	1
2	9.22
3	17.1
4	59.2
5	0.844
8	4.1
9	0.0568
10	1
17	0.0426

To purify the PCR amplicons, we utilised the GeneJET PCR Purification Kit manufactured by Thermo Fisher Scientific (catalogue number K0701)., followed by DNA quantification using the Qubit™ dsDNA BR (cat. no. Q32850, Q32853) Pub. No. MAN0002325 Rev. B.0. Thermo Fisher Scientific Assay Kit. The Qubit working solution was prepared and analysed per the manufacturer's instructions. Using two PCR tubes for the standard for all groups of samples, prepare the Qubit working solution by diluting the Qubit BR Reagent 1:200 in BR Buffer. Then, add 10µL of each Qubit standard to each of the sample tubes of purified DNA to make a total volume of ~200 µL.

Table 1. This table shows the original DNA concentrations from purifying the DNA before sequencing.

Amplicon Sequencing

DNA concentration samples were standardised and mixed in equimolar amounts for amplicon sequencing. Reviewing back to the protocol of 16S Barcoding Kit 1-24 (cat.no. SQK-16S024), Oxford Nanopore, UK, and using the equipment U MinION MK1B and

SpotON Flow Cell (FLO-MIN106, ID, FAX81391). Sequencing ran for 72 hours, with real-time base-calling by MinKNOW software.

R Statistical Analysis

Data was processed in R studio Markdown, version 4.0.4 (2021-02-15) using packages:

"readr" (Wickham *et al*., 2022),
"dplyr" (Wickham *et al*., 2022),
"biocmanager" (Bioconductor Core Team, 2022),
"phyloseq" (McMurdie and Holmes, 2022),
"lattice" (Sarkar, 2022),
"ggplot2" (Wickham, 2022),
"tidyr" (Wickham *et al.*, 2022),
"purrr" (Henry and Wickham, 2022),
"tibble" (Müller and Wickham, 2022) ,
"stringr" (Wickham, 2022)
"forcats" (Wickham, 2022),
"magrittr" (Bache and Wickham, 2022),
"ggrepel" (Slowikowski, 2022),
"vegan" (Oksanen *et al*., 2022),
"igraph" (Csardi and Nepusz, 2022).

The data was then sorted into relevant categories, removing low-quality and ambiguous reads and retaining only barcodes 01, 02, 03, and 04, and unclassified for analysis. Taxonomy data was categorised into species, genera, families, orders, and classes. Microbial community abundance was visualised in bar charts comparing garden and university samples, and an NMDS ordination plot. Differences in alpha diversity (ACE (Abundance-based Coverage Estimator) and Shannon were analysed using a Welch Two Sample T-test. Taxonomic abundances were compared across barcodes, and a microbiome network graph was produced. The Kruskal-Wallis test, Tukey's Honest Significant Difference (HSD) test and ANOVA were performed for significance assessment. A copy of the R script used for the complete analysis can be found in the appendix's link.

Results

The samples from the university campus show that Alphaproteobacteria dominates Barcode02 with a high abundance level (Figure 2,c; Hypothesis one), a significant difference in Acidobacteria phyla in the HSD test adjusted p-value of 0.0030551. In contrast, Barcode04 (garden sample) has a lower count, with Epsilonproteobacteria and Bacilli phyla dominating. This suggests an ecological variation between the two barcodes. However, an unusually high abundance of Epsilonproteobacteria could indicate an environmental imbalance due to nutrient pollution (Soininen *et al*., 2021).

The Kruskal-Wallis rank sum test has a significant p-value of 0.01481 in at least one university sample compared to other barcodes (figure 2); however, the output from further analysis from Turkey's Honest considerable difference test (HSD) test adjusted p-values the

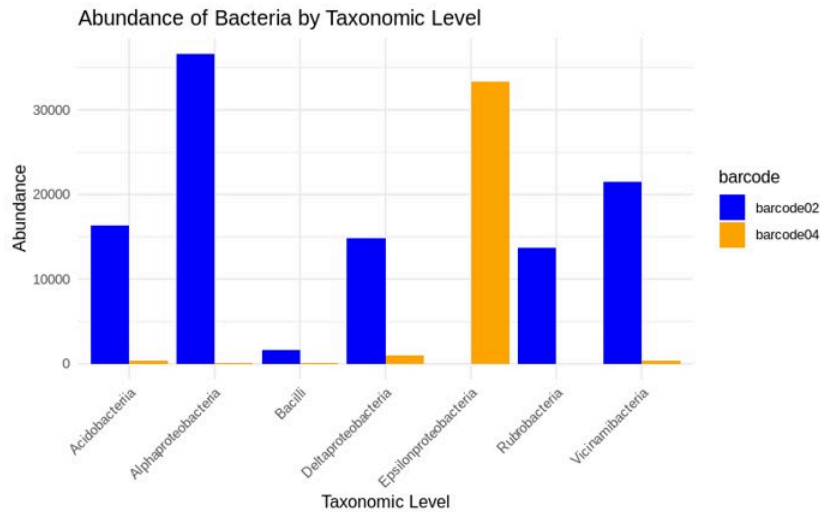


Figure 2. The bar chart represents the occurrence count of bacteria across diverse taxonomic classifications. The X-axis represents the taxonomic levels of sampled bacteria. The Y-axis indicates the abundance of bacteria, determined by the sample counts. Each bar corresponds to the abundance of bacteria at a specific taxonomic level for a given barcode, with the tallest bars denoting the most abundant taxonomic groups within each barcode category. The blue colour represents barcode 02, and the yellow colour represents barcode 04. Notably, Alphaproteobacteria was the most abundant group in barcode 02, while Epsilonproteobacteria was the most abundant in barcode 04.

value is above 0.05, it means that there is not a substantial distinction between the two variable means of university groups. (c; hypothesis 1).

Hypothesis one suggests that university samples have a higher overall abundance than the university sample, which is meant by barcode 3 (figure 3) having a higher abundance overall. However, Barcode 01 (garden) has more Bacilli that enhance plant growth, possibly from fertiliser. In contrast, Barcode 03 has a higher abundance of Alphaproteobacteria and Verrucomicrobia that play a role in the nitrogen and carbon cycles (Tsoy *et al*., 2016). Rubrobacteria is also more abundant in the university sample and aids in decomposing organic compounds.

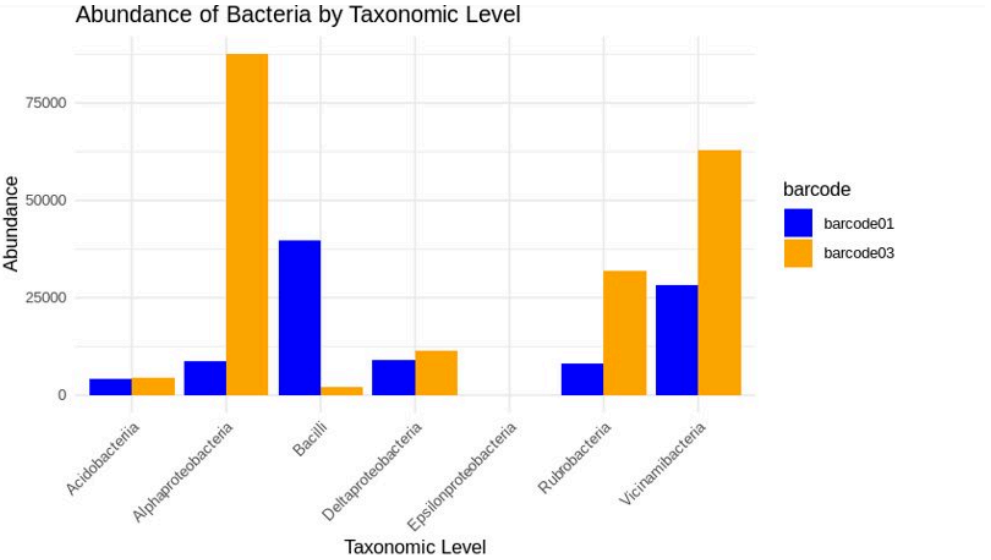


Figure 3. The bar chart represents the occurrence count of bacteria across diverse taxonomic classifications. The X-axis represents the taxonomic levels of sampled bacteria. The Y-axis indicates the abundance of bacteria, determined by the counts within the sample. Each bar corresponds to the abundance of bacteria at a specific taxonomic level for a given barcode, with the tallest bars denoting the most abundant taxonomic groups within each barcode category. The blue colour represents barcode 01, and the yellow colour represents barcode 03. Notably, Alphaproteobacteria was the most abundant group in barcode 03, while Bacilli was the most abundant in barcode 01.

However, in the Kruskal-Wallis rank sum test to determine the significance of species and garden barcodes 1 and 4, the p-value equalled 0.334, suggesting no significance in phylum and garden samples. Furthermore, the HSD test-adjusted values are close to 1, indicating insignificant. (c; hypothesis 2)
Comparing both Garden samples (barcodes 1 and 4) and university samples (barcodes 2 and 3) significantly with the Tukey HSD test, the P-value was 0.2043657, above the threshold of 0.05 (c; hypothesis 2).

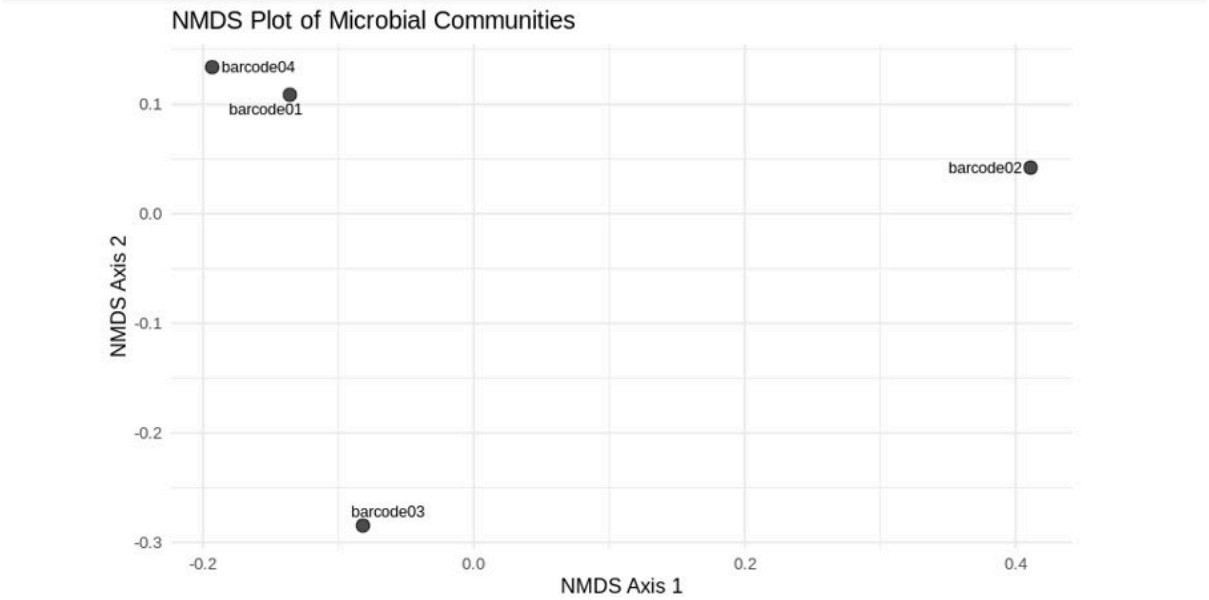


Figure 4. The NMDS ordination plot represents a non-metric multidimensional scaling analysis, plotting the similarity of microbial communities across samples identified by barcodes. Points on the graph correspond to samples (barcodes), with proximity indicating similar microbial composition. Axes NMDS 1 and 2 display gradients of dissimilarity, differentiating the microbial communities. The closer the two points are on the plot, the more similar their microbial community composition is. The clustering of 'barcode01' and 'barcode04' suggests a likeness between their microbial profiles, contrasting with the more distinct communities represented by 'barcode02' and 'barcode03.'

NMDS algorithm generated a plot (figure 4) to visualise the differences in microbial populations between samples. Barcode 2 (a university sample) was found to be distant from other points along NMDS axis 1, while barcode 3 (another university sample) was isolated along both axes. In contrast, barcodes 1 and 4 (both garden samples) were proximate, suggesting similar microbial communities, soil conditions, and human activities.

Table of results for Richness and evenness of Alpha Diversities

	ACE	se.ACE	Shannon	Group
barcode01	2519.54	24.8695	5.20053	Garden
barcode03	2555.84	25.0501	5.75529	University
barcode04	2324.02	24.2625	4.88438	Garden
barcode02	3100.11	27.675	5.26029	University

Table 2. This table shows the alpha diversity measures of richness and evenness within microbial communities from different environments, designated as "Garden" and "University". The ACE column lists the estimated species richness for each sample, indicating the total number of species present. The "se. ACE" column reflects the ACE index's standard error, indicating the variability and reliability of the richness estimates. The "Shannon" column reports the Shannon index values, a measure of community diversity that accounts for the abundance and evenness of species present. The "Group" column categories the samples into two environments: "Garden" and "University", signifying the microbial samples' origin and for further statistical significance analysis. The barcodes ("barcodes01,02,03,04") uniquely identify each sample, allowing for specific references to the data collected. This table summarises ecological metrics for assessing biodiversity within the sampled microbial communities.

Barcode 3 is the most diverse sample, with the highest species richness and evenness(hypothesis 1, table 1). Barcodes 4 and 3 represent samples with more extraordinary species richness, with Barcode 4 having a high species richness but a lesser evenness. Meanwhile, other barcodes exhibit a more even species distribution in their populations, as indicated by their higher Shannon indices.

Both alpha diversity tests were insignificant. The ACE results from the Welch Two Sample T-test showed a t-value of -1.4047 and a p-value of 0.3576 when comparing the university with garden results. Similarly, when comparing university and garden samples, the Shannon index yielded a non-significant p-value of 02.749. Therefore, the difference in species richness between urban gardens and urban universities is insignificant (hypothesis 2, table 1).

In Figure 5, we can see the comparison of two diversity indices - ACE and Shannon Indexes. Barcode02 has the highest species richness with an ACE index of 2635.2, while the evenest species distribution can be seen in sample barcode03 with a Shannon index of 5.75. Sample barcode04 has the lowest diversity and evenness with an ACE index of 2355.4 and a Shannon index of approximately 4.8. Therefore, the university samples have a higher microbial richness and evenness.

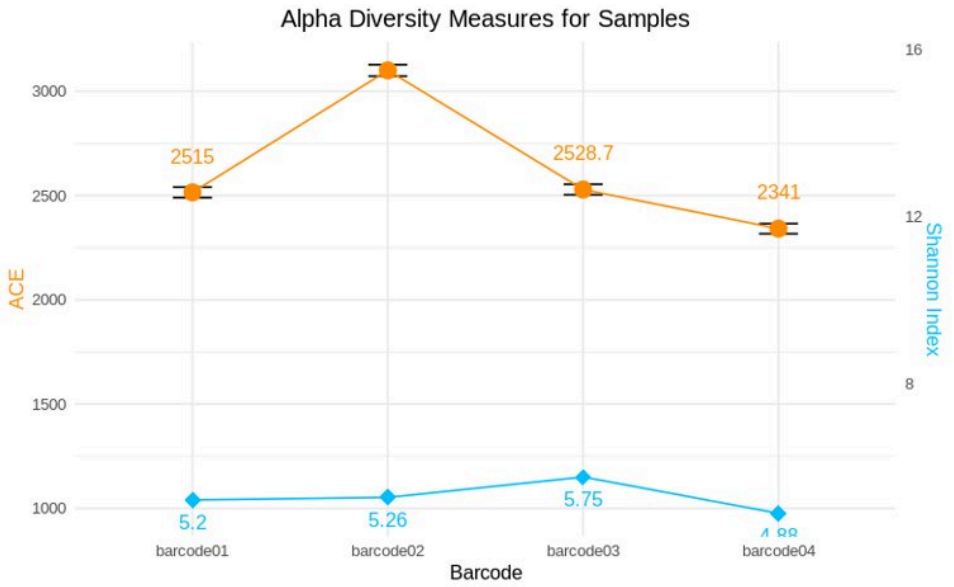


Figure 5. The graph is a dual-axis combination chart that presents a comparative analysis of microbial diversity across four samples, each point denoted by barcodes ranging from barcode01 to barcode04. On the horizontal axis marked "Barcode," the samples are individually listed. At the same time, the vertical axes represent two key diversity indices: the ACE index on the left and the Shannon index on the right. The ACE index, shown by an orange line with square markers, estimates the species richness within the microbial community. Conversely, the blue line with diamond markers traces the Shannon index, capturing species' diversity and evenness.

Error bars associated with each data point reflect the standard error of the mean, highlighting the reliability of the measurements. The graph reveals notable differences between the samples, with barcode02 exhibiting the highest species richness according to the ACE index, whereas barcode03 has the highest Shannon diversity, indicating a more even spread of species. In contrast, barcode04 has the lowest values for both indices, implying a reduced richness and evenness.

Genus Haloarcula and Acidilipia connect to all other nodes, suggesting keystone species within the network and multiple interactions with other species. Although this is a simple network with four nodes, the edges show the interaction and correlations between taxa; this co-occurrence or possible metabolic exchanges could result in both urban environments being insignificant (hypothesis 2, figure 6) and species thriving under similar conditions.

Acidicapsa and Abditibacterium are less connected, which implies fewer interactions. Haloarcula" is known to thrive in high-salt environments.

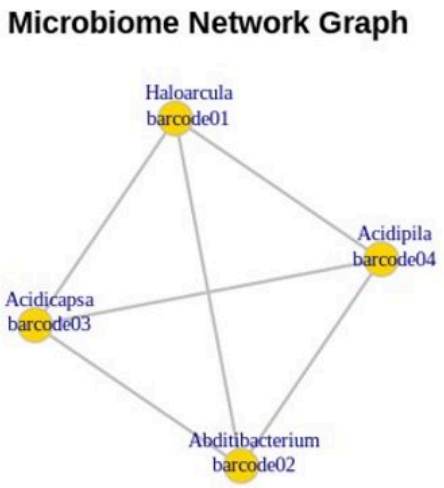


Figure 6. This microbiome network graph demonstrates the relationships between microbial taxa within a community. Each node on the graph represents a unique microbial taxon, taxa represented: Haloarcula (barcode01), Abditibacterium (barcode02), Acidicapsa (barcode 03), and Acidipila (barcode 04). The edges connecting the nodes suggest potential interactions of correlations between these taxa. The positioning of the nodes with one another is based on the strength and number of their connections, reflecting the complexity of their interactions.

Discussion

Our investigation of microbial communities in urban university settings and garden areas has revealed some interesting findings. Our study suggests that urban university settings harbour a more abundant and diverse microbial community. This aligns with the hypothesis that increased human activity may contribute to microbial communities' proliferation and diversity (table 1), especially in universities compared to urban gardens.

Our findings align with prior research outcomes, such as Li G *et al*. (2018), who reported similar trends in the Richness of microbial communities in urban spaces. It is essential to note that urbanisation could even give rise to antibiotic-resistant genes, which can negatively impact human health.

Although our results suggest a higher microbial abundance in university environments, the functional implications of this diversity, particularly in critical nutrient cycles like nitrogen and carbon, still need to be fully understood.
While Alphaproteobacteria were prevalent in university samples (as shown in Figures 2 and 3) and may enhance nutrient cycling processes due to their role in nitrogen fixation. (Tsoy et al., 2016) The garden sample from barcode one (Figure 2) showed a higher abundance of Epsilonproteobacteria, known for their role in the nitrogen cycle, which could be related to over-fertilisation of turfgrass (Soininen et al., 2021).
However, to gain deeper insights, future research would require an analysis of fertiliser application rates, other soil chemistry parameters, and functional genes involved in the nutrient cycle, as done by Ouyang et al. (2018). These findings from our research highlight

the importance of exploring these microbial communities' metabolic activities and ecological functions in future projects.

Microorganisms are essential for nutrient cycling. They decompose organic matter, fix nitrogen, and perform other vital functions for soil fertility and ecosystem health (Prasad *et al*., 2020). However, an imbalance in
The microbial community, whether due to environmental stressors or human activities, can disrupt these cycles and potentially lead to soil degradation (Cavicchioli *et al*., 2019).

For instance, increased salinity favouring halophiles like Haloarcula (figure 7) can indicate and contribute to an imbalance in the soil ecosystem (Talaat, 2018). It can suppress other beneficial microbes, alter soil chemistry unfavourably for plant growth, or accumulate toxic compounds.
On the other hand, microbes like Acidicapsa (figure 7) might indicate naturally acidic soils, which do not necessarily affect the ecosystem negatively, as previously studied by Kalam *et al*. (2020).

The overall impact of these microbes on soil and environmental health and their abundance alone does not indicate a positive or negative effect. It is their interaction with the environment.
The network graph (figure 7) is typically a starting point for further analysis. Future works could explore the significance of the interactions that need to be combined with additional data, such as their metabolic capabilities, to draw more concrete conclusions about their ecological roles and implications for urbanisation.

The methodological approach of our study, although effective in assessing microbial diversity through 16S amplicon sequencing, needed to explore the abundance of microbial communities. There, however, is a limit to this research. Only targeting the 16S rRNA gene has a downfall in finding new or 'unclassified' species ubiquitously (Clarridge, 2004). Our future research could integrate different target gene sequencing like De Filippis *et al.* (2017) did with 18S ribosomal small subunits. Such studies could also benefit from including fungal communities, providing a more comprehensive picture of microbial diversity.

Our results further raise questions for future studies about the urban activities influencing microbial distribution and abundance compared to rural settings. One hypothesis for future testing is the difference between rural environments and urban environments' microbial diversity, comparing ecosystems and comparing for statistical significance. A methodological shift towards a rural perspective would enable us to address different questions regarding the effects of human actions on microorganism populations and the influence of urban development on a broader scope.

Another limitation of our study is the assumption that microbial abundance equates to ecosystem functionality. Additionally, our focus was on bacterial communities, and including fungal communities in future research could provide a more holistic view of microbial diversity and ecosystem functionality (Wagg *et al*., 2019).

Furthermore, another limitation is that the results need to tell us about the effect of urbanisation alone directly. To determine how urbanisation affects microbial communities, we

need a more controlled comparison where the groups are defined by levels of urbanisation, not just the phyla present in the microbial communities.

Our study found no significant statistical differences between urban university and garden settings in microbial abundance and diversity. However, it is important to note that this does not necessarily mean that minor variations do not exist. Future studies that consider a variety of graphs, such as the NMDS graph with a stress level calculation, may reveal a different significance level.
Additionally, investigating different soil types, vegetation cover, or specific human activities unique to each setting over an extended period of time could shed more light on the impact. It is possible that the insignificance of this study could be attributed to similar levels of human activity, pollution, or habitat disturbance in both urban locations. Therefore, it is crucial to continue exploring this topic and conducting further research to understand microbial communities in different environments comprehensively.

In conclusion, our research adds to the body of knowledge on the impact of urbanisation on microbial communities. It highlights the importance of considering these communities in urban planning and restoration (Pavao-Zuckerman, 2008). By understanding the complex relationships between urbanisation, microbial ecology, and environmental health, we can better manage microbial diversity for the sustainability of urban ecosystems. Our study underscores the potential of microbial communities as indicators of environmental health and as agents for ecological restoration and resilience in urban settings.

Appendixes

R markdown the entire HTML file found online at https://rpubs.com/CT27/1138428.

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