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Tiffany Scholier

Cities and Their Effects on Free-Living and Host-Associated Microbes

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ABSTRACT

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Microbes are essential for all life on Earth and can be found in the environment or in association with host organisms, where they perform essential tasks either needed for the health of ecosystems or their hosts. Humans impact almost every habitat on our planet through various processes such as loss and degradation of habitat including urban development, pollution and climate change. As a result, microbial communities (also referred to as the microbiota, including bacteria and fungi) are expected to respond to these selective pressures by adjusting to a changing environment. In this way, human alterations of the natural landscape have the power to impact diverse microbiota (both free-living and host-associated), that in turn may affect the delivery of the services they provide to ecosystems and their hosts. Using an innovative combination of extensive cross-sectional surveys, longitudinal field experiments, DNA metabarcoding techniques and stable isotope analyses, my doctoral thesis focuses on the impacts of human activities on free-living and host-associated microbiota, with the aim to quantify the specific variation in bacterial and fungal (1) forest soil and (2) rodent (the bank vole) gut microbial communities in the context of urbanisation, and (3) the level of resistance (*i.e.,* mechanism by which microbial communities do not change after habitat alteration) and plasticity (*i.e.,* mechanism by which microbial communities change to match the novel environment after habitat alteration) displayed by the rodent bacterial gut microbiota in response to a change in the environment (host transfer between urban and rural forests). I found both (1) urban soil microbiota and (2) urban rodent gut microbiota to be distinct from those occurring in forests that are less impacted by urbanisation. Notably, soil pH and a dietary switch were identified as important factors in shaping the soil and bank vole gut microbiota, respectively. Additionally, I found that (3) both past (resistance) and present (plasticity) habitats influence the gut microbiota composition in a wild rodent. This thesis summarises the effects of the urban environment on microbial communities in two different systems and hereby demonstrates the far-reaching effects of urbanisation on microbial life forms.

Keywords: Bacteria, fungi, gut microbiota, rodent, soil microbiota, urban, wildlife.

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TIIVISTELMÄ

Scholier, Tiffany
Kaupungit ja niiden vaikutukset vapaasti eläviin ja isäntään liittyviin mikrobeihin
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Mikrobit ovat oleellisen tärkeitä elämälle maapallolla, ja niitä esiintyy vapaina ympäristössä tai isäntäorganismeissa tukien ekosysteemien ja/tai isäntien normaalia toimintaa. Ihminen vaikuttaa lähes kaikkiin planeettamme elinympäristöihin aiheuttaen niiden häviämistä ja huonontumista (mukaan lukien kaupunkien rakentaminen) sekä ympäristöjen saastumista ja ilmastonmuutosta. Mikrobiyhteisöjen (kutsutaan myös mikrobiotaksi, mukaan lukien bakteerit ja sienet) odotetaan reagoivan näihin ihmisen aiheuttamiin valintapaineisiin sopeutumalla muuttuvaan ympäristöön. Siten ihmisen aiheuttamat muutokset ympäristössä voivat muovata mikrobiyhteisöjä (sekä vapaasti eläviä että isäntiin liittyviä) muuttaen mikrobiyhteisöjen vaikutuksia ekosysteemeissä ja isännissä. Väitöskirjassani tutkin ihmistoiminnan vaikutuksia ympäristössä vapaasti eläviin ja luonnonvaraisissa isäntäeläimissä esiintyviin mikrobiyhteisöihin käyttämällä erilaisia tutkimusasetelmia, mukaan lukien laaja-alaiset poikkileikkausaineistot ja kokeelliset työt sekä DNA-metaviivakoodaus ja isotooppianalyysit. Väitöskirjatyöni tavoitteena oli määrittää kuinka kaupungistuminen vaikuttaa (1) metsämaaperän ja (2) jyrsijöiden (metsämyyrän) suoliston bakteeri- ja sieniyhteisöihin. Lisäksi tarkastelin, kuinka (3) metsämyyrän suoliston mikrobiota säilyy muuttumattomana (resistenssi) tai muuttuu (plastisuus) vastaamaan uutta ympäristöä elinympäristön vaihtumisen jälkeen (isännän siirto kaupunkien ja maaseudun metsien välillä). Tulosteni perusteella molemmat, sekä 1) metsän maaperän mikrobisto, että 2) metsämyyrän suolistomikrobisto eroavat kaupunkimetsien ja kaupunkien ulkopuolisten metsien välillä. Erityisesti erot maaperän happamuus ja jyrsijöiden ruokavaliossa tunnistettiin tärkeiksi tekijöiksi maaperän ja metsämyyrän suolen mikrobiotan muovautumisessa. Lisäksi havaitsin, että elinympäristön muutosta edeltävä ympäristö (resistenssi) ja uusi ympäristö (plastisuus) vaikuttavat suoliston mikrobiotan koostumukseen luonnonvaraisessa jyrsijässä. Väitöskirjani tulokset osoittavat, että kaupunkiluonto vaikuttaa mikrobiyhteisöihin kahdessa eri järjestelmässä ja osoittavat täten kaupungistumisen kauaskantoiset vaikutukset mikrobien elämänmuotoihin.

Avainsanat: Bakteerit, jyrsijä, kaupunki, luonnoneläimet, maaperän mikrobisto, sienet, suolen mikrobisto.

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ORIGINAL PAPERS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–III.

I Scholier T, Lavrinienko A, Brila I, Tukalenko E, Hindström R, Vasylenko A, Cayol C, Ecke F, Singh NJ, Forsman JT, Tolvanen A, Matala J, Huitu O, Kallio ER, Koskela E, Mappes T, Watts PC. 2022. Urban Forest soils harbour distinct and more diverse communities of bacteria and fungi compared to less disturbed forest soils. *Molecular Ecology* 32: 504-517.

II Scholier T, Lavrinienko A, Brila I, Tukalenko E, Hindström R, Cayol C, Ecke F, Singh NJ, Forsman JT, Tolvanen A, Matala J, Huitu O, Kallio ER, Koskela E, Mappes T, Watts PC. 2023. Dietary change alters the gut microbiota in urban rodents. Manuscript.

III Scholier T, Lavrinienko A, Kallio ER, Watts PC, Mappes T 2023. Effects of past and present habitat on the gut microbiota of a wild rodent. Submitted manuscript.

The following table shows authors' contributions to the original papers.

Study	I	II	III
Study design	TS, AL, IB, ET, CC, FE, NS, JF, AT, JM, OH, ERK, EK, TM, PW	TS, AL, IB, ET, CC, FE, NS, JF, AT, JM, OH, ERK, EK, TM, PW	TS, AL, ERK, TM, PW
Data collection	TS, AL, IB, ET, RH, AV, TM	TS, AL, IB, ET, RH, TM	TS, AL, TM
Laboratory work	TS	TS	TS
Data analyses	TS	TS, AL	TS
Manuscript writing	TS, AL, PW	TS, AL	TS, PW

Tiffany Scholier (TS), Anton Lavrinienko (AL), Phillip C. Watts (PW), Tapio Mappes (TM), Eva R. Kallio (ERK), Ilze Brila (IB), Eugene Tukalenko (ET), Rasmus Hindström (RH), Andrii Vasylenko (AV), Claire Cayol (CC), Frauke Ecke (FE), Navinder J. Singh (NS), Jukka T. Forsman (JF), Anne Tolvanen (AT), Juho Matala (JM), Otso Huitu (OH), Esa Koskela (EK)

1 INTRODUCTION

1.1 Urbanisation as an anthropogenic pressure

Currently, 56% of humans live in cities and this number is expected to increase to 70% by 2050 (Gross 2016). This makes urban areas the most common interface where humans interact with their surroundings. When natural environments are transformed to urban areas, usually homogenisation of the natural species assemblage occurs with the specific loss of sensitive and specialist species (McKinney 2006). In contrast, generalist species such as many rodents can thrive in these artificial environments (Ducatez *et al.* 2018). Notably, most of what we know about the effect of cities on wild living organisms is constrained to macroscopic species such as plants and animals. However, it is also crucial to understand how the urban environment impacts wildlife that is invisible to the naked eye or microbial life forms. Microbes are versatile, and while some are free-living, others are characterised by a host-associated lifestyle. Even though, microbes are hidden from plain sight, they perform important tasks essential for proper ecosystem functioning (Fierer 2017), as well as for a healthy human life (Rooks and Garrett 2016, Dearing and Kohl 2017). In this thesis, I aim to address some simple but very important questions about the impact of urban environments on microbial life (both free-living and host-associated), targeting both bacteria and fungi.

1.2 Microbes in the urban environment

1.2.1 Environmental microbes

Microbial communities, commonly referred to as the microbiota, can be found in almost all environments on Earth. These communities typically consist of bacteria, fungi and other microorganisms (McFall-Ngai *et al.* 2013). Environmental microbes such as those found in soil are known to be of great

importance because they perform various ecological functions (*e.g.,* primary production, decomposition, carbon cycling and nutrient mineralisation (Fierer 2017, Delgado-Baquerizo *et al.* 2020)). Besides their vital role in ecosystem functioning, there is also an established link between diverse microbial communities in the environment and animal health. For example, humans that have little contact with natural environments (*e.g.,* people with an urban lifestyle) decrease their interactions with biodiversity on a general scale and are thought to experience a lower exposure to naturally occurring microbes (*i.e.,* biodiversity hypothesis (Von Hertzen *et al.* 2011)). However, the proper development of the human immune system relies on this exposure to learn how to distinguish harmful from benign particles entering the organism. When this process is hampered, for example when humans do not experience sufficient contact with nature in childhood, chances of developing atopic diseases (*e.g.,* allergies and asthma) later in life increases (Hanski *et al.* 2012). This general detachment from nature has been proposed as one of the explanations to why such diseases are more common in industrial populations (Von Hertzen *et al.* 2011). As such, it is now clear that free-living microbes are important not only for proper ecosystem function but also for human health and well-being. Thus, it is crucial to understand the processes that affect the free-living microbial communities.

By comparing soil microbial communities originating from urban and non-urban areas, numerous studies have shown that the urban environment can impact free-living microbiota and change their compositions. Notably, bacterial alpha diversity (for background details, see Section 2.4.1.) was found to be higher in cities (Hui *et al*. 2017, Naylo *et al*. 2019, Tan *et al*. 2019), whereas fungal diversity has been reported to decline (Andrew *et al*. 2019, Abrego *et al*. 2020) or remain stable (Tan *et al*. 2019, Tedersoo *et al*. 2020). However, many of these studies suffer from limitations in the sampling design, and for instance compare urban gardens to non-urban forests, or provide little to no information about surveyed habitats. In addition, bacteria and fungi are usually studied separately which makes it difficult to directly contrast taxon-specific patterns.

In my thesis, I addressed these issues by utilising the features of the typical Finnish landscape, where cities have natural forest patches within their borders, to study soil bacteria and fungi in the same soil samples (**I**). This approach made it possible to directly compare "real" forest soil microbiota (in comparison to city parks) between urban and non-urban areas.

1.2.2 Host-associated microbes

Microbes play an important role in biology of their host organisms. For example, microbial communities inhabiting the gastrointestinal tract of animals, (*i.e.,* gut microbiota) provide important services to their hosts such as aiding digestion, securing nutrient supply (Dearing and Kohl 2017), regulating the immune system (Rooks and Garrett 2016) and protecting against pathogens (Suzuki 2017). Moreover, recent evidence indicate that the gut microbiota can even expand animal biology, for example by degrading dietary toxins (Kohl *et al.* 2014), modulating energy metabolism (Sommer *et al.* 2016), and even influencing host

behaviour (Trevelline and Kohl 2022). However, studies on animal gut microbiota have mainly focused on the bacterial component, creating a knowledge gap concerning the role of gut fungi (Richard and Sokol 2019). Yet, gut fungi are also important players in host-microbiota interactions, and, for instance, play a vital role in the development of the host immune system (van Tilburg Bernardes *et al.* 2020).

Gut microbial communities can be affected by many environmental variables (*e.g.*, diversity of environmental free-living microbes (Wang *et al.* 2017, Grieneisen *et al.* 2019), the amount of (microbes associated with) other encountered species and environmental pollution (Coolon *et al.* 2010)), yet host diet is thought to be one of the most important factors (David *et al.* 2014). Host-related variation, such as host genetics can also influence the gut microbiota composition, however relative to environmental factors, their contribution is typically less strong (Rothschild *et al.* 2018). Although the majority of gut microbiota studies are focusing on humans and laboratory animals (Pascoe *et al.* 2017), diet and environment-related factors are also thought to play an important role in determining the gut microbiota structure in wild animals. Given that the composition of the gut microbiota can impact the health and performance of their hosts in nature, it is crucial to understand the exact impacts of environmental changes on host-associated microbiota of wild animals, particularly in the context of environmental changes caused by human activities.

Urban environments are associated with a change in the composition of the bacterial gut microbiota of various animals, with some animals exhibiting alpha diversity levels (for background details, see Section 2.4.1.) that are higher (Littleford-Colquhoun *et al*. 2019, Gadau *et al*. 2019), lower (Teyssier *et al*. 2018, 2020, Fuirst *et al*. 2018, Murray *et al*. 2020) or remain unchanged (Anders *et al.* 2022) in comparison to their non-urban conspecifics. These contrasting outcomes might be explained by differences in environment features at a local scale: presence of suitable habitats and habitat heterogeneity (Fuirst *et al*. 2018, Murray *et al*. 2020), and/or (seasonal) availability of food (Littleford-Colquhoun *et al*. 2019, Gadau *et al*. 2019, Teyssier *et al*. 2020) rather than their urban status *per se* (Teyssier *et al.* 2018). Dietary variation is thought to be a major factor in shaping the urban microbiota diversity and community composition with a shift towards higher sugar metabolism in urban animals (Littleford-Colquhoun *et al.* 2019, Gadau *et al*. 2019). Such dietary shifts in urban animals could be due to access to different dietary items (*e.g.*, anthropogenic foods, (Knutie *et al*. 2019, Littleford-Colquhoun *et al*. 2019, Teyssier *et al*. 2020, Sugden *et al*. 2020, Anders *et al*. 2022)) and/or altered behaviour (*e.g.*, predation, (Mazza *et al.* 2020)). A strong effect of host diet was confirmed by an experimental study (Teyssier *et al.* 2020) that carried out cross-feeding trials in birds, such that urban birds were fed a typical rural diet and vice versa and found that diet was sufficient to alter the gut microbiota in both urban and rural birds (in both directions). It is important to note that all aforementioned studies have only examined the bacterial component of the gut microbiota, which emphasises the scarcity of data on fungal microbiota in wildlife and highlights the need to study the impacts of urbanisation on gut fungi.

In this thesis, I built on the existing knowledge and investigated the impact of living in an urban environment on the gut microbiota of a small rodent (*Clethrionomys glareolus*, formerly *Myodes glareolus*, in Finnish: metsämyyrä, (Kryštufek *et al.* 2020)), focusing on both bacteria and fungi (**II**). Moreover, by sampling animals from multiple replicated areas along a gradient of habitat disturbance (urban, suburban, managed and natural forests in national parks, see Section 1.3.), I place the effects of urbanisation into the appropriate contrast with natural habitat. Overall, I tested whether changes in the gut microbiota between urban and non-urban animals could be detected and whether those differences could be linked to variation in the long-term host diet (through stable isotope analysis, see Section 2.2.4.). Additionally, I investigated a potential link between the presence of bacterial and fungal microbes in the living environment (*i.e.,* soil, **I**) and those found inside the bank vole gut.

1.2.3 Plasticity of gut microbial communities

A growing body of evidence suggests that the gut microbiota can modulate host capacity to adapt and survive in a changing environment (Alberdi *et al.* 2016; Michel *et al.* 2022). Consistently, there is considerable interest in understanding how the gut microbiota can respond to environment change, especially in wild animals. In general, it is established that the immediate environment determines the gut microbiota of wild animals (see Section 1.2.2), but this raises the question of: *"What happens to the gut communities of wild animals when they experience a new environment (e.g., move or are being translocated between different habitats)?"*. One of the potential response mechanisms of the gut microbiota would be *plasticity* (Fig. 1A), whereby the gut microbial communities would adjust to a new habitat and its specific characteristics (*e.g.,* available food sources). In this scenario, the gut microbiota of the migrated/translocated animals are expected to resemble the gut microbiota of animals native to this new habitat, thus enabling the host to adapt more efficiently to the environment change (Alberdi *et al.* 2016; Michel *et al.* 2022). The opposite mechanism is called *resistance* (Fig. 1B), by which the gut microbiota communities would not shift in response to environment change but instead would retain their original composition, potentially hindering the host capacity to adapt to their new environment. While plasticity is thought to be mostly driven by changes in the host diet (Alberdi *et al.* 2016; Michel *et al.* 2022), resistance can be favoured in the presence of strong priority effects (*i.e.,* the formerly established microbes prevent further colonisation of (specific) microbes, (Robinson *et al*. 2010, Obadia *et al*. 2017, Björk *et al*. 2018)).

Currently, there is some debate on whether the gut microbiota of wild animals generally respond to changes in the environment by exhibiting plasticity (Fig. 1A (Alberdi *et al.* 2016)) or resistance (Fig. 1B (Allison and Martiny 2008)). Additionally, the gut microbiota communities could display both plasticity and resistance (retaining features of the original microbiota and acquiring portion of the microbiota from the new environment, Fig. 1C) or even an interaction between the two mechanisms (microbiota of different origin would respond in a different way, Fig. 1D). As an alternative, no patterns could be found in the gut

microbiota due to high stress levels experienced by the host during the migration/translocation (Fig. 1E (Zaneveld *et al.* 2017)).

Experimental studies with longitudinal sampling can offer powerful insights into the dynamic nature of the important processes of the gut microbiota assembly. In this context, reciprocal translocation (RT) experiments are particularly useful, as this experimental approach features translocation of animals between different habitats. And yet, the RT experiments with full factorial design are rare in the wild, and have only been performed with aquatic host species (Bletz *et al*. 2016, Chen *et al*. 2017, Uren Webster *et al*. 2020). These studies have either confirmed the importance of plasticity in the gut microbiota (Uren Webster *et al.* 2020) or highlighted interaction effects between resistance and plasticity based upon the habitat of origin (Bletz *et al*. 2016, Chen *et al*. 2017).

By carrying out the first reciprocal translocation experiment in a terrestrial system (with pre- and post-transfer faecal sampling) (**III**), I was able to investigate the longitudinal changes that occur in the gut microbiota of a wild rodent in response to a change in the host environment. Specifically, wild bank voles were translocated among forests that differ in their levels of anthropogenic disturbance (urban and rural forests). With this experimental design, I quantified and compared the relative effects of host origin (resistance) and the immediate environment (plasticity) on the assembly of the post-transfer gut microbiota.

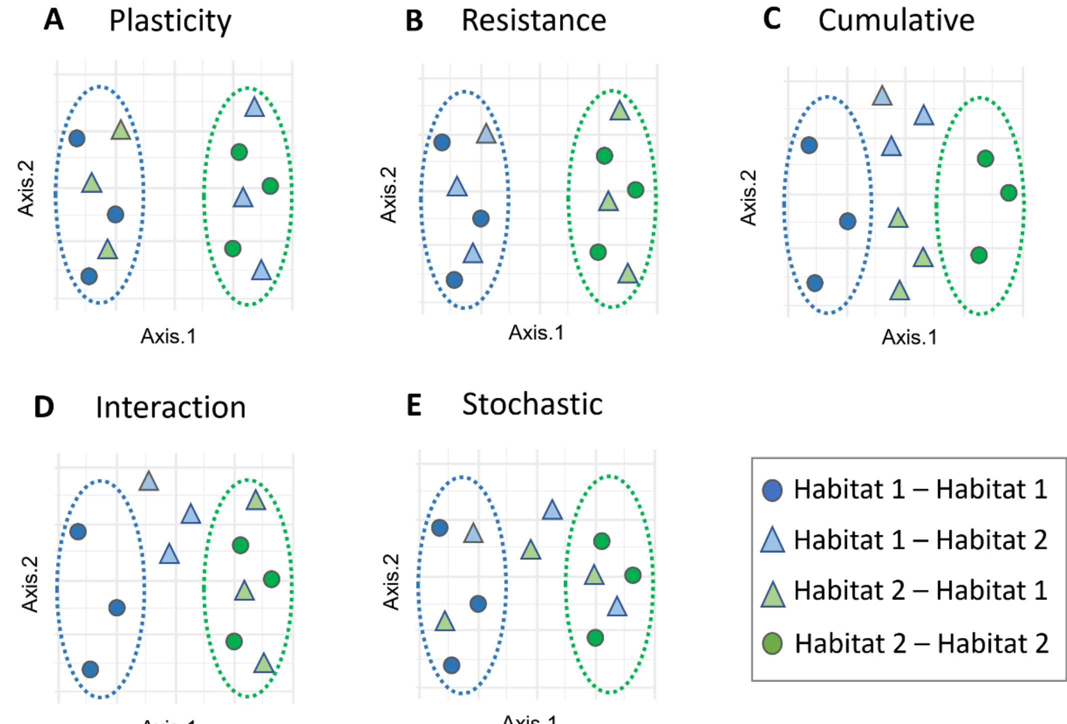

FIGURE 1 Microbiota response scenarios under changing environmental conditions. As a response to change in the environment of the host organism, the associated gut microbes change their composition according to one of the following scenarios: (A) plasticity where the microbiota matches the microbiota of animals native to the new environment, (B) resistance where the microbiota resists change and (C) cumulative and (D) interaction effects between these two mechanisms. When no pattern is observed, it is likely that the gut microbiota either have no specific response or that the host organism is experiencing stress (E). Dashed lines represent 99% confidence ellipses. Horizontal axes explain the variation between the 'native' microbiota of animals of the two different habitats while the vertical axes show the variation within those two groups. Figure taken from Manuscript III.

1.3 Study system

Forest patches in Finnish cities are abundant and not extensively managed. Such urban lay-out provides the opportunity to directly study the differences between urban and non-urban forests without the risk of confounding habitat types by comparing urban city parks with more natural forests. To expand the anthropogenic disturbance gradient, I incorporated urban forests, suburban (managed forests adjacent to a city), managed (managed forests close to a national park, located away from cities) and the most pristine forests that can be found in industrialised countries (national parks) into our study design. To minimise spatial bias in the cross-sectional datasets (**I-II**), I sampled three cities (Jyväskylä, Kuopio, Mikkeli) and their corresponding suburban forests and seven national parks and adjacent managed forests. Additionally, each of these sample

locations was represented by multiple replicate sampling sites (*n* sampling sites=382). In the longitudinal experiment (**III**), I included twenty urban and twenty suburban (also referred to as rural) sampling sites in and around the city of Jyväskylä (*n* sampling sites=40). All sampling sites were dominated by Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*), and silver and downy birch (*Betula pendula* and *B. pubescens*), with bilberry (*Vaccinium myrtillus*) and lingonberry (*V. vitis-idaea*) as undergrowth.

In this thesis, soil samples and bank voles (C*lethrionomys glareolus*, formerly *Myodes glareolus*, in Finnish: metsämyyrä, (Kryštufek *et al.* 2020)) were collected at each sampling site. Soil microbes were used to study the urban impact on free-living environmental microbes (Fig. 2, **I**) while bank voles were used to investigate the effect of urban lifestyle on the gut microbiota of a wild rodent (Fig. 2, **II-III**). The bank vole is one of the most widespread and abundant small mammals in Northern Europe and is commonly used as a model species for ecological and evolutionary research (Schneider *et al.* 2021). As such, their physiology and life history traits are well studied and their gut microbiota have been described (Lavrinienko *et al*. 2018, 2020, Brila *et al*. 2021). Wild bank voles are also easily bred in laboratory settings (Lonn *et al.* 2017), which enables large sample sizes and provides the possibility to conduct experimental work. Moreover, the bank vole is an ecologically relevant model since it is a common host organism for various zoonotic disease agents (*i.e.,* disease that can be transmitted between animals and humans (Han *et al.* 2015)) that can impact human health (*e.g.,* Puumala hantavirus (Voutilainen *et al.* 2016)).

The research on wild animals was conducted in accordance with the relevant laws and all procedures performed had an ethical committee approval (ESAVI/3981/2018).

FIGURE 2 Two types of study systems used in this thesis. Environmental microbes associated with the organic layer of forest soil (left) and host-associated microbes residing in the gut of wild bank voles (right) were studied in this thesis. Photo credits to Piko Rautio.

1.4 Objectives

This thesis contributes to a better understanding of the effects of the urban environment on the health of ecosystems and wild mammals through its interactions with free-living and host-associated microbes. I address key knowledge gaps, by conducting large-scale surveys and field experiments and by considering both bacterial and fungal components of the soil and animal gut microbiota.

Specifically, my thesis addresses the following research questions (Fig. 3):

I. Does the urban environment impact bacterial and fungal communities in soil (in a similar way)?

II. Does urban lifestyle impact the bacterial and fungal gut microbiota in bank voles (in a similar way)?

a. If yes, do the changes in the gut microbiota composition reflect the changes in microbial communities from the immediate living environment (soil)?
b. If yes, do changes in the gut microbiota composition associate with a shift in host diet?

III. Is the composition of the bacterial gut microbiota in wild bank voles more influenced by its current or past environment?

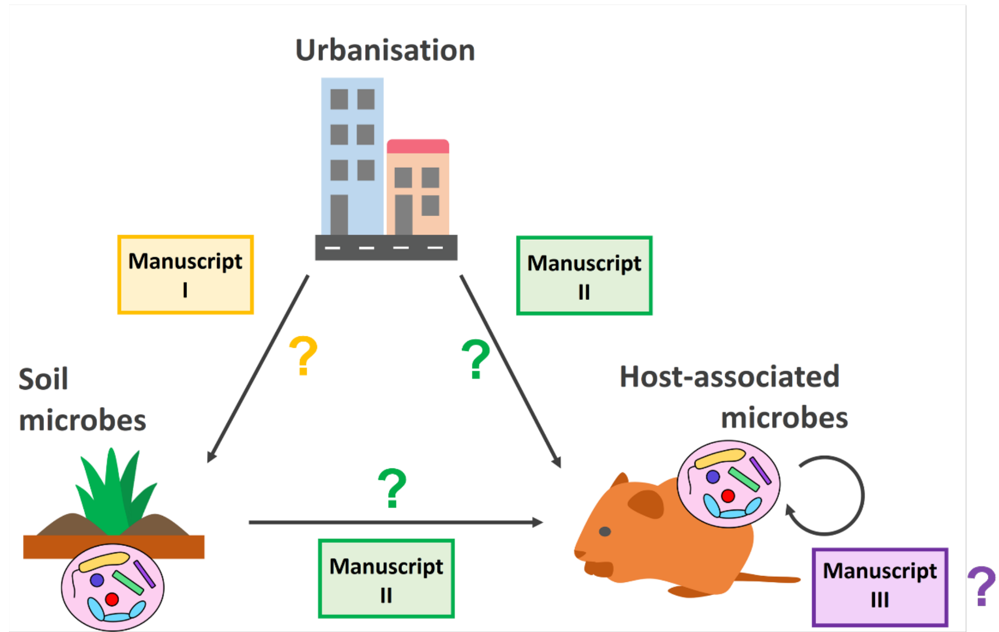

FIGURE 3 The diagram provides an overview of the research questions that have been studied in the manuscripts included in this thesis.

2 METHODS

2.1 Field work and sample collection

2.1.1 Cross-sectional soil and faecal samples

In the summer of 2019, large-scale field work was carried out in twenty sampling locations across Central-Finland. The sample locations were divided into four forest types that had different levels of anthropogenic disturbance (Fig. 4). Specifically, seven national parks and adjacent managed forests, together with three cities (Jyväskylä, Kuopio, Mikkeli) and adjacent suburban forests were included in this survey (**I-II**). Numerous replicate sites per each sample location (12-22 replicates, Fig. 1) were used in the study design to minimise spatial bias.

To validate this categorical division of forest types, the Human Influence Index (HII) was calculated for every sampling site by importing the Global Human Influence Index Dataset (https://doi.org/10.7927/H4BP00QC) into ArcGIS v.10.8.1 software. The HII summarises nine data layers that reflect the level of anthropogenic habitat disturbance, including human population density, human land use, infrastructure (built environment, nighttime lights, land use/land cover), and human access (coastlines, roads, railroads, navigable rivers). I found that the HII was sufficient to successfully differentiate between three out of four forest types (Fig. 4), with HII levels being the highest in urban areas, intermediate in suburban forests and lowest in managed forests/national parks ($p<0.01$). In contrast, managed forests and national parks had similar values for HII (likely due to the lack of human settlements/infrastructure around these areas). This indicates that the HII is a useful tool to distinguish between types of human impact that alter the topography of a landscape (*e.g.*, urbanisation).

Soil samples were collected in every forest site (*n* total soil=312, Fig. 5) with a metal core instrument (diameter=3cm, depth=10cm), after which the mineral and leaf litter layers were removed, and the remaining organic layer was immediately put on dry ice in the field and transferred to the -80 °C freezer until sample processing.

During the same field work effort, soil sampling sites were used to capture wild bank voles by live trapping (*n* total bank voles=382, Fig. 5). Fur samples were collected by trimming the upper thorax of each bank vole and were stored on room temperature. All animals were euthanised by cervical dislocation in the field, put on dry ice and stored in the -80 °C freezer until dissections.

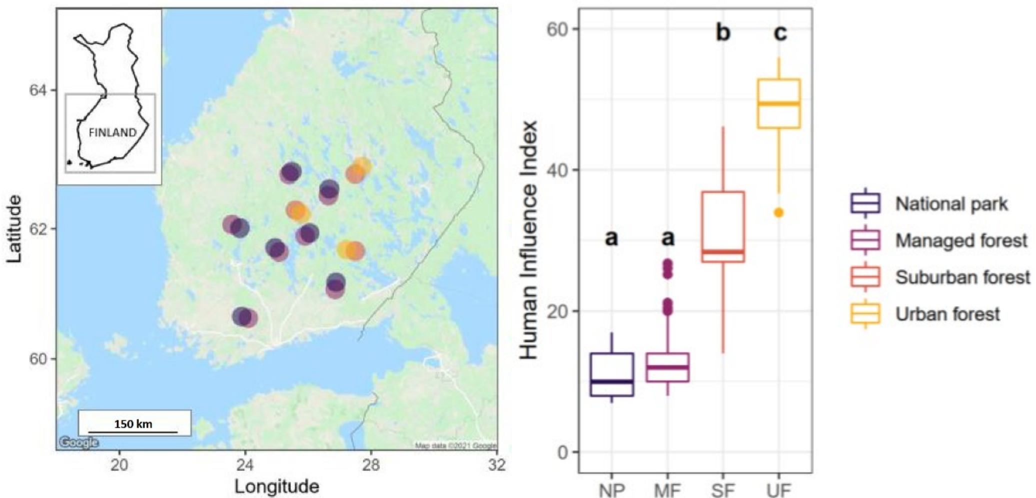

FIGURE 4 Forest soil sampling and study design. The figure on the left shows an overview of the sampling locations: seven national parks (dark purple), adjacent managed forests (light purple), suburban (orange) and urban (yellow) forests of three cities (Jyväskylä, Kuopio and Mikkeli). Every point on the map represents 12-22 forest sites where soil samples and bank voles were collected. The figure on the right shows the association between the Human Influence Index (HII) and the four types of forests surveyed in this thesis. Figures taken from Manuscript I.

Plant species that were common in all forest types (*Sorbus*, bilberry and lingonberry) were collected as well and stored in dry paper envelopes at room temperature until further processing (Fig. 5).

Additionally, I also quantified the variability of the environment through structural habitat surveys (modified from (Ecke *et al.* 2002) for a subset of the sampling sites (*n*=178, Fig. 5). The surveys described twenty-three (a)biotic properties of the habitat, ranging from categorical classification of the above ground vegetation to the number of fallen trees (see Manuscript **I** for more details).

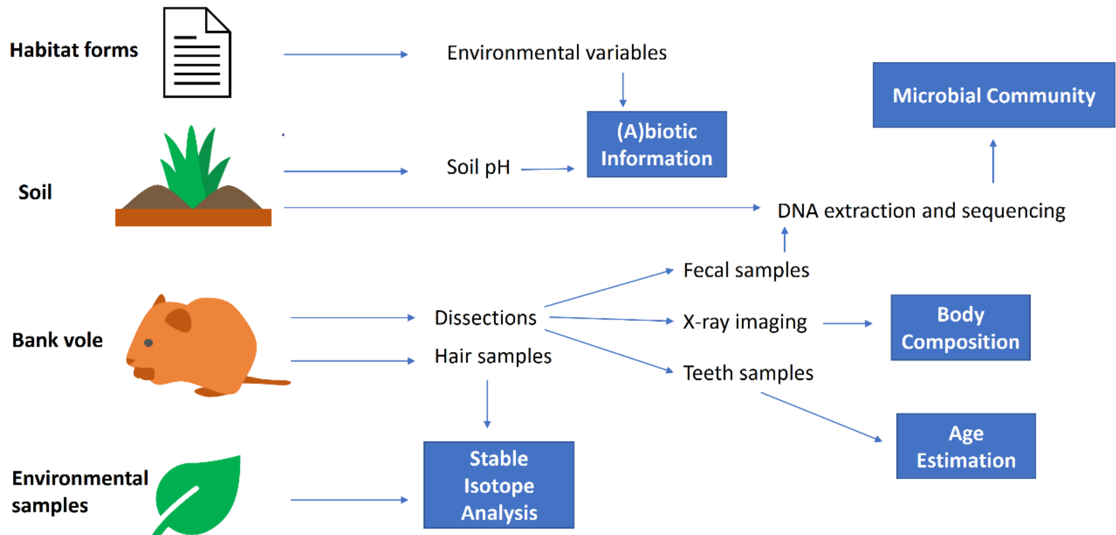

FIGURE 5 Overview of the multiple data layers analysed in this thesis (**I-II**). On the left, figures symbolise the work that was done in the field during the summer of 2019. Terms in black colour represent work that was done in the lab, while terms in white colour and blue boxes represent the outcomes and data retrieved.

2.1.2 Experimental faecal samples

In the summer of 2020, a large-scale reciprocal transplant experiment was carried out, where bank voles were transferred between twenty urban and twenty rural (referred to as suburban forests in other studies, **I-II**) forests in and around the city of Jyväskylä (**III**). Pregnant females were live trapped in urban and rural forests with the use of Ugglan traps and brought to the facilities at JYU for housing until they gave birth. Then, the nursing mothers with their newborn offspring were transferred to a forest site that was different from their origin site, such that four experimental groups were created as part of the experiment (Fig. 6). Transferring mothers with newborn offspring increased the chances that the female adult would not disperse but would rather stay in the area until her young would be fully nursed. By the end of the nursing time, we recaptured the mothers (average nursing time for bank voles ~3-4 weeks).

In this study, every experimental individual was sampled twice: 1) the initial pre-transfer sampling after which the animal was kept in captivity until the birth, and 2) the post-transfer sampling after which the animal was released back into the field as soon as possible. During both sampling sessions in the field, bank voles were immediately put into plastic boxes (sterilised with ethanol) for transport. At the JYU facilities, sterilised tweezers were used to collect the faecal matter from the boxes into sterile tubes that were then transferred to the -80◦C freezer until further processing. At the end of the experiment, I successfully collected pre- and post-transfer faecal samples for 28 mothers (*n* experimental faecal samples=56).

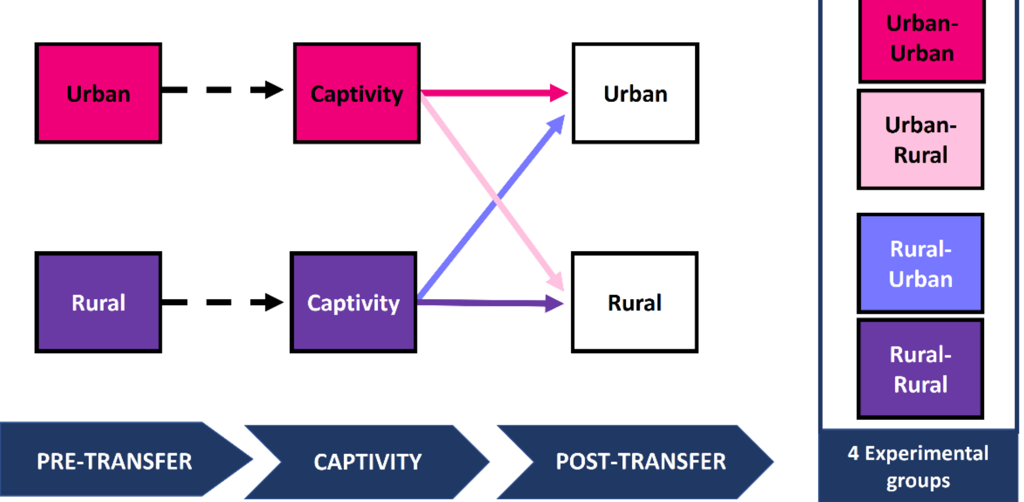

FIGURE 6 Reciprocal transplant experiment design. Forty urban and forty rural adult female bank voles were captured in the field and brought to the JYU facilities to give birth. Pre-transfer faecal samples were collected on the day of their initial arrival to the lab. Two days after birth, the mothers were transferred to the wild together with their offspring. Mothers never returned to their exact site of origin. Mothers were recaptured after spending 3-4 weeks in their new forest habitat, brought back to the JYU facilities where the post-transfer faecal samples were collected. Figure taken from Manuscript III.

2.2 Laboratory analyses

2.2.1 Animal dissection and x-ray imaging

All bank voles that were captured as a part of the cross-sectional dataset (*n* cross-sectional faecal samples=382) were dissected at the JYU facilities. The distal part of the colon (~2cm) was removed to collect faecal matter which was immediately stored on dry ice and then transferred to -80°C until DNA extractions. Jaws including teeth were also collected and stored in ethanol. Body mass, sex and breeding characteristics were recorded for each animal. Body composition, including bone area (in cm^2), total tissue mass, and percentage of body fat were measured for each bank vole using the dual energy x-ray imaging (Lunar Piximus, General Electric).

2.2.2 DNA extraction and sequencing

The DNA from all faecal samples (*n* soil samples=312 (I), *n* cross-sectional faecal samples=382 (**II**), *n* experimental faecal samples=56 (**III**)) was extracted at the JYU facilities using a Qiagen DNeasy PowerSoil Pro Kit following the manufacturer's instructions. Sequencing of the DNA was performed using an Illumina HiSeq at the Beijing Genomics Institute (BGI, https://www.bgi.com/global/), using the 515F/806R primer pair (Caporaso *et*

al. 2011) for amplification of the V4 region of the 16S ribosomal RNA (rRNA) gene in bacteria (**I-III**), and the ITS3/ITS4 primer pair (White *et al.* 1990) to target the ITS2 region in fungi (**I-II**). The exact number of reads acquired through the process of amplicon sequencing differed between studies, thus detailed information can be retrieved from each individual manuscript.

2.2.3 Soil pH measurements

The soil pH was measured (**I**) from all soil samples by oven drying the samples at 38°C for three days, after which the dry mass was diluted in deionised water (1:3 ratio, soil to water). The solution was mixed on a shaker platform for 1 hour before using a combination pH electrode (Mettler Toledo, InLab® Expert Go, Vantaa, Finland) to determine the soil pH.

2.2.4 Stable isotope analysis

The isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) were quantified in both bank vole fur and environmental vegetation material using stable isotope analysis (Crawford *et al.* 2008). The isotopes in bank vole hair reflect the long-term diet of the animal since isotopes are assimilated in newly grown hair, and the composition correspond to the dietary intake (Kurle *et al.* 2014). Since hair is a metabolically inert material, the hair shaft contains the isotopes that are assimilated over a long period of time (~1-2 months prior to capture). Additionally, the isotopes present in the collected vegetation material (*Sorbus*, bilberry, lingonberry) can be used to approximate the level of elements present in the environment and in putative food sources (Butet and Delettre 2011). Hence, the isotopes in the vegetation were used to examine the baseline variation in nitrogen and carbon among the four forest types (Balčiauskas *et al.* 2018).

Fur samples collected during field work were processed to remove the lipids using a 2:1 chloroform:methanol solution (Blight and Dyer 1959). Environmental samples were homogenised by TissueLyser II (Qiagen) and steel beads. All samples were oven dried on 60°C C for 24h before 0.5-1.2 mg of each sample was analysed for carbon and nitrogen isotopes by using a Thermo Finnigan DELTAplus Advantage stable isotope ratio mass spectrometer connected to a Carlo Erba Flash EA1112 elemental analyser. Calibration of the results was done according to the method described in Lavrinienko *et al*. 2020.

2.2.5 Bank vole age estimation

Jaw samples obtained during the dissections were boiled to facilitate the removal of individual teeth. The first lower molar tooth on the right and left mandible were selected. The mean length of the root was used as a proxy for age since in bank voles the roots continue to grow throughout life. The protocol was carried out using an Olympus SZ51 stereomicroscope according to the methods described in Meri *et al*. (2008).

2.3 Bioinformatic analyses

All sequence data were processed using QIIME2 (the implemented versions of QIIME2, associated plugins and reference databases differed between studies and are described in each individual manuscript (Bolyen *et al.* 2019), following the same workflow. First, adaptor sequences were removed with the CUTADAPT plugin (Martin 2011). Then, the DADA2 plugin (Callahan *et al.* 2016) was used to trim off primers, truncate the 3'end of low-quality reads (exact cut-offs differed between studies and are described in each individual manuscript), to merge the paired reads, and filter out chimeric sequences. These steps generated feature tables of amplicon sequence variants (ASVs). Next, non-target (*e.g.,* Archaea, Eukaryota (when working with bacteria), mitochondria, chloroplasts and sequences without assigned Kingdom) and low-frequency ASVs (*i.e.,* less than 10 reads overall) were filtered from the feature tables. After this step, rarefaction was applied to ensure that all samples contained the same number of total read sequences (Weiss *et al.* 2017). To gain taxonomic information, I assigned taxonomy to each ASV by training the Naive Bayes classifiers on the SILVA database for the V4 region of 16S rRNA in bacteria (Quast *et al.* 2012), and on the UNITE database for fungi (Nilsson *et al.* 2019). For both kingdoms, clustering of reference sequences was applied according to the 99% sequence similarity threshold. Phylogenetic midpoint rooted trees were constructed with the use of the FASTTREE plugin (Price *et al.* 2010), but only for bacteria, since the ITS2 fungal region evolves too rapidly to be useful for phylogeny-based analyses (Nilsson *et al.* 2008).

Functional traits were assigned to bacterial and fungal ASVs in soil by implementing FAPROTAX (Louca *et al.* 2016) and FUNGUILD (Nguyen *et al.* 2016), respectively (**I**). The latter program was also used as an additional tool to manually curate the fungal ASVs found in the bank vole gut into likely non-resident fungi (*e.g.,* groups of fungi known to grow fruiting bodies, (Lavrinienko *et al.* 2021)) and potential resident fungi after which the first group was filtered out of the dataset (**II**).

To study the temporal differences in the gut microbiota communities of the experimental bank voles (**III**), I used the Q2-LONGITUDINAL plugin (Bokulich *et al.* 2018b) within QIIME2. The output (*i.e.,* paired-differences and paired-distances) was loaded into R for further analysis. To clarify, paired differences correspond to the difference in alpha diversity (see Section 2.4.1) between the pre-transfer and post-transfer faecal gut microbiota of the same individual, while paired distances equal the amount of compositional change (beta diversity, see Section 2.4.2) that occurred within the gut microbiota of a single individual between the pre- and post- transfer sample collection times.

2.4 Statistical analyses

2.4.1 Background information on key microbiota metrics

Microbial communities are usually studied in terms of their diversity and composition, and the two metrics that are commonly used to describe these patterns are alpha diversity and beta diversity. Alpha diversity represents the within-sample diversity, and the simplest way of calculating it is by estimating the number of unique ASVs (or microbial species) per sample (*i.e.,* richness). In contrast to alpha diversity, beta diversity represents the between-sample diversity. Therefore, this metric is measured on the group level as opposed to the individual sample level. The simplest beta diversity metric measures the difference in microbial composition among samples (dissimilarity), making it possible to compare the inter-sample diversity between different study groups or treatments. The value for beta diversity ranges between 0 (complete overlap in the composition of different samples) to 1 (no overlap in the composition of different samples). Beta diversity can be measured with different distance metrics, with each metric focused on a different aspect of the community. For example, the Bray-Curtis metric is the ideal metric to use when relative abundances of taxa should be taken into account (abundant taxa will influence the calculations more than rare taxa) but it does not consider the phylogenetic relationship between these taxa. When both relative abundances and phylogenetic information are important, the Weighted UniFrac metric can be implemented instead. The corresponding metrics for the Bray-Curtis and the Weighted UniFrac, yet that give more weight to rare taxa, are the Jaccard Index and the Unweighted UniFrac metric, respectively. Data on alpha diversity are mostly summarised using boxplots or scatter plots, while beta diversity is typically visualised by ordination plots (with samples that have similar microbial compositions being plotted closer together). Information about the alpha and beta diversity gives an overview of the regional diversity in samples (diversity within each sample and the diversity between samples). The goal of many microbiome studies is to identify environmental or host-associated variables that can (at least partially) explain the patterns and any differences in alpha and beta diversity between different groups of samples. For example, in this thesis, I examined whether forest type (urban vs non-urban) influences the microbiota alpha and beta diversity of microbiota found in soil (**I**) and the bank vole gut (**II**).

2.4.2 General analyses

The feature tables were transformed into phyloseq objects with the PHYLOSEQ package (McMurdie and Holmes 2013) in R (the implemented versions of R and individual packages differed between studies and are described in each individual manuscript (R Core Team 2020)), and the phyloseq objects based on the rarefied data were used to calculate the alpha and beta diversity metrics. Next, the importance of variables of interest (*e.g.,* forest type) were tested by

running PERMANOVA tests with the *adonis2* function in the R package VEGAN (Oksanen *et al.* 2020). The output of these tests showed how much of the variation in the composition of the microbiota found in soil/gut samples (beta diversity) could be explained per factor and included the effect size (R^2) and significance level ($p<0.05$). The alpha diversity estimates were compared between the different forest types using the Kruskal-Wallis (KW) test with a Benjamini–Hochberg adjustment (BH) for multiple comparisons with the DUNN.TEST R package (Dinno 2015). Lastly, differential abundance analyses were performed on the unrarefied feature tables with the DESEQ2 (**I** (Love *et al.* 2014)) and ALDEX2 (**II** (Fernandes *et al.* 2014)) packages in R.

2.4.3 Analyses specific to Manuscript I

To study the effects of the environmental variables (attained from the habitat surveys and pH measurements, **I**), I first removed collinearity in the data by examining correlations between variables with the Spearman's rank correlation tests (when ranked categorical variables were compared) or the Pearson's correlation coefficient (when numeric variables were compared). The remaining variables were fitted into a linear model with the use of the *lm* function, with alpha diversity as the response variable, after which the *dredge* function in the MUMIN R package (Barton 2020) was applied for model selection. The output of this workflow yielded the list of important variables influencing alpha diversity. To examine which variables impacted beta diversity the most, I used additional PERMANOVA tests with the *adonis2* function to compare the portion of explained variation (R^2) associated with each variable. Additionally, differences in the bacterial and fungal functional traits between different forest types were examined using the DUNN.TEST R package (KW test with BH correction).

2.4.4 Analyses specific to Manuscript II

The relative contribution of soil microbes (**I**) to the total composition of the gut microbiota in bank voles (**II**) was examined using the QIIME2 plugin SOURCETRACKER2 (Knights *et al.* 2011). The dietary niche width per bank vole and the dietary niche overlap between bank voles was calculated with the SIBER ((Jackson *et al.* 2011) and RJAGS (Plummer 2003) packages in R. A plugin implemented in QIIME2 (supervised learning classifier plugin (Bokulich *et al.* 2018a)) was used to determine the accuracy with which the machine learning models could successfully predict the forest type solely based on the bank vole gut microbiota composition. Differences in bank vole host variables (*i.e.,* weight, age, body composition data, fur isotope data) and environmental vegetation isotope data between forest types were tested using the DUNN.TEST R package (KW test with BH correction).

2.4.5 Analyses specific to Manuscript III

Appropriate QIIME2 outputs (*i.e.,* paired-differences (alpha diversity) and paired-distances (beta diversity)) were used to test if alpha and beta diversity changed within an individual throughout the experiment, and whether the magnitude of these changes differed between animals belonging to different experimental groups ('Urban-Urban', 'Urban-Rural', 'Rural-Urban' and 'Rural-Rural', **III**). Specifically, linear models were constructed by using the *lm* function with either paired-differences or paired-distances as response variables and the site of origin, the site of transfer, and their interaction as explanatory variables. Inter-group comparisons were calculated with Tukey Honest Significant Differences with the *aov* and *TukeyHSD* functions in R.

Besides studying the magnitude of change in the gut microbiota, I was also interested in understanding the directionality of these changes. For example, I examined whether the post-transfer gut microbiota of animals transferred between different forest types (*e.g.,* 'Urban-Rural') more resembled the gut microbiota of animals associated with the native microbiota of their origin site ('Urban-Urban') or their transfer site ('Rural-Rural'). Therefore, I used the alpha diversity data from the post-transfer gut microbiota and implemented the same workflow as described above with the use of the *lm*, *aov* and *TukeyHSD* functions. To gather information about the directionality of the beta diversity, I ran PERMANOVA tests that included a distance metric based upon the post-transfer compositions within the *adonis2* function to examine whether the site of origin, site of transfer and/or their interaction was more important for explaining the composition of the post-transfer gut microbial communities. Additionally, the *ordination* function with a priori given hypothesis (*i.e.,* site of origin + site of transfer) within the PHYLOSEQ package was used to create Constrained Analysis of Principal Coordinates plots to aid the visualisation of the beta diversity patterns in the bank vole gut microbiota.

3 RESULTS AND DISCUSSION

3.1 Key findings

By quantifying the bacterial and fungal microbial communities, I found that the urban environment shapes both free-living microbiota in soil, and host-associated microbiota residing in the gut of wild bank voles. For soil communities, soil pH (more alkaline soil in cities) was identified as an important factor influencing the community composition (Fig. 7, **I**), while an apparent shift in host diet is likely responsible for the changes observed in the gut microbiota of urban bank voles (Fig. 7, **II**). Additionally, I did not find evidence for frequent or abundant microbial spill-over between microbes in the soil and microbes in the gut microbial communities of bank voles. In addition, I found that for animals translocated between urban and rural forest sites, both the past and present habitat have an influence on the gut microbiota composition. This suggests that urban and rural environments not only shape the gut microbiota directly, through the presence of different dietary items, but also prime bank voles during their early life. In other words, the effects on the gut microbiota of living in an urban (or rural) environment are not entirely reversible in wild rodents (Fig. 7, **III**).

Taken together, the three manuscripts included in my doctoral thesis provide a comprehensive view on the impacts of anthropogenic habitat disturbance on free-living and host-associated microbiota (Fig. 7).

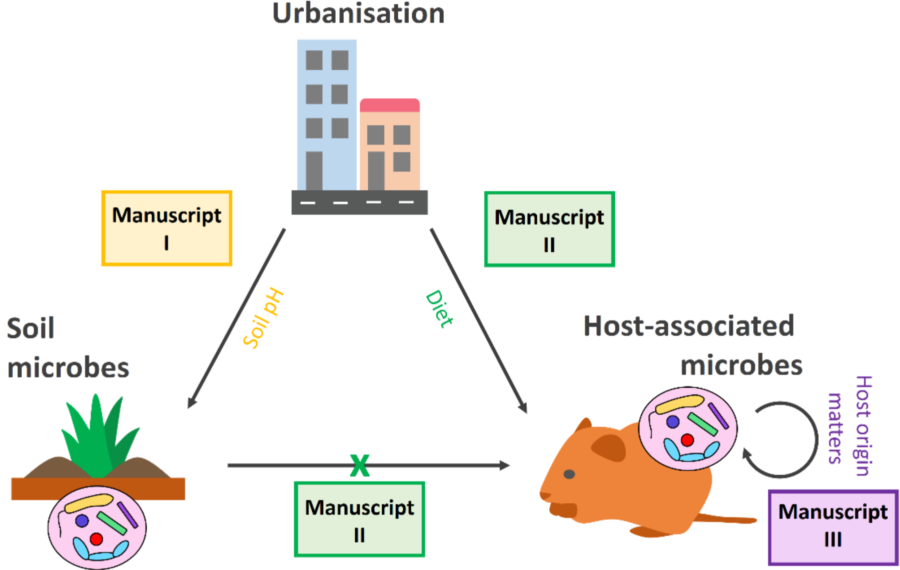

FIGURE 7 The diagram provides an overview of the results that have been found in the manuscripts included in this thesis.

3.2 Urban forest soils harbour distinct and more diverse communities of bacteria and fungi (I)

In this study, I found that the composition of forest soil microbiota changes depending on the level of anthropogenic disturbance, with urban forests having the most distinct soil microbiota in comparison to other forest types (*i.e.*, suburban, managed forests, and national parks, **I**). Alpha diversity of soil communities was positively associated with the level of anthropogenic disturbance, such that urban soil samples contained the highest alpha diversity (Fig. 8). From all studied environmental variables, I found soil pH to be the strongest predictor of the microbial alpha (Fig. 9) and beta diversity in forest soil. In accordance, I also found a consistent increase in soil pH with the level of anthropogenic disturbance. This pattern can be explained by rain run-off through alkaline concrete material such as streets and gutters (Davies *et al*. 2010, Nugent and Allison 2022). Indeed, soil alkalisation is most likely an inherent property of the built environment (Delgado-Baquerizo *et al.* 2021), which contributes to the differences observed between soil microbial communities in urban and natural forests.

In contrast to bacteria, where I found little difference in the prevalence of functional traits between urban and non-urban forests, I identified a decline in the relative abundance of ectomycorrhizal fungi in urban forests. One potential reason for this could be a concurrent decline in ectomycorrhizal plant species in urban areas (Tedersoo *et al*. 2020, Delgado-Baquerizo *et al*. 2021).

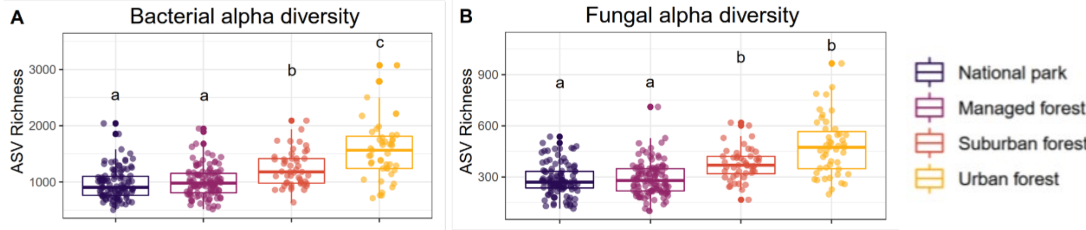

FIGURE 8 Boxplots showing the alpha diversity (ASV richness) of soil microbial communities with regard to the disturbance level of the forest, for bacteria (A) and fungi (B). Higher levels of bacterial and fungal alpha diversity are observed in forests with higher disturbance. Letters refer to significance levels. Figure taken from Manuscript I.

With these results, I demonstrate that even when habitats are comparable, urban and non-urban areas can still be distinguished from one another solely based on the composition of soil microbiota. As such, one implication of these findings is that rewilding cities (through the development of urban green spaces, the 'rewilding paradigm' (Mills *et al.* 2017)) is likely to be insufficient for recreating 'natural' microbial communities in urban areas.

Interestingly, the highest levels of microbial diversity were found in the least pristine habitats which suggests that the diversity measures commonly used for preserving macrospecies (*i.e.,* higher diversity means higher conservation status) do not work equally well on the microbial level. One somewhat surprising implication is that humans living a rural lifestyle are not necessarily exposed to the highest diversity of soil microbes in comparison to humans living in cities. In this context, perhaps exposure to specific microbial taxa is likely to be more important for an adequate immune system stimulation (rural lifestyle is linked to lower incidence of atopic diseases, see Section 1.2.1.), rather than exposure to more diverse microbial communities *per se*. Alternatively, the key microbial exposure might occur through other pathways, such as frequent contact with domestic/ companion animals or via ingestion of more natural vs. processed foods (von Mutius and Vercelli 2010), rather than through direct contact with environmental microbes found in soil.

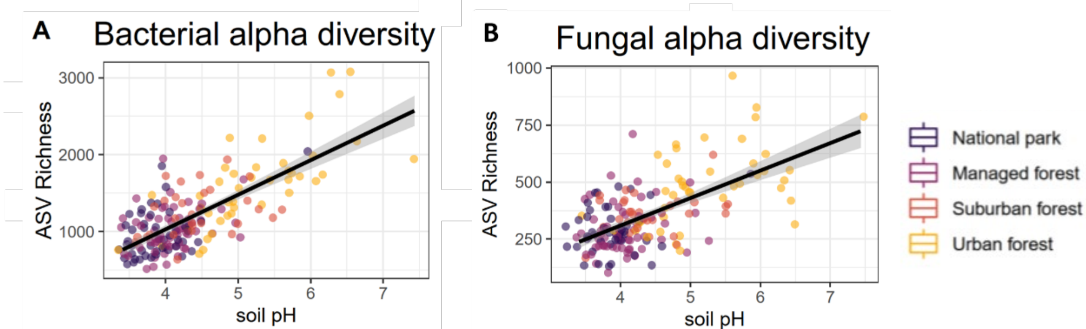

FIGURE 9 Correlation between soil pH and the ASV richness of bacterial (A) and fungal soil communities (B). A positive association between soil pH and bacterial and fungal diversity can be observed. Colours correspond to the disturbance level of the forest. Figure taken from Manuscript I.

3.3 Dietary change alters the gut microbiota in urban rodents (II)

I found that the bacterial and fungal gut microbiota of bank voles differed depending on the level of habitat disturbance (Fig. 10, **II**), with urban bank voles harbouring the most distinct gut microbiota in comparison to animals inhabiting any other forest type. Indeed, the machine learning algorithm successfully assigned nearly all urban samples to their origin solely based on the bank vole gut microbiota composition (100% of samples for gut bacteria, and 96% for gut fungi). I did not find significant differences in the alpha diversity of the bacterial gut microbiota among forest types. In contrast, I found a negative association between the alpha diversity of the gut fungi in bank voles and the disturbance level in their site of origin.

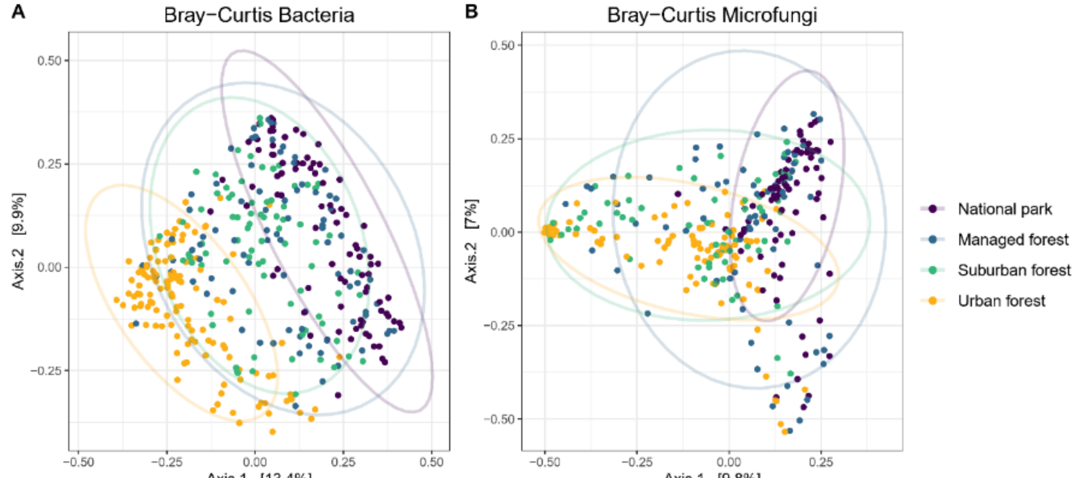

FIGURE 10 Ordination plots showing the composition of the bacterial (A) and fungal (B) communities residing in the gut of wild bank voles. Each dot represents a single faecal sample, coloured according to the forest type. The visualised ordinations are based upon the Bray-Curtis distance metric. Figure taken from Manuscript II.

These gut microbiota patterns are likely driven by a change shift in the bank vole diet. Indeed, the dietary niche width was 29-44% higher in urban bank voles and only had an overlap of 29-40% with the dietary niches of bank voles inhabiting any other forest type. Moreover, in comparison to bank voles living in the national parks, urban bank voles also had a 5% increase in fat percentage, and a significant increase in nitrogen levels found in their fur (which could not be explained by an increase in environmental nitrogen, as shown by comparing vegetation samples, Fig. 11), as well as higher proportions of microbial genera associated with low-quality processed foods. Interestingly, taken together these data suggest that urban bank voles experience a dietary shift towards a typical "western diet" (Dillard *et al.* 2022).

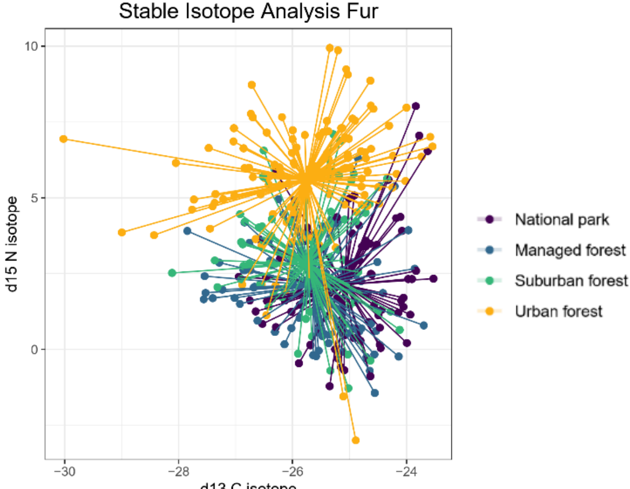

FIGURE 11 Starplot visualising the nitrogen and carbon content in the fur of wild bank voles. Each dot represents a single sample, coloured according to the forest type. Figure taken from Manuscript II.

The small dietary niche overlap with bank voles inhabiting other forest types, higher body fat percentage, and higher proportions of microbial genera related to processed foods could potentially be attributed to urban animals encountering a greater spectrum of novel anthropogenic and energy-rich food items (Anders *et al.* 2022). Indeed, an increase in nitrogen in the bank vole fur also suggests that these animals increase their protein intake, Fig. 11 (Kelly JF 2000)). In practice, this can potentially be explained by an enhanced predatory behaviour or broader changes in foraging of urban voles, which for instance could lead to a more insect rich diet or more frequent consumption of fungal fruiting bodies (Galetti *et al.* 2016). Additionally, using microbial source tracking analysis, I did not find a clear link between the microbes found in the soil and the gut microbiota of bank voles living in the same forest patch. However, I found a discrepancy in microbial source tracking between bacteria and fungi, with soil fungal ASVs contributing more to the bank vole gut microbiota than soil bacteria. This differences in recovery rate of ASVs between bacteria and fungi could be due to fungal spores exhibiting resistance to the acidic gastric passage (Coluccio *et al.* 2008) which would suggest that many of the observed fungal ASVs are not functional members of the gut mycobiota. Nevertheless, such overall negligible contribution of soil microbes to the bank vole gut microbiota is somewhat surprising and suggest that even with their soil-dwelling lifestyle, soil is unlikely to be a significant source of microbes for adult bank voles. In accordance with the results of this study, I highlight the importance of studying wildlife in the context of both urban and non-urban settings since their lifestyle, their gut microbiota and perhaps their behaviour can be different in cities, which subsequently can change natural dynamics of host populations.

3.4 Effects of past and present habitat on the gut microbiota of a wild rodent (III)

In this study, I demonstrate that the bank vole gut microbiota are shaped by the cumulative effects of resistance and plasticity (Fig. 1C, Fig. 12, **III**), with both mechanisms explaining roughly equal amount of variation present in the gut microbiota (~5%). Indeed, the non-phylogenetic beta diversity metrics (Bray-Curtis, Jaccard) detected both signs of resistance (site of origin) and plasticity (site of transfer) while the Unweighted UniFrac metric (see Section 2.4.1. for more information) only identified resistance as an important mechanism. There were no differences between experimental groups on the level of abundant phylogenetically dissimilar taxa (Weighted UniFrac metric). Taken together, these results suggest that the past habitat (site of origin) of bank voles mostly determines the presence of rare phylogenetically different taxa, while the present habitat (site of transfer) influences the distribution of abundant and phylogenetically similar taxa.

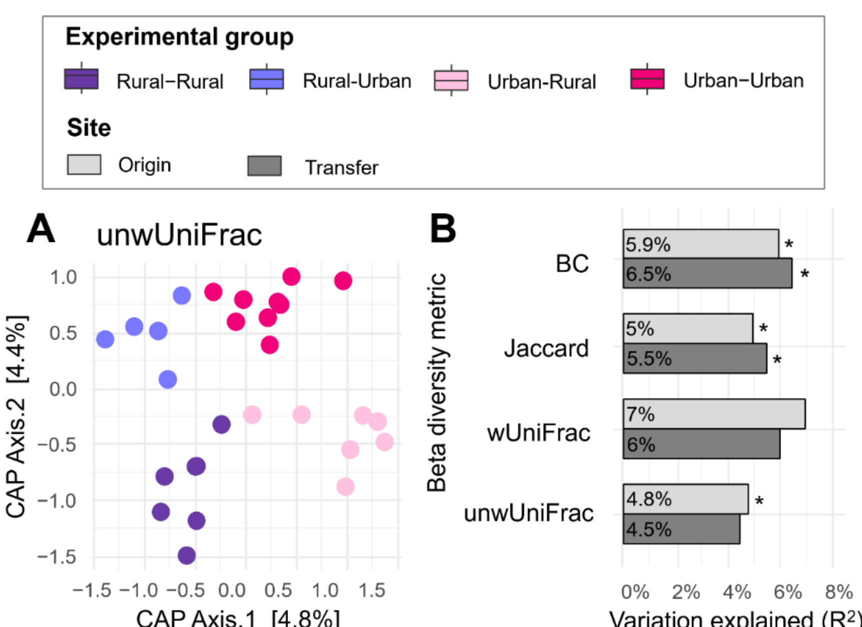

FIGURE 12 Effect of site of origin and site of transfer on the post-transfer gut microbiota of bank voles. Ordination plot showing the composition of the post-transfer bacterial communities residing in the gut of wild bank voles, coloured by the experimental group they belong to (A). Each dot represents a single faecal sample. The ordination is based upon the Unweighted UniFrac distance metric. The x axis separates animals originating from different forest habitats while the y axis divides the animals according to the different type of forest they were transferred to. The exact amount of variation in the composition explained by the two variables of interest is shown per distance metric (B). Asterisks refer to significant results (p<0.05). Figure taken from Manuscript III.

The implication of these findings is that after translocation, bank voles retain part of their original gut microbiota (Allison and Martiny 2008), but also gain features

of the 'typical' microbiota associated with the new transfer environment (Ren *et al.* 2016). While reasons and exact mechanism behind these observations remain unknown, this pattern could be driven by priority effects that hinder complete plasticity (Robinson *et al*. 2010, Obadia *et al*. 2017, Björk *et al*. 2018). In the context of urban effects, I found a trend towards a higher turnover in the gut microbiota of bank voles originating from urban forests in contrast to rural forests (Fig. 13). In addition, bank voles originating from urban forests also had a higher level of alpha diversity in their post-transfer gut microbiota.

This study demonstrates the importance of considering previous exposures when assessing gut microbiota and adaptive responses of wild animals experiencing environment change, and highlights the relevance of rare taxa. I also show that both living in a natural (rural forests) or an artificial environment (urban forests) can have a long-lasting effect on the wildlife gut microbiota. These findings make a significant contribution to the field of evolutionary ecology and microbiome research, and in the long term could also provide an impact in a more applied dimension, by informing decision-making in the field of conservation, especially with regard to translocation of animals (Carthey *et al*. 2019, van Leeuwen *et al*. 2020).

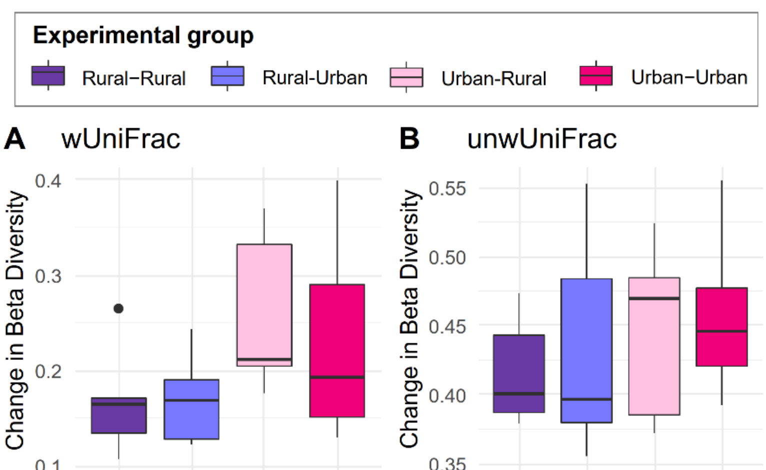

FIGURE 13 The turnover in the bacterial gut composition within the same individual bank vole between the pre- and post-transfer gut samples, summarised per experimental group. There is a trend towards a higher turnover in the bacterial gut composition in bank voles originating from urban forests in contrast to animals from rural forests. Turnover rates are shown for both weighted UniFrac (A) and unweighted UniFrac (B) distances. The colours represent the different experimental groups. Figure taken from Manuscript III.

4 CONCLUSIONS AND FUTURE DIRECTIONS

Through the indirect effect of building cities, humans have the capacity to change microbial communities found in the environment and inside the gastrointestinal tract of wild urban animals. Concrete materials in urban areas associate with alkaline soil which impacts soil communities (Delgado-Baquerizo *et al.* 2021), while access to anthropogenic foods can potentially create a shift in the diet of wild bank voles and hence their gut microbiota communities (Dillard *et al*. 2022, Anders *et al*. 2022). Such changes in the gut microbiota appear to be long-lasting, as urban animals retain urban-specific rare taxa in their gut microbiota even after spending considerable amount of time in a rural forest. Differences between free-living microbes in urban and non-urban areas suggest that the microbial exposure experienced by humans in cities is different from that experienced by people living in more natural environments. This brings unknown repercussions for human health (Sonnenburg and Sonnenburg 2019) and the functioning of the human immune system and could be linked to a higher prevalence of atopic diseases in more urbanised human populations (Von Hertzen *et al*. 2011, Hanski *et al*. 2012). At the same time, animals that roam freely in cities have a different gut microbiota than their conspecifics living in more natural habitats, likely because they have access to novel food items of anthropogenic origin. It is possible that the changes in the gut microbiota of urban animals can interfere with the animal host health and can stimulate the overgrowth of certain pathogenic microbial strains. This in turn could also have important consequences for disease risk in humans, especially if such urban host species are common reservoirs for zoonotic diseases (such as rodents). As such, humans, animals, microbes and the environment are undeniably interconnected.

Although key findings in my thesis are based on robust data, these broader conclusions regarding human and animal health remain to be constrained by the limitations of largely correlative observations. For example, further mechanistic studies are needed to investigate direct links between environmental microbial communities and the incidence of atopic diseases in humans to identify microbial taxa important for proper human immune development. In my research on wild animals, some key limitations include the absence of data on short-term diet (*e.g.*,

through diet metabarcoding), direct measurements of health impacts (*e.g.,* immune markers), pathogen/parasite burden data, and the lack of assessments of long-term fitness parameters (*e.g.,* survival rates and reproduction). If time and resources would not be an issue, one potential approach to study the fitness consequences of urbanisation upon the bank vole gut microbiota would be carrying out a faecal microbiota transplant experiment between urban and rural animals to evaluate changes in host health and physiology, both in laboratory and (semi-)natural settings (*e.g.,* outdoor enclosures). It would also be interesting to gain more knowledge and resolution about the identity and function of urban microbes, and to examine the relevance of functional redundancy (*i.e.,* different taxonomic taxa perform the same functions), for instance via integration of multi-omics techniques (*i.e.,* shotgun metagenomics and metabolomics). Finally, one other crucial next step would be to investigate how general the findings in this thesis are, for instance by examining multiple host species that differ in life-history traits and/or lifestyle, and surveying cities that differ in sizes (and thus have different levels of disturbance) and biomes.

While this thesis generates a number of new questions and hypotheses that can be tested in future studies, it is clear that the microbial communities found in urban soil and in the gut of urban animals differ from those found in natural forests. Although health consequences of such differences remain to be quantified, I would kindly suggest that all people start to invest in reconnecting with nature since it is entirely possible that the benefits of being in nature extend far beyond having a breath of fresh air.

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ORIGINAL PAPERS

I

URBAN FOREST SOILS HARBOUR DISTINCT AND MORE DIVERSE COMMUNITIES OF BACTERIA AND FUNGI COMPARED TO LESS DISTURBED FOREST SOILS

by

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ORIGINAL ARTICLE

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Urban forest soils harbour distinct and more diverse communities of bacteria and fungi compared to less disturbed forest soils

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Abstract

Anthropogenic changes to land use drive concomitant changes in biodiversity, including that of the soil microbiota. However, it is not clear how increasing intensity of human disturbance is reflected in the soil microbial communities. To address this issue, we used amplicon sequencing to quantify the microbiota (bacteria and fungi) in the soil of forests ($n = 312$) experiencing four different land uses, national parks (set aside for nature conservation), managed (for forestry purposes), suburban (on the border of an urban area) and urban (fully within a town or city), which broadly represent a gradient of anthropogenic disturbance. Alpha diversity of bacteria and fungi increased with increasing levels of anthropogenic disturbance, and was thus highest in urban forest soils and lowest in the national parks. The forest soil microbial communities were structured according to the level of anthropogenic disturbance, with a clear urban signature evident in both bacteria and fungi. Despite notable differences in community composition, there was little change in the predicted functional traits of urban bacteria. By contrast, urban soils exhibited a marked loss of ectomycorrhizal fungi. Soil pH was positively correlated with the level of disturbance, and thus was the strongest predictor of variation in alpha and beta diversity of forest soil communities, indicating a role of soil alkalinity in structuring urban soil microbial communities. Hence, our study shows how the properties of urban forest soils promote an increase in microbial diversity and a change in forest soil microbiota composition.

KEYWORDS
bacteria, biodiversity, forest management, fungi, national park, urban

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1 | INTRODUCTION

An accelerating rate of habitat conversion is a prominent feature of the Anthropocene. This change in landscape typically comes at the cost of degradation and loss of natural habitats and biodiversity (McDonald et al., 2020; Seto et al., 2010). The effects of habitat conversion on the soil are often neglected but are of vital importance given that healthy ecosystem functioning depends on the communities of soil-associated microbes, via processes such as primary production, decomposition, carbon cycling and nutrient mineralization (Fierer, 2017). Hence, changes in land use might impact microbial biodiversity and ecosystem functions.

Two important drivers of habitat conversion are expansion of urban areas and the increase of natural resource exploitation (Kuipers et al., 2021). To mitigate against habitat loss, land can be set aside, for example as national parks or urban greenspaces, to provide putative benefits of recreational use (Li et al., 2021; Marselle et al., 2021; Siikamäki et al., 2015) and climate regulation (Mexia et al., 2018), and/or to act as biodiversity refugia (Lehmann, 2021; Mills et al., 2017; Siikamäki et al., 2015). Of course, the emphasis on type of land use differs among national parks and urban greenspaces, with the former areas primarily directed towards biodiversity conservation (Siikamäki et al., 2015) and the latter more towards recreational use and human health/well-being (Li et al., 2021). Inevitably, urban greenspaces also experience greater anthropogenic impacts through direct use and by virtue of being located within or adjacent to an urban area. Indeed, it is, for example, known that not all (macro)species can tolerate or adapt to life in an urban area (Faeth et al., 2011; Parsons et al., 2018; Spotswood et al., 2021) and that the (macro)biodiversity of urban habitats typically differs from that in more natural areas (e.g., national parks). Such changes in assemblages associated with cities might spread to adjacent suburban areas to create a gradient of biodiversity and associated ecosystem services (Spotswood et al., 2021). Outside urban areas, intensive forest management practices, such as clear-cutting, cause a disturbance that degrades forests and impacts various groups of biodiversity (Fisher & Wilkinson, 2005; García-Tejero et al., 2018; Thompson et al., 2013). Even after decades of recovery, certain managed forests can harbour different communities of (macro)species when compared to more pristine forests (Fisher & Wilkinson, 2005; García-Tejero et al., 2018; Thompson et al., 2013).

Urban habitats are also associated with a change in the composition of bacterial and fungal soil microbiota. In soil bacteria, changes manifest in an increased alpha diversity (Hui et al., 2017; Naylo et al., 2019; Tan et al., 2019) and an apparent lack of convergence (i.e., a process that makes communities become more similar) in the bacterial community composition (Schmidt et al., 2017). By contrast, there is a lack of consensus about the differences among soil fungal communities in urban and natural areas, with studies reporting either negative (Abrego et al., 2020; Andrew et al., 2019) or neutral (Tan et al., 2019; Tedersoo et al., 2020) impacts of urban habitat on fungal diversity. Nonetheless, Schmidt et al. (2017) reported convergence of fungal communities within cities, but only when natural reference sites were compared with construction sites within the urban matrix. Such changes in fungal soil microbiota were associated with the loss of ectomycorrhizal taxa (ECM) in cities (Schmidt et al., 2017), supporting the idea of fungal convergence in urban areas due to the loss of sensitive species (McKinney, 2006). Also, forest management outside cities is thought to have a weak positive effect on the divergence (i.e., a process that makes communities become more dissimilar or dispersed) of bacterial and fungal communities (Lee-Cruz et al., 2013). However, the impacts of anthropogenic disturbance upon the soil microbiota in urban forests are expected to outweigh those in managed forests (Lee & Eo, 2020; Lee-Cruz et al., 2013).

A key limitation with many studies that have attempted to quantify effects of urban land use on soil microbial community composition is that land use is either ambiguously defined or confounded with habitat type. For example, studies examining urban soil microbiota do not clearly describe the habitat type (such as whether the soils were grassland or forest; Andrew et al., 2019; Pouyat et al., 2015; Schmidt et al., 2017; Tedersoo et al., 2020), or the habitat types differ between the urban and nonurban sample locations (such as sampling gardens and parkland within urban areas and sampling forests outside urban areas; Abrego et al., 2020; Hui et al., 2017; Tan et al., 2019; Wang et al., 2018). Sampling comparable habitats is an important issue to consider given the general association between habitat type and the composition of soil microbiota (Baruch et al., 2020, 2021; Hui et al., 2017; Mills et al., 2020).

In this study, we used amplicon sequencing to examine the impacts of anthropogenic disturbance on the forest soil microbiota (bacteria and fungi) where we specifically define disturbance as human-induced changes to the environment that affect the natural structure of ecosystems, including that of microbial communities (adapted from Sergio et al., 2018). To capture the multifactorial nature of anthropogenic impacts, we first calculated a standardized geospatial index—the Human Influence Index (HII; https://doi.org/10.7927/H4BP00QC)—under the assumption that greater proximity to humans and their built settlements is associated with a higher degree of chronic disturbance to natural systems (Arnan et al., 2018). The HII differentiates among three forest types (Table S1; Figure S1): urban forests, suburban forests and forests located away from the built environment. Because the HII does not account for the effects of commercial forestry (Danneyrolles et al., 2019), we further partitioned the last category of forests into either managed forests or national parks. As protected areas, forests in national parks have some of the lowest levels of chronic anthropogenic disturbance and forest management that is possible to find in Northern Europe. National park samples thus serve as an ideal contrast to quantify possible legacy effects of commercial forestry (Hartmann et al., 2013) and to examine effects of biodiversity conservation (Siikamäki et al., 2015) independently

from urban impacts. Hence, here we combined quantitative and qualitative information to make a distinction between four levels of human-induced forest disturbance which broadly represent a gradient: (i) urban forests (areas located entirely within an urban area), (ii) suburban forests (commercially managed forests that are adjacent to urban areas), (iii) managed forests (commercially managed forests located away from the built environment) and (iv) national parks (unmanaged and protected forests away from the built environment). To the best of our knowledge, no study has placed the urban soil microbiota in a wider context of extensive nature conservation areas with long-term protection status such as national parks.

Building upon results of previous studies on soil microbiota, we hypothesized that (i) proximity to urban areas will increase the alpha diversity of soil bacteria (Hui et al., 2017) while forestry practices will have little long-term impact (Lee-Cruz et al., 2013). In contrast, we did not expect to find clear associations between the fungal alpha diversity and the level of forest disturbance (Tedersoo et al., 2020). Additionally, we predicted to find (ii) distinct soil microbiota profiles between forests of different disturbance levels, where the dispersion in beta diversity changes between soil fungal communities but not between soil bacterial communities (Schmidt et al., 2017). Specifically, we expected to see greater dissimilarities between communities in suburban and managed forests in comparison to communities in cities and national parks for soil fungi. Finally, we predicted that changes in community composition would elicit (iii) distinct functional traits in soil microbiota from forests that differ in disturbance levels, with a noticeable decline of ECM fungi in urban soils.

2 | MATERIAL AND METHODS

2.1 | Study sites

As explained in the Introduction, we studied four forest groups of land use that differ in their level of anthropogenic disturbance: (i) urban forests, (ii) suburban commercially managed forests, (iii) nonurban commercially managed forests and (iv) natural forests located in national parks. We note here that although urban forests were not used for commercial harvest, they are not exempt from low-impact management practices. All soil samples (total $n = 312$, urban $n = 47$, suburban $n = 48$, managed forest $n = 112$, national park $n = 105$) were collected from 20 sample locations (three urban and three suburban forests, seven managed forests and seven national parks, Figure 1) where each sample location was represented by multiple soil replicates (12–22 soil samples per sample location; see Tables S2 and S3 for metadata). All soil samples were collected in a period of 4 weeks in July–August 2019. Urban and suburban forest sites were located within and around three Finnish cities: Jyväskylä, Kuopio and Mikkeli (Figure 1). All urban forest sites were at least 500 m^2 in area and enclosed by houses and roads. The suburban forest sites were located on the periphery of the urban areas with a minimum distance of 200 m to the nearest detached house and at least 500 m from multihouse settlements. The distance between managed forest sites and their corresponding protected forest sites within national parks ranged from 5 to 19 km. All forest sites were located within the boreal forest zone, with habitats dominated by Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*), and silver and downy birch (*Betula pendula* and *B. pubescens*), with bilberry (*Vaccinium myrtillus*) and lingonberry (*V. vitis-idaea*) as undergrowth.

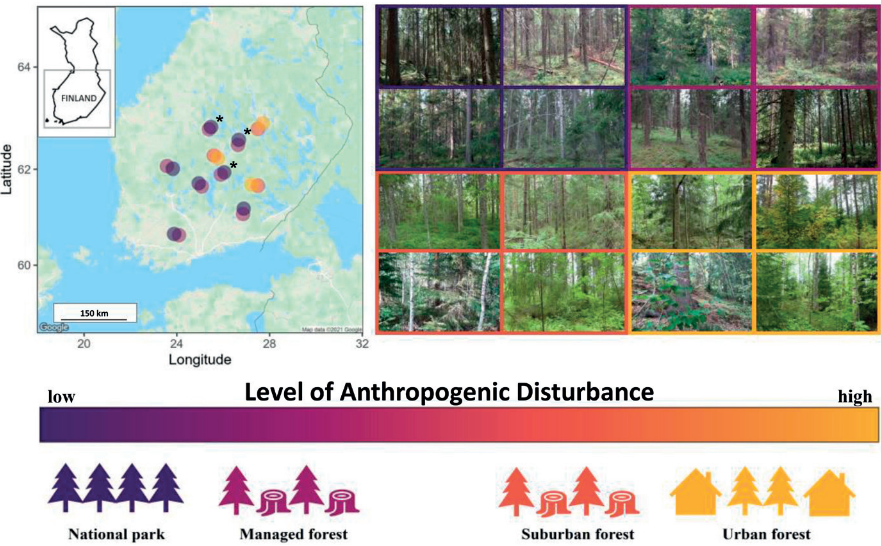

FIGURE 1 Forest soil sampling and study design. The upper left panel provides an overview of the sample locations: Seven national parks (dark purple), their surrounding managed forests (light purple), and suburban (orange) and urban (yellow) forests of three cities (Jyväskylä, Kuopio and Mikkeli). Every point on the map represents 12–22 forest sites where soil samples were collected (total $n = 312$). The upper right panel shows a mosaic of forest photographs with colour frames matching the corresponding forest disturbance level. The bottom panel shows the position of the four studied forest types along a gradient of anthropogenic disturbance that increases from national parks, managed forests, to suburban and urban forests. National parks and managed forests marked with an asterisk are included in the subset of data used for beta diversity analyses ($n = 195$). The map (upper left) was created with the *ggmap* package in R (Kahle & Wickham, 2013). [Colour figure can be viewed at wileyonlinelibrary.com]

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Twenty-three structural habitat factors were quantified for 178 sites during our habitat survey at the time of soil sampling using a method similar to that described by Ecke et al. (2002) (see Tables S2 and S3). These data aimed to describe the biotic and abiotic properties of the habitat using categorical or numerical scales, including the above-ground vegetation (ferns, grass, lichens), abundance of coarse and fine woody debris, and stones. To examine the levels of anthropogenic habitat disturbance in our sampling sites, we calculated the HII for each sample location using the data from the Global Human Influence Index Dataset (https://doi.org/10.7927/H4BP00QC) in ARCGIS version 10.8.1 software. The HII summarizes nine data layers, including population density, land use/land cover, built environment, roads, railroads and other factors reflecting anthropogenic habitat disturbance. The HII differentiates between three out of four forest types with HII levels being highest in urban areas, intermediate in suburban forests and lowest in managed forests ($p < .01$, for details on statistics see the Methods section, Table S1, Figure S1). In contrast, managed forests and national parks had similar HII values.

2.2 | Sample collection and processing

At each sampling site, soil samples (total of ~30 g) were collected at a depth of 10 cm below the ground surface using a metal core instrument (diameter = 3 cm). The precise sample location was picked at random in the sparsely vegetated part of the forest patch (away from roads, forest paths, large trees and/or other dense vegetation). After discarding the upper leaf litter layer and the lower mineral layer, the intermediate organic soil layer was sealed into a sterile plastic bag and mixed thoroughly. When necessary, several cores were taken next to each other to provide enough material per sampling site. Samples were collected wearing gloves and all the equipment was surface sterilized with ethanol before use. All the samples were immediately put on dry ice and stored at −80°C until further processing.

The total genomic DNA was extracted from 100–200 mg of soil homogenate ($n = 312$) using the Qiagen DNeasy PowerSoil Pro Kit following the manufacturer's instructions. Measures were taken to avoid contamination (i.e., working under a laminar flow hood, sterilization of surfaces and tools by UV light, usage of sterile filter tips and plastic ware [according to Eisenhofer et al., 2019]). Soil samples from different study sites were processed in a random order to avoid any systematic bias and possible batch effects. Negative controls containing sterile water ("blanks") were included during DNA extraction. The remaining soil of each original sample ($n = 306$) was oven dried at 38°C for at least 72 hr. Ten grams of dried soil was diluted in deionized water (1:3, soil to water ratio) and mixed thoroughly using a shaker platform for 1 hr prior to measuring pH using a combination pH electrode (Mettler Toledo, InLab Expert Go).

2.3 | Amplicon sequencing and read data processing

The DNA samples were amplified and sequenced using an Illumina HiSeq at the Beijing Genomics Institute (BGI, https://www.bgi.com/global/). Briefly, the 515F/806R (Caporaso et al., 2011) and the ITS3/ITS4 (White et al., 1990) primer pairs were used to amplify the V4 region of the 16S ribosomal RNA (rRNA) gene in bacteria (with 250 bp paired-end [PE] reads), and the ITS2 region in fungi (with 300 bp PE reads) The PE reads were demultiplexed by BGI before being processed with QIIME2 version 2020.8 (Bolyen et al., 2019). We used CUTADAPT (Martin, 2011) to remove adaptor sequences and any resulting short reads. The DADA2 plugin (Callahan et al., 2016) was used to trim primers, truncate the 3′ end of the reads when the median quality score dropped below 39 for bacterial reads and 35 for fungal reads (forward and reverse reads at 227 bp for bacteria, reverse read at 257 bp for fungi), merge reads, filter out potential chimeric sequences with the consensus chimera detection method, and call amplicon sequence variants (ASVs) using default parameters in QIIME2. We removed ASVs that did not get a taxonomic designation, and the ASVs that were classified as archaea, mitochondria or chloroplasts. Next, we filtered the low-frequency (<10 reads) ASVs from the data set. After filtering, a total of 27,815,087 reads (48,797–233,051 reads per sample) and 40,924 ASVs were recovered for soil bacteria, and 35,829,831 reads (44,457–292,035 reads per sample) and 18,894 ASVs were recovered for soil fungi. The final ASV feature-tables were generated after rarefaction (Weiss et al., 2017) to 48,979 and 44,457 reads per sample in the bacterial and fungal data sets, respectively, and were used for analyses unless stated otherwise.

The Naive Bayes classifiers (Bokulich et al., 2018) were trained to assign taxonomy to representative sequences using the V4 region of the 16S rRNA gene (matching the 515F/806R primers) of the SILVA database version 138 for bacterial ASVs (Quast et al., 2012), and the full-length ITS region from the UNITE version 8.0 database for fungi (Nilsson et al., 2019). Both bacterial and fungal reference sequences were clustered at a 99% sequence similarity threshold. We parsed the ASVs through FAPROTAX version 1.2.4 (Louca et al., 2016) and FUNGUILD version 1.1 (Nguyen et al., 2016) to infer diversity of functional traits of bacteria and fungi, respectively. FAPROTAX assigned functional traits to 9251 out of 40,726 bacterial ASVs (22.72%), while FUNGUILD assigned fungal functional traits to 6723 out of 18,735 ASVs (35.88%) with a confidence level of "Probable" or "Highly Probable."

2.4 | Statistical analyses

The soil microbiota alpha diversity was estimated using three different metrics (ASV richness, Shannon diversity index [referred to as Shannon diversity for clarity] and Faith's Phylogenetic diversity) in QIIME2.

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Phylogenetic diversity was not calculated for fungi as the ITS2 region evolves too rapidly to be useful for phylogeny-based analyses (Nilsson et al., 2008). Changes in alpha diversity (and HII values) between different forest disturbance levels were assessed using the Kruskal–Wallis (KW) test, with a Benjamini–Hochberg adjustment for multiple comparisons using the DUNN. TEST version 1.3.5 package (Dinno, 2015) in R version 4.0.2 (R Core Team, 2020).

The soil microbiota beta diversity for each forest disturbance level was visualized by principal coordinate analysis (PCoA), based on Bray–Curtis and Jaccard distances generated by the PHYLOSEQ version 1.34.0 package in R (McMurdie & Holmes, 2013). In order to verify potential bias due to geolocation (latitudinal or longitudinal clines), we analysed a subset of samples ($n = 195$) that included all urban and suburban samples but only the three nearest (in relation to cities) pairs of national parks and managed forests (Konnevesi, Pyhä-häkki and Leivonmäki; Figure 1). The Bray–Curtis distances were recalculated for this subset of samples and the spread of variance was assessed by the *betadisper* and *permutest* (n permutations = 9999) functions in VEGAN version 2.5–7 (Oksanen et al., 2020). PERMANOVA tests implemented by the *adonis2* and *pairwise.adonis2* functions in VEGAN (n permutations = 9999) were then used to examine whether forests with different disturbance levels differ significantly in terms of sample grouping and to determine if disturbance level had a higher explanatory value (R^2) than location per se (i.e., latitude and longitude). The prerequisite of dispersion homogeneity for the *adonis2* function was not met (i.e., the within-group variation differed between the four disturbance levels which could confound the results). However, our results are still valid since we analysed a subset of samples with a balanced design (Anderson & Walsh, 2013). To further examine whether geographical distance between soil samples may explain variation in the beta diversity patterns, we fitted bacterial and fungal distance–decay models per disturbance level with a negative exponential function by implementing the *decay.model* function using the BETAPART version 1.5.6 R package (n permutations = 9999) with both the Bray–Curtis and Jaccard metrics (Baselga et al., 2022).

Differential abundance of bacterial and fungal ASVs (from the most abundant phyla with >0.01 relative abundance only) between forest disturbance levels was calculated using the *deseq* function in DESEQ2 version 1.30.1 (Love et al., 2014) using the unrarefied feature-tables. We also calculated the relative proportions of ASVs grouped at the phylum level to identify significant differences in proportions of the most abundant phyla (with >0.01 relative abundance) between forest groups. This was done by first constructing general linear models with a quasibinomial distribution and then applying the Tukey's multiple comparison test with the *glht* function in the MULTCOMP version 1.4.20 R package (Hothorn et al., 2008). Successfully assigned bacterial (FAPROTAX) and fungal functional traits (FUNGUILD) were converted to relative abundances, with low relative abundance traits (<0.05 for all four disturbance levels) removed to aid plotting. Differences in the relative abundance of these most abundant functional traits between the four forest groups were examined with the same statistical methods as for taxonomic proportions.

To examine how environmental variables (23 structural habitat factors and soil pH, Tables S2 and S3) correlate with alpha and beta diversity, we first assessed their collinearity. We used the Spearman's rank correlation coefficient in R to identify correlations involving ranked categorical variables, whereas the Pearson's correlation coefficient was used for correlations between numerical variables (Table S4). To identify variables with the strongest impact on the community structure, we used constrained analysis of principal coordinates (CAP) through the *ordinate* function within the PHYLOSEQ version 1.34.0 R package and extracted the values from the first CAP axis (Table S5). Correlated variables with little impact on community structure were removed, and six remaining structural habitat factors (grass, boulders, fine woody debris, stumps, lichens and shrubs for bacteria; stone holes instead of boulders for the fungal analyses; Tables S2 and S3) and soil pH were included in the subsequent analyses. For the analyses of alpha diversity, we verified that the linear model had a variance inflation factor that was lower than 2 for all these selected variables (Johnston et al., 2017). The alpha diversity model including these seven variables was inserted into the model selection tool provided by the *dredge* function within the MUMIN version 1.43.17 R package (Barton, 2020) and was based on the subset of the samples for which environmental data were available ($n = 178$). The most parsimonious model that was within two AIC (Akaike information criterion) units of the model with the lowest AIC value was considered the best model. Additionally, either Spearman's rank correlations (involving categorical variables) or Pearson's correlations were used (involving continuous variables) to test for significant associations between the variables in the final model and soil microbiota alpha diversity. We also simultaneously examined the effects of the seven environmental variables selected above on the soil microbiota beta diversity (Bray–Curtis dissimilarity, $n = 178$) using a PERMANOVA test implemented by the *adonis2* function in VEGAN version 2.5-7 (n permutations = 9999).

Given a well-established association between pH and soil microbiota (Fierer & Jackson, 2006), we have examined the relative importance of soil pH and habitat disturbance (i.e., HII) in explaining the variation in alpha and beta diversity. We compared the Pearson's correlation coefficients for soil microbiota alpha diversity. For beta diversity, we ran PERMANOVA tests with the *adonis* function to compare the amount of variation (R^2) explained by either the soil pH or the HII in separate models. These analyses were run on two data sets: (i) on all soil samples with pH data ($n = 306$), and (ii) on a subset of soil samples from two contrasting habitat disturbance levels that had comparable levels of soil pH ($n = 50$). Specifically, we selected the 25 soil samples collected from managed forests with the highest pH (range: 4.34–5.08; a comparable subset of national parks did not result in equivalent levels of soil pH with urban soil samples) and the 25 soil samples from urban forests with the lowest pH (range: 3.52–5.01). The comparable levels of soil pH between the two forest groups were confirmed by a nonsignificant *wilcox. test* ($p > .05$). Thus, this smaller data set allowed us to examine the effects of habitat disturbance in isolation from the effects of soil pH.

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3 | RESULTS

3.1 | Forest soil microbiota alpha diversity is positively associated with anthropogenic disturbance

The level of alpha diversity (e.g., ASV richness) present in forest soil samples varied greatly between bacteria and fungi with the former having a range of 504-3078 ASVs and the latter having a range of 101-966 ASVs. For both bacteria and fungi, we found that the forest disturbance level was an important explanatory factor for the variation in soil microbiota alpha diversity (e.g., ASV richness: χ^2 = 74.5207, df = 3, p < .001 for bacteria; χ^2 = 76.5905, df = 3, p < .001 for fungi). Specifically, we found that all three alpha diversity metrics differed significantly between forest groups (p < .02, KW, Table S6), except between the forests in national parks and managed areas, and between urban and suburban forests for fungal ASV richness (p > .025, KW, Table S6). Interestingly, alpha diversity of both bacterial and fungal soil microbiota was lowest in the national parks and managed forests, intermediate in suburban forests, and highest in urban forests (Figure 2; Figure S2; Table S6). Urban forest soil samples had on average 58% more bacterial and 57% more fungal ASVs than the soil samples from national parks.

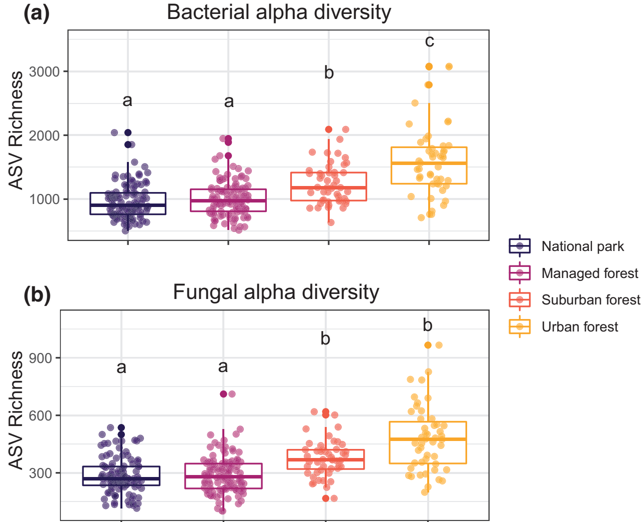

FIGURE 2 Differences in the alpha diversity of soil microbiota according to the forest disturbance level. An increase in diversity is visible from national parks (dark purple) towards urban forests (yellow) for bacterial (a) and fungal ASV richness (b). National parks (dark purple) and managed forests (light purple) are not significantly different from one another. Suburban forests (orange) have intermediate levels of microbial alpha diversity when compared to less disturbed forests (i.e., national parks and managed forests) and urban forests (yellow). The letters correspond to the significance levels between groups based on Kruskal–Wallis tests, with a Benjamini–Hochberg adjustment (p < .025). [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Urban forest soil microbiota communities look alike irrespective of geolocation

Both bacterial and fungal soil microbiota were separated principally along the first PCoA axis, but it explained a greater amount of variation for bacteria (17.2%) than for fungi (4.5%; Figures S3 and S4). Urban soil samples formed a separate cluster with distinct sample grouping in the PCoA (and CAP) ordinations (Figures S3, S4 and S5). In contrast, the soil samples from national parks and managed forests tend to overlap considerably in the ordination space. The importance of anthropogenic disturbance level was greater than that of geolocation, which had little notable impact on variation in beta diversity (Table S7). For example, disturbance level explained almost 10% of the variation in bacterial beta diversity (F = 6.865, R^2 = .094, p < .001, based on Bray–Curtis) which is six times greater than the explanatory power of latitude (F = 3.28, R^2 = .015, p < .001), while longitude did not explain a statistically significant proportion of the variation. Similarly, 4% of the variation in beta diversity of forest soil fungal microbiota was explained by the habitat disturbance level (F = 2.767, R^2 = .041, p < .001, based on Bray–Curtis) which is five times more than the variation explained by latitude (F = 1.552, R^2 = .008, p < .002) and almost seven times more than the variation explained by longitude (F = 1.221, R^2 = .006, p < .05). Similar patterns were observed when analyses were run based on the Jaccard distance metric (Table S7, Figure S4).

Differences in dispersion of soil samples between the forest groups was found for both bacteria (F = 8.562, p < .001, based on Bray–Curtis) and fungi (F = 5.375, p < .002, based on Bray–Curtis). Permutation tests with pairwise comparisons revealed that dispersion in bacterial communities was significantly higher between soil samples from urban forests (in comparison to all the other forest groups; Figure 3; Table S7). In the case of fungi, the highest levels of dispersion were found for soil samples from national parks (Figure 3; Table S7). This pattern differed significantly from the dispersion of soil samples from managed and suburban forests (p < .002) but not in comparison to the dispersion of soil samples from urban forests (p > .05).

Additionally, we found evidence of a significant increase in assemblage dissimilarity occurring with greater distance separating samples for bacteria and fungi (p < .05 for all disturbance levels, except urban fungi). The strength of the slopes representing this relationship gradually decreased with anthropogenic disturbance (steepest slopes were found in national parks for bacteria and fungi, Figures S6 and S7, Table S8). However, the strength of all slopes can be considered very weak (all slopes $<3\times10^{-6}$) making microbial communities located further apart only slightly more dissimilar than nearby located microbial communities.

3.3 | Impacts of anthropogenic disturbance on forest soil microbiota composition

Five phyla of bacteria (Acidobacteriota, Proteobacteria, Planctomycetota, Actinobacteriota and Verrucomicrobiota) and two phyla of fungi (Ascomycota, Basidiomycota) comprised ~80%–90%

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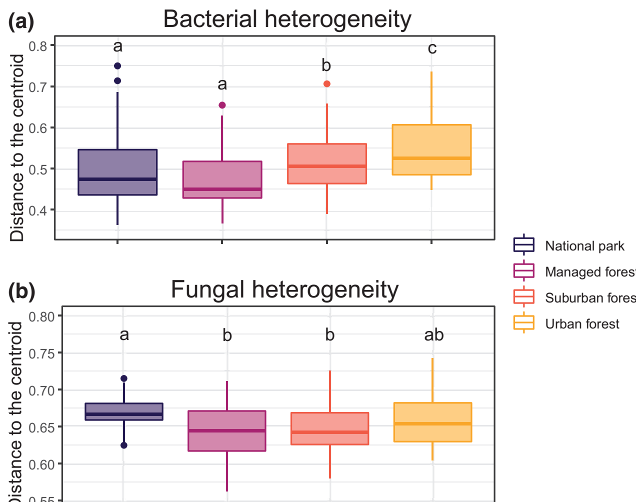

FIGURE 3 Dispersion in microbial communities according to the forest disturbance level. Box plots represent the dispersion in the microbial communities calculated over the entire multidimensional space (all axes included) and based upon the bray–Curtis metric ($n = 195$). The variation in the bacterial community positively correlates with increasing anthropogenic disturbance reaching its peak in urban forests (a). The lowest variation in the fungal community is found in managed and suburban forests, followed by urban forests and reaching its highest level in national parks (b). The letters correspond to the significance levels between groups based upon permutation-based tests of multivariate homogeneity of group dispersions ($p < .05$). [Colour figure can be viewed at wileyonlinelibrary.com]

of the total microbial community. Soil microbiota composition varied markedly among forest disturbance levels, with greater taxonomic changes observed in bacteria than in fungi. The proportions of all five dominant bacterial phyla differed significantly between the two most contrasting forest groups (national parks and urban forests), supporting the observed variation in beta diversity (Figure 4a,c; Table S9). For example, when compared to national parks, the urban soil comprised relatively less Acidobacteriota (−37.3%) and Planctomycetota (−25.4%) and relatively more Actinobacteriota (+37.8%), Proteobacteria (+23%) and Verrucomicrobiota (+81.5%). Interestingly, suburban forest soils reflected a composition of bacterial phyla that is intermediate to the soils of natural forests and urban forests, thus reinforcing the patterns observed for alpha and beta diversity.

The frequencies of 1306 bacterial ASVs represented by a total of 13,174,114 sequences were significantly different between national parks and urban forests (3.2% of the total 40,924 nonrarified ASVs and 47.36% of the total 27,815,287 nonrarified reads), with the majority of these ASVs assigned to one of the five dominant phyla, and thus driving the interphyla differences between national parks and urban forests (Figure 4b,d). The orders belonging to Acidobacteriota and Planctomycetota that experienced the greatest reduction in urban areas were Acidobacterales (Acidobacteriota) and Isosphaerales (Planctomycetota). Simultaneously, the orders belonging to Actinobacteria, Proteobacteria and Verrucomicrobiota that had the highest gains in urban areas were Gaiellales, Microtrichales and Solirubrobacterales (Actinobacteria), Burkholderiales and Rhizobiales (Proteobacteria), and Chthoniobacterales (Verrucomicrobiota) (Table S10).

In contrast, the two dominant fungal phyla had no significant changes in their proportion between national parks and urban forests although there was a trend towards a decline in proportion of Basidiomycota (−11.7%) in urban forests (Figure 4a,c; Table S9). Nonetheless, we identified 325 fungal ASVs represented by a total of 10,528,620 sequences that were differentially abundant between national parks and urban forests (1.7% of the total nonrarified 18,894 ASVs and 29.39% of the total 35,829,831 nonrarified reads), and 80 ASVs were assigned to the phylum Basidiomycota (Figure 4b,d). The orders of Basidiomycota that experienced the highest reduction in urban areas were Agaricales, Atheliales and Russulales (Table S10).

3.4 | Impacts of urban forests on the composition of functional traits associated with soil microbes

The five most common functional traits of bacterial communities (>5% relative abundance for at least one of the four disturbance levels) were aerobic chemotrophs, chemotrophs, intracellular parasites, animal symbionts and cellulolytic species, whereas the two chemotrophic groups account for ~60% of the present functional traits (Figure 5). The fungal functional traits were dominated by ECM (ranging from 47% to 67%), with the other most common traits being either saprotrophs and/or endophytes (Figure 5). The relative proportion of the five most abundant predicted functional traits showed more apparent changes for fungi than for bacteria when compared between different forest groups (Figure 5; Table S11). For clarity, only the forest groups with the highest difference in disturbance level (i.e., national parks and urban forests) will be further discussed in detail. Urban soils had a significantly lower proportion of cellulolytic bacteria (−25.3%) and intracellular parasites (−60.41%) than soil from national parks. Urban soil fungal microbiota had significantly less ECM (−27.84%) and ECM-endophytes (−69.6%), whereas the proportions of endophytes–litter saprotrophs (+84.9%) and undefined saprotrophs (+152.6%) were significantly increased in comparison to national parks (Table S11).

3.5 | Impacts of environmental factors on soil microbiota alpha and beta diversity

Much of the variation in the alpha and beta diversity of bacteria and fungi was associated with changes in soil pH. Although this was true for both bacteria and fungi, the effect of soil pH was much stronger for bacteria than for fungi (Figure 6; Figure S8; Tables S12 and S13).

Soil pH was the sole explanatory factor for bacterial ASV richness and bacterial Shannon diversity (Table S12). The best models explaining fungal ASV richness included pH and the abundance

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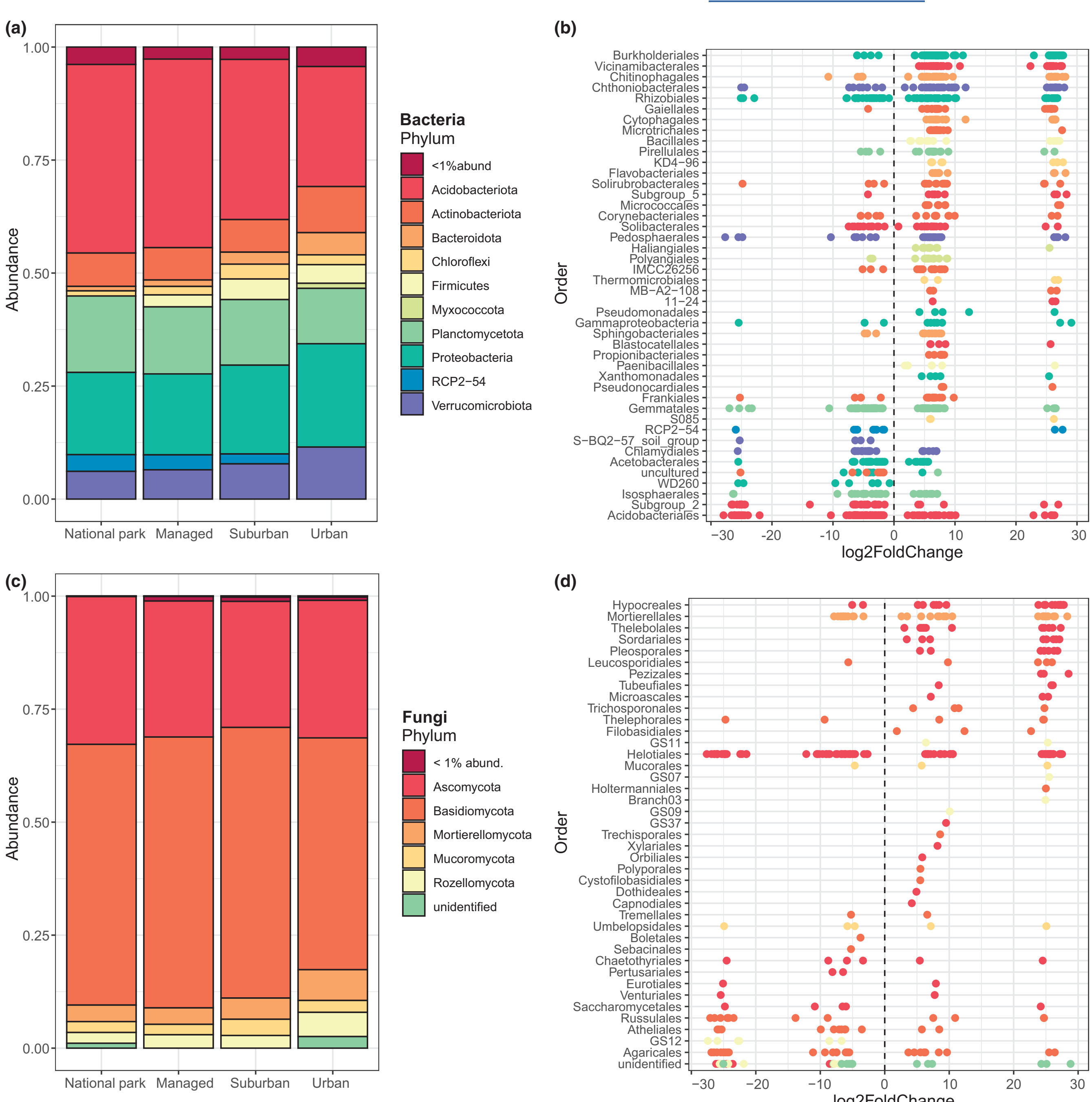

FIGURE 4 Taxonomic changes in the soil microbiota according to the forest disturbance level. The two stacked bar plots on the left show the average relative abundances of microbial phyla in soils from forests that differ in disturbance level: Bacteria (a) and fungi (c). Only the phyla that constitute at least 1% of the total abundance are shown. The remaining phyla are summarized under the category "<1% abund." The corresponding differential abundance plots on the right, bacteria (b) and fungi (d), provide an overview of which underlying orders are driving shifts in the microbial community composition. Every order is categorized by one or several ASVs (closed points) that are either significantly more abundant in urban soils (positive values) or significantly more abundant in soil of national parks (negative values). A summation of all ASVs per order makes the order either increase (orders at the top of the graph, e.g., Burkholderiales) or decrease (at the bottom of the graph, e.g., Acidobacteriales) in abundance in urban forests. The colours of phyla between the panels on the left and right are matched for bacteria and fungi. To aid plotting, only the bacterial orders with the highest differential change ($|\text{Log}_2\text{FoldChange}| > 38$) are shown (b). [Colour figure can be viewed at wileyonlinelibrary.com]

of shrubs, while pH, the abundance of shrubs and the amount of stumps determined the fungal Shannon diversity (Table S12). Using Spearman's and Pearson's correlations, we found that soil pH is the only significant variable correlating with fungal ASV richness and fungal Shannon diversity, making the effect of the other explanatory variables (i.e., shrub abundance and amount of stumps) negligible. More specifically, soil pH and all studied alpha diversity metrics are characterized by a strong positive correlation for both bacteria (e.g., ASV richness: $r = .73$) and fungi (ASV richness: $r = .61$; Figure 6a,c; Table S13).

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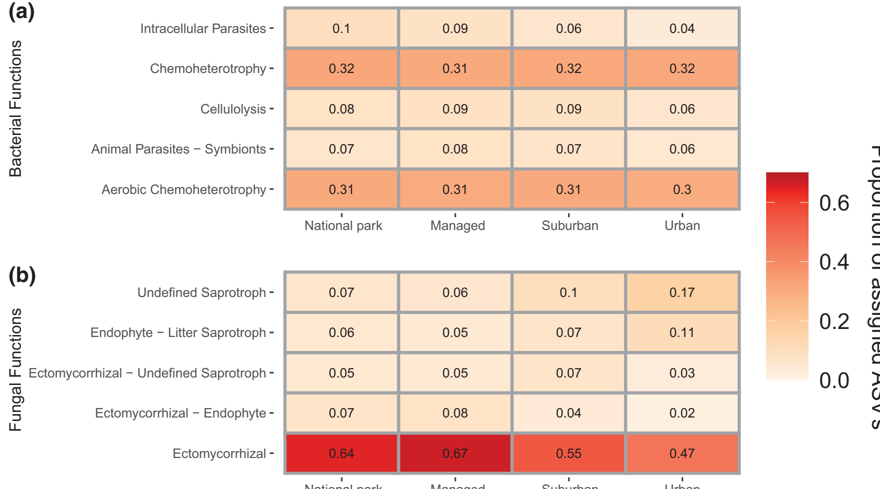

FIGURE 5 Changes in predicted functional traits of the soil microbiota according to the forest disturbance level. The heatmaps visualize the relative abundance of functional traits of the soil microbiota according to the forest disturbance level in bacteria (a) and fungi (b). Only the five most abundant functional traits (>0.05 relative abundance for at least one of the four disturbance levels) for both bacteria and fungi are shown for clarity. Numbers represent relative abundance in percentages, ranging from 0.1 (10%) to 0.7 (70%). [Colour figure can be viewed at wileyonlinelibrary.com]

In terms of explaining the observed variation in beta diversity for both bacteria and fungi, soil pH also appeared to be the most important environmental variable. About 16% of the total variation in the bacterial beta diversity could predominantly be explained by soil pH ($F = 29.3185$, $R^2 = .136$, $p < .001$, based on Bray–Curtis; Table S13), and less by the abundance of shrubs ($F = 1.421$, $R^2 = .026$, $p < .05$, based on Bray–Curtis; Table S13). In contrast to bacteria, only ~6% of variation in fungal beta diversity was explained by the combination of soil pH ($F = 5.758$, $R^2 = .031$, $p < .001$, Bray–Curtis) and the abundance of grass ($F = 1.175$, $R^2 = .025$, $p < .01$, Bray–Curtis; Table S13). These patterns were consistent when analyses were run based on the Jaccard distance metric (Table S13).

Notably, some environmental factors used in the analyses were confounded within the disturbance level. For example, soil pH showed consistently higher alkaline levels in forest areas with higher levels of anthropogenic disturbance (Figure 6b,d). This pattern was further confirmed by a strong positive correlation between pH and the HII ($r = .65$, Figure S9). For the complete data set ($n = 306$), we found that these two variables have comparable explanatory power in terms of explaining alpha diversity in fungi and beta diversity in both bacteria and fungi (although the overall explained variation is ~4 times higher for bacteria in comparison to fungi; Table S14). In contrast, bacterial alpha diversity correlated more strongly with soil pH ($r = .70$ for ASV richness) than with the HII ($r = .54$ for ASV richness; Table S14). We found the same trend with our smaller preselected data set ($n = 50$) where the two variables explained the beta diversity within bacteria and fungi more or less equally. Interestingly, the bacterial alpha diversity correlated more strongly with soil pH ($r = .39$ for ASV richness) while the fungal alpha diversity was more influenced by the HII ($r = .25$ for ASV richness; Table S15).

4 | DISCUSSION

Forest soil microbes are important as they are essential for proper ecosystem functioning (Fierer, 2017). Here, we used amplicon sequencing to characterize bacterial and fungal communities in forest soils that differ in their level of anthropogenic disturbance (national parks, managed, suburban and urban forests). In accordance with our hypotheses, we found that (i) the alpha diversity of forest soil bacteria increases with proximity to urban areas with no apparent impact of forest management. However, we unexpectedly also found the same pattern for the diversity of soil fungi. The prediction that (ii) the communities of bacteria and fungi would be impacted by anthropogenic disturbance is compatible with our data, although the underlying patterns of dispersion were not foreseen. The greatest dispersion in beta diversity for soil bacterial communities was found in urban forests while the pattern for fungi was the opposite, as we observed the greatest dispersion in national parks. We found (iii) little evidence that variation in bacterial communities of urban and other forest soils elicits a major change in functional traits, but the variation in fungal communities was indeed associated with a decline in relative abundance of ECM in urban areas. Additionally, our data revealed a strong association between the intensity of habitat disturbance (measured by the HII) and soil pH, identifying important factors underlying the variation in forest soil microbiota diversity and composition.

4.1 | Urban soil microbiota have a higher level of alpha diversity in comparison to less disturbed soil microbial communities

That the level of anthropogenic disturbance is positively associated with both bacterial and fungal diversity (Figure 2; Figure S2) is in accordance with earlier studies on soil bacteria (Hui et al., 2017; Naylo et al., 2019; Tan et al., 2019), but not with many studies on soil fungal communities (Abrego et al., 2020; Andrew et al., 2019; Tan et al., 2019; Tedersoo et al., 2020). A possible reason for this discrepancy is that other studies often confounded habitat type and urban location (i.e., sampling soil from forests in nonurban areas but from gardens and parks in urban areas). Suburban forests characterized by an intermediate level of alpha diversity lend support to the concept of an apparent biodiversity gradient from natural to urban forests (Spotswood et al., 2021). Our data from soils of managed forests are consistent with the idea that long-term effects of forest management

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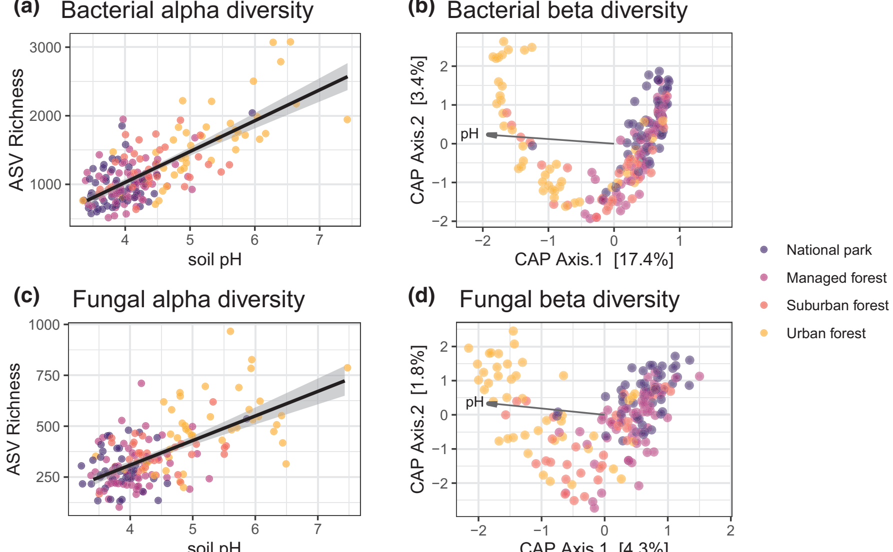

FIGURE 6 The association between forest soil microbiota alpha and beta diversity and soil pH. The Pearson's correlation between the pH of forest soil and the alpha diversity of the forest soil microbiota is shown for bacteria (a) and fungi (c) in terms of ASV richness ($n = 178$). The constrained analysis of principal coordinates (CAP) plots based on the subset of soil samples with the environmental data available are shown for bacteria (b) and fungi (d). Bray–Curtis distances were used for both ordinations. The length of the arrows corresponds to the strength of its association with the beta diversity of the microbial community. Only the most important arrow (for soil pH) has been added to this graph to facilitate readability. CAP plots with arrows for all the 23 recorded environmental variables and pH are shown in Figure S5. Point colour matches the corresponding forest disturbance level. [Colour figure can be viewed at wileyonlinelibrary.com]

do not interfere much with microbial alpha diversity (Lee & Eo, 2020; Lee-Cruz et al., 2013). On the other hand, it is possible that the immediate effects of commercial forestry management (i.e., compaction and removal of the upper organic layer) can cause disruptions in the soil microbial communities shortly after timber harvest.

Although Finnish national parks were established with the principal aim to act as a refuge for (macro)diversity (Siikamäki et al., 2015), it is unclear whether such biodiversity policy is equally efficient at conserving microbial biodiversity. Our data show that the microbiota communities in soils from national parks have the lowest degree of alpha diversity, which indicates that the biodiversity of macro- and microspecies are not necessarily following the same patterns. This also suggests that certain physicochemical properties of urban soil enable a wider range of microbes to coexist on smaller geospatial scales (Tedersoo et al., 2020; Wang et al., 2017).

4.2 | Soil microbial communities exhibit parallel shifts in urban forests

The anthropogenic disturbance of forests alters soil microbial communities, with urban forests consistently harbouring the most distinct communities (relative to other areas, Figures S3 and S4). This implies that there are strong parallel environmental stressors associated with urban forests that markedly shape both bacterial (Wang et al., 2017) and fungal (Tedersoo et al., 2020) microbial communities.

Furthermore, we highlight the contrasting patterns for bacteria and fungi in terms of the dispersion in their community profiles. In contrast to our hypotheses, we observed that urban landscapes promote higher dissimilarities between bacterial communities, while national parks increase the dissimilarities between fungal communities (Figure 3). Generally, there are two potential mechanisms supporting higher dispersion patterns: (i) greater dispersal limitation between fragmented forest patches, and (ii) a higher variety of microhabitats with different selection pressures for colonization (Wang et al., 2016). Interestingly, the distance decay analyses did not show strong support for dispersal limitation being a major contributing factor in explaining the observed patterns in bacteria and fungi (Figures S6 and S7, Table S8). Thus, it is more likely that urban forests and national parks provide diverse microhabitats for bacteria and fungi, respectively. These results challenge the idea that the concept of urban biotic convergence (McKinney, 2006) can be generally applied to model urban impacts on soil microbes. Additionally, it also underlines the potential conservation value that national parks have by sustaining natural variation among fungal communities, probably through variation in the accumulation of deadwood (different decay stages and/or plant species; Dudley & Vallauri, 2005).

4.3 | Urban forests associate with changes in bacterial and fungal species and functional traits

The composition of microbial communities differs markedly between urban and natural forests (Figure 4). For bacteria in cities, these changes are linked to shifts in the proportions of several phyla characteristic of forest degradation such as the decreased ratio of Acidobacteriota to Proteobacteria (Zhou et al., 2018). One of the

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major changes in urban soil fungal communities included a relative (but nonsignificant) decrease in Basidiomycota, a phylum that has many ECM members (Tedersoo et al., 2020). This reduction in Basidiomycota might reflect the lower percentage of conifers in urban forests (i.e., "forest order"; Figure S5, Table S3).

From the viewpoint of functional traits, urban forests have higher proportions of fungal saprotrophs while having lower proportions of cellulolytic bacteria and ECM in comparison to national parks (Figure 5). Only small changes in the relative proportion of bacterial functional traits were detected among the four forest groups, which implies little change in the diversity of functional traits. In contrast, the proportion of ECM in the total community is much lower in urban areas (47%) in comparison to national parks (64%). Such community changes resulted in a higher level of fungal saprotrophy in urban soil. The main limitation of these findings lies in the predictive nature of this method, and that the greater part of the sequences was not assigned any functional traits. While we cannot validate these results with the available data, the major decline in the proportion of ECM fungi in our samples from urban areas is indeed consistent with previous studies (Abrego et al., 2020; Schmidt et al., 2017).

4.4 | Association between soil pH, habitat disturbance and changes in soil microbiota

Soil pH is important in explaining the differences in soil microbiota between forests of different anthropogenic disturbance levels (Figure 6; Tables S12 and S13) although we did find that bacteria are more responsive to changes in soil pH than fungi (Rousk et al., 2010; Shen et al., 2020). Despite this strong relationship, it remains difficult to distinguish the individual effects of environmental factors since many are collinear (Figure S5). For example, we found that soil pH and the HII correlate strongly with one another (Figure S9) with higher soil pH found in forests with higher values for the HII. As such, our data uncover an important association between the proximity to the built environment and soil pH. Interestingly, even when accounting for differences in soil pH between forest groups (i.e., selecting samples with similar pH ranges), our analyses suggest that the HII and soil pH are still relevant in shaping the alpha and beta diversity of soil microbiota (Table S15). This suggests that although soil pH and the HII generally correlate, they both have independent effects on the soil microbiota.

Future experimental studies are needed to establish directionality of the intriguing association between soil pH and the proximity to the built environment and detect the underlying mechanisms to the changes observed in bacterial and fungal communities in urban areas. One potential explanation for this interaction could be that high soil alkalinity is an inherent part of a typical built environment (Pouyat et al., 2015) due to rainwater passage through concrete materials such as pipes and street gutters (Davies et al., 2010; Nugent & Allison, 2022). Although no causal inferences could be made with the available data, our study raises important questions in relation to any attempt to implement the "Microbiome Rewilding Hypothesis," which postulates that degradation of microbial communities in cities can simply be counteracted by restoration and rewilding of urban green spaces (Mills et al., 2017).

AUTHOR CONTRIBUTIONS

All authors took part in conceiving the project design; Tiffany Scholier, Tapio Mappes, Anton Lavrinienko, Rasmus Hindström, Eugene Tukalenko and Ilze Brila conducted field surveys and sample collection for the experiments. Tiffany Scholier, Anton Lavrinienko and Andrii Vasylenko carried out work necessary for the pH measurements. Tiffany Scholier performed laboratory work and completed data analysis, with additional bioinformatics support provided by Anton Lavrinienko and Phillip C. Watts. Tiffany Scholier wrote the manuscript with significant contributions from Anton Lavrinienko and Phillip C. Watts and critical input from all other authors. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The raw sequences and associated metadata have been deposited with the National Center for Biotechnology Information (NCBI) under accession no. PRJNA823643 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA823643/).

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SUPPORTING INFORMATION

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II

DIETARY CHANGE ALTERS THE GUT MICROBIOTA IN URBAN RODENTS

by

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III

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