Vom Fachbereich VI Raum- und Umweltwissenschaften der Universität Trier zur Verleihung des akademischen Grades ‘Doktor der Naturwissenschaften’ (Dr. rer. nat.) genehmigte Dissertation

Biodiversity in a bottle – metabarcoding environmental samples from long-term monitoring archives for retrospective assessment of changes in biotic communities in the Anthropocene

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Datum der wissenschaftlichen Aussprache: 18.07.2025

Trier, 2025

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Acknowledgements

First of all, I would like to thank my supervisor, Prof. Dr. Henrik Krehenwinkel, for taking me as your PhD student. During my time in your working group, I have learnt a lot, not only professionally but also about myself. I really appreciate the ongoing dialogue with you and your constructive criticism, and I think it's great that you always find time for everyone in your busy schedule.

I would also like to thank all the collaborators, colleagues and students involved in the thesis. Special thanks go to the employees of the ESB project group in Trier, who collected the great samples I was able to analyse in this thesis.

My special thanks also go to my colleagues from the Department of Biogeography, who have become true friends over the years. I truly appreciate the time we spent together, filled with laughter, deep talks and great moments. I would like to thank Lukas K., Nico F., Manuel S., Lisa M., Judith P., Lina F., Arndt S., Yannis S., Anja M., Sven W., Lisa R., Sarah G., Philipp B., Amadeus P., Till M. and Sophie O. for helping me to get through this time. Special thanks to Susan K., I appreciate your help since my master's. A heartfelt thank you also goes to my friends outside academia, you always knew how to help me clear my mind when I needed it most. Even if there are too many names to write down, I hope that you feel seen and valued. I would especially like to thank my dear friend Isabelle J. We started this PhD journey together and shared so many experiences along the way. I'm already looking forward to everything the future holds for us.

Of course I would like to thank my family. My parents, Karin and Klaus, who have always supported and loved me, and my siblings, Florian, Lukas and Victoria. Thank you for being there and for being you.

Finally, I would like to thank my partner, Tobias. Your support in all situations and patience with me has brought me to where I am today. I am incredibly grateful for you and am glad to have you in my life.

“I worked really hard for this and I deserve it”

Barbie

Summary of this thesis

Biotic communities experienced significant changes in recent decades. Climate change, the overexploitation of natural resources and the immigration of invasive species are major drivers for this change and present unknown challenges for communities worldwide. To assess the impact of these drivers, standardised long-term studies are required, which are currently lacking for many species and ecosystems. Analysing environmental samples and the DNA of associated organisms using metabarcoding and high-throughput sequencing provides a cost-efficient and rapid way to generate the high-resolution biodiversity data which is so direly needed.

In this thesis, I demonstrate the great potential of using samples from the German Environmental Specimen Bank (ESB), a long-term monitoring archive that has been collecting and cryogenically storing highly standardised environmental samples since 1985. Modern analytical methods enable retrospective long-term biodiversity monitoring using these samples.

In the first chapter, I illustrate metabarcoding as a central method, discussing its strengths and drawbacks, how to avoid them, and new application approaches. This chapter provides the methodological basis for the following studies.

In subsequent chapters, I present time series analyses of communities associated with these environmental samples. While for Chapter two the focus is on terrestrial arthropod communities, in Chapter three aquatic and terrestrial communities across the tree of life are analysed. A null model was developed for this survey for robust conclusions. The studies covered the last three decades and revealed substantial compositional changes across all ecosystems. These changes deviated significantly from the model, indicating that the changes are occurring faster than expected. Moreover, a trend toward homogenization in many terrestrial communities was uncovered. Climate change and the immigration of invasive species in combination with the loss of site-specific species are suspected to be the main drivers for this.

In a follow-up study, changes of arthropod communities in German and South Korean terrestrial ecosystems were compared using ESB leaf samples from these two countries. Since both ESBs are harmonised in sample collection and processing, comparative analyses were applicable. This research covered the last decade and revealed substantial declines in species richness in Korea. Abiotic and biotic factors are discussed as potential drivers of these results.

Finally, the possibility of assessing tree health by analysing changes in functional fungal groups using German ESB samples was investigated. The results indicate that increasing infestation of specific functional groups is a proxy for declining tree health, with further analyses planned.

In this dissertation, I present the great potential of samples from long-term monitoring archives to conduct retrospective biodiversity trend analyses across the tree of life. As technologies evolve, these samples will help to understand past and predict future ecosystem changes.

Contributions

For this thesis, many people were involved in the sample collection, storage, data processing and data analysis over the last four years (Table 1).

Table 1: Contribution for all included publications. Percentages reflect the amount of work done by myself, mentioned authors share the rest of the respective category.

Co-Authors and people involved and/or collaborated in the composition in the different studies of this thesis:
Henrik Krehenwinkel (HK), Isabelle Junk (IJ), Benoît Perez-Lamarque (BPL), Hélène Morlon (HM), Manuel Stothut (MS), Sven Weber (SW), Anja Melcher (AM), Lisa Mahla (LM), Susan R. Kennedy (SRK), Rieke Broekmann (RB), Rüdiger Wolf (RW), Axel Hochkirch (AH), Jan Koschorreck (JK), Andrea Körner (AK), Sven Künzel (SK), Christoph Müller (CM), Rebecca Retzlaff (RR), Martin Paulus (MP), Roland Klein (RK), Diana Teubner (DT), Sonja Schanzer (SS), Thomas Udelhoven (TU), Michael Veith (MV), Elisabeth Gold (EG), Caroline Schubert (CS), Alice Schumacher (AS), Nina Schmitt (NS), David Chung (DC), JangHo Lee (JHL), JungKeun Oh (JKO)

Paper	Study design	Data compilation	Data Analysis	Manuscript composition
Metabarcoding for Biodiversity Estimation	**5%** HK	**10%** **JH**, IJ, LM, AM, MS, SRK	**5%** HK, **JH**, IJ, LM, AM, MS, SRK	**5%** HK, **JH**, IJ, LM, AM, MS, SRK
Environmental DNA from archived leaves reveals widespread temporal turnover and biotic homogenization in forest arthropod communities	**5%** HK, MV, SW	**5%** SW, AM, RB, RW, **JH**, DT, SK, SS, CM	**5%** HK, SW, RW, RR, TU, SS	**5%** HK, SW, SRK, MP, RK, AH, RR, DT, TU, JK, MV
Archived natural DNA samplers reveal four decades of biodiversity change across the tree of life	**30%** IJ, **JH,** HK, BP, HM	**30%** IJ, **JH,** AM, SW, MS, EG, CS, AS, NS, SRK	**30%** IJ, **JH,** HK, BPL, HM	**25%** IJ, **JH,** HK, BPL, HM, SRK, MP, RK, DT, JK
Arthropod diversity decline in South Korean, but not in German forest ecosystems	**60%** HK, **JH**	**60%** **JH,** MS, EG, CS, AM	**80%** **JH,** HK, MS, TU	**75%** **JH,** HK, MS
Shifts in functional groups of tree-associated fungi reveal decreasing tree health in German forest ecosystems	**50%** HK, **JH,** MS	**60%** **JH,** MS, EG, CS	**40%** **JH,** HK, MS, BPL	**80%** **JH,** HK, MS

General Introduction

Biodiversity and ecological functions of selected groups

The term biodiversity encompasses the variety of all living organisms across the three major domains of life: Eukaryota, Bacteria and Archaea. Each of these groups harbours an immense number of species, forming the foundation of the planet's biological and ecological richness. This great diversity allowed for the formation of highly complex interaction networks, which are present in every ecosystem. Within these networks, remarkable processes emerged, like the co-evolutionary adaptation between the Malagasy orchid and its pollinator, the hawkmoth, or the close mutualistic relationships between trees and their microbial symbionts in the rhizosphere (Nilsson *et al.* 1985; Steidinger *et al.* 2019). Biodiversity is however not evenly distributed across the globe and varies considerably between regions. For example, in the Amazonian region, 775 mammal species are currently documented, while in Europe around 231 mammal species have been detected to date (Spironello *et al.* 2023; Temple & Terry 2009). This phenomenon can be partly explained by the latitudinal biodiversity gradient, where the diversity of many species groups decreases from equatorial to polar regions (Mannion *et al.* 2014). Although European ecosystems harbour fewer species in comparison to global biodiversity hotspots, they nonetheless support numerous different species of plants, invertebrates and vertebrates. For example, bacterial communities were even found to exhibit the highest diversity in forest ecosystems of temperate regions (Guerra *et al.* 2022). The diversity of all these communities worldwide contributes to the overall biodiversity, which is important for pivotal functions like ecosystem stability, ecological resilience and disease regulation (Keesing *et al.* 2010; Oliver *et al.* 2015; Tilman *et al.* 2006). Analysing the composition and structure of biological communities provides critical insights into the mechanisms that link biodiversity to ecosystem functioning and sustain ecosystem health under ongoing environmental change.

Biodiversity, reflected in the multitude of species and their roles in ecosystem services across the tree of life, demonstrates the complexity of life on Earth. Organisms of all kinds are constantly interacting with each other, whether through mutualistic symbiosis, parasitic relationships or by acting as ecosystem engineers. These interactions are of central importance, as they not only determine the structure and function of ecosystems but also promote the adaptation and evolution of the species involved. Biotic communities should therefore be understood as living networks, whose dynamics are characterised by the interactions among their members, rather than strict collections of individuals.

This work focuses on changes and trends within the groups of arthropods, fungi and bacteria and will first point out their ecological importance in the environment. Subsequently, effects and drivers of biodiversity loss in the Anthropocene affecting these groups are discussed, followed by an overview of monitoring methods with their respective benefits and limitations. Finally, time series analysis using state-of-the-art techniques and valuable, highly standardized collected samples are presented as powerful tools to assess biodiversity trends.

Diversity within the arthropod phylum

Arthropods are a particularly striking part of biodiversity within the Eukaryotes. This extremely species-rich phylum is the most diverse group of animals in the world (Giribet & Edgecombe 2012) and plays important roles in numerous ecosystem processes. Approximately 1 million species are currently described, with estimates of up to 10 million species worldwide (Ødegaard 2000; Thorp 2009). The unique lifestyles and habitat preferences of some organisms, especially those inhabiting highly remote or inaccessible locations, contribute significantly to this knowledge gap. For example, in a small area of tropical forest, thousands of arthropod species were successfully detected (Basset *et al.* 2012). These forests are often difficult to access due to dense vegetation, which makes it difficult to assess species diversity. Other issues, like the requirement of special expertise for taxonomic identification and the sometimes cryptic lifestyles of arthropods can add another layer of complexity. Cryptic species often share similar phenotypic traits despite being genetically distinct, which can lead to an underestimation of biodiversity (Struck *et al.* 2018).

Understanding the diversity within this phylum is crucial when analysing the ecological functions of arthropods in the environment. Pollination is one of the most important functions of arthropods for ecosystems. Sometimes remarkable co-evolutionary adaptations between plants and their pollinators have emerged. A particular striking example in European habitats is the relationship between the globeflower (*Trollius europaeus*) and its pollinating fly (*Chiastocheta* genus) (Ferdy *et al.* 2002). Within the flower, multiple fly species of the same genus coexist due to contrasted oviposition behaviours. While the larvae feed on the seeds, the flower is pollinated by the flies' visits. With this mutualistic interaction the larvae gain access to a food supply while the plant secures reproductive success. This co-evolutionary adaptation represents a fascinating example of close interaction between a plant and its pollinator. The value of pollinators can also be quantified in economic terms. The services provided by pollinators have been estimated at several hundred billion US dollars, clearly demonstrating their environmental and economic value (Breeze *et al.* 2016). In addition to pollination, arthropods form the base of terrestrial trophic pyramids and serve as a food source for numerous other species (Klein *et al.* 2022) and are equally essential in the decomposition of organic matter (Wall *et al.* 2025). Shredding litter optimizes the decomposition by other organisms, for example bacteria and fungi. Their vertical movements in the soil improve aeration and water storage capacity and thereby promote plant growth (Culliney 2013). Further positive effects on plants and entire ecosystems result from the role of arthropods as natural pest control, acting as predators, parasites and parasitoids. Complex parasite-host interactions can significantly regulate pest populations, whereas the absence of such regulators can have profound effects on entire regions. Each year, herbivorous arthropods cause economic losses totalling hundreds of billions of US dollars (Culliney 2014). In agricultural areas where synthetic chemicals are frequently used, natural predator-prey balances are disrupted. This can result in pest outbreaks, with severe ecological and economic implications (Crowder *et al.* 2010). Consequently, environmentally friendly yet targeted control strategies are essential to safeguard both crop yields and regional biodiversity.

In conclusion, arthropods contribute significantly to ecosystem health and functioning, by playing key roles as pollinators, in nutrient cycles and as natural pest control. Particularly in times of ongoing global biodiversity loss, it has become apparent that this ecologically and economically important group is experiencing a significant decline, especially in terrestrial ecosystems (van Klink *et al.* 2020). Impacts on other groups and ecosystems by this loss are inevitable, yet comprehensive data to assess these trends are still largely lacking (Bálint *et al.* 2018).

Diversity within the fungal kingdom

Another highly diverse group within the Eukaryotes are fungi. Estimates of species richness range from 2.2 to 3.8 million species worldwide, of which approximately 150,000 species have been taxonomically described to date (Hawksworth & Lücking 2017; Rudramurthy & Kaur 2024). The fungal kingdom exhibits a remarkable diversity of morphological and physiological characteristics, enabling a pronounced ecological adaptability to varying conditions (Debeljak & Baltar 2023). Besides terrestrial habitats, such as forests and grasslands, fungi are present in limnic and marine environments. Further, specialists can thrive in extreme habitats, like hydrothermal vents, caves and cold or hot deserts (Coleine *et al.* 2022; Vanderwolf *et al.* 2013).

Among their many ecological roles, one essential function of fungi in the environment is as symbionts. A prominent example is the mycorrhizal relationship between fungi and plants in the rhizosphere. Here, fungi colonize either roots and sometimes migrate into plant cells. With the formation of fine structures, the plant's ability to acquire vital nutrients, such as organic nitrogen and phosphorus, is significantly improved (Schüßler 2009). Moreover, through this mutualistic relationship, the plant benefits from an increased resistance against phytopathogens. This resistance arises from the direct competition of pathogens and fungi for resources in the rhizosphere or by directly inhibiting pathogen growth due to fungal activity (Faria *et al.* 2021). In addition to being important symbiotic partners in the environment, fungi play a crucial role in biological pest control. Entomopathogenic fungi offer a sustainable alternative to conventional agricultural practices, such as pesticide application (Shah & Pell 2003). Despite their beneficial functions, fungi can also cause considerable environmental and economic damage as phytopathogens. Documentation of crop damage caused by pathogenic fungi and plant diseases dates back to ancient times (Doehlemann *et al.* 2017). Modern advances have led to the scientific description of numerous pathogens, including those responsible for various forms of plant diseases causing wilting or root and stem rot (Fernandez & Conner 2011; Pandey *et al.* 2022). In addition to crops, plant-pathogenic fungi can also harm higher plants, such as trees. Plant health is a critical factor in pathogen colonization, with stressed or weakened trees often exhibiting higher pathogen infestations. Normally, trees produce defence substances such as phenols or phytoalexins. Weakened or stressed trees reduce or stop the production of these compounds. Wounds in the bark or roots can serve as entry for fungi, allowing them to penetrate quickly into the xylem and phloem, where they absorb nutrients, and spread throughout the host (La Fuente *et al.* 2022). Drought stress, mechanical damage or previous pest infestation can further strain the defence mechanisms of trees and thus favour a secondary infection (Pandey & Senthil-Kumar 2019). The implementation of new agricultural practices and extensive forest plantations led to novel pathogen-insect interactions, recently promoting this mechanism (Ghelardini *et al.* 2016).

These examples illustrate the central position of fungi in the environment. They act as decomposing organisms in the nutrient cycle, as symbionts in mutualistic relationships or as pathogenic components and thereby contribute to dynamics within ecosystems. Like arthropods, fungal diversity is threatened by ongoing biodiversity loss. Limited and incomplete data hinder our ability to fully assess the scope of this decline (Mueller & Schmit 2007). Nonetheless, the impacts of losing undiscovered yet ecologically significant species in other systems are evident.

Diversity within the bacterial domain

Alongside arthropods and fungi, bacteria are an important part in the tree of life. Within this group, the classification into distinct species is extremely challenging. As many bacterial species are difficult or even unable to be cultivated, a phenotypic and genetic analysis is largely limited (Lewis *et al.* 2021). However, estimates suggest that there may be few millions to potentially billions of bacterial species in the environment (Wiens 2023). Their high adaptive capacity enables them to thrive globally and living communities were even found in ancient permafrost samples, dating back thousands of years. Constant and stable environmental conditions enabled perfect preservation of these communities (Vorobyova *et al.* 1997).

Their adaptability allows them to exist in every ecosystem, where they fulfil numerous important roles. A key function lies in the nitrogen cycle, in which nitrogen compounds are transformed into bioavailable forms for plants, such as ammonium and nitrate, supporting plant nutrition and promoting plant growth (Hayat *et al.* 2010; Kuypers *et al.* 2018). Moreover, the decomposition of organic litter in the rhizosphere is mainly conducted by bacteria and fungi, which significantly accelerates nutrient bioavailability for numerous organisms. This interaction between microbes enhances the energy flow within ecosystems, highlighting their complementary roles in maintaining soil fertility and ecological stability (Krishna & Mohan 2017). In addition to the nitrogen cycle, bacteria actively participate in the carbon and phosphorus cycles, further pivotal processes for entire ecosystems (Luo *et al.* 2020). Despite these examples of symbiotic relationships, interactions with bacteria are not invariably beneficial for both partners. For example, phytopathogens can cause considerable damage to their hosts. Although knowledge is scarce about this group of bacteria, genera like *Pseudomonas*, *Agrobacterium* and *Xanthomonas* are known to include multiple phytopathogenic bacteria. For the genus *Xanthomonas*, for example, almost 400 plant hosts have already been identified in which disease symptoms are triggered by an interaction with these bacteria (Ryan *et al.* 2011).

Further research is required to fully understand all mechanisms and interactions of bacteria in the environment. However, these examples illustrate the importance of bacteria as symbiotic partners, pathogens and ecosystem engineers in the environment. Like other organism groups, bacterial communities are expected to already have experienced significant biodiversity losses in recent years (Zhu & Penuelas 2020). These declines are expected to have substantial impacts across ecosystems and trophic levels.

Biodiversity change and potential drivers in the Anthropocene

Abiotic and biotic processes are constantly shaping the environment, driving changes in biodiversity at local and global scales. Throughout Earth's history, species extinctions have been a natural part of this dynamic. Five significant mass extinctions have been documented to date, each with profound consequences for biodiversity (Ceballos & Ehrlich 2018). Mass extinctions are defined by the loss of more than 75% of biodiversity within a geologically short period (Cowie *et al.* 2022). The prevailing theories attribute these events to major environmental transformations, such as anoxia, ocean acidification, or global warming, affecting thousands of species (Bond & Grasby 2017). Recent scientific studies suggest that the Earth is currently undergoing a sixth mass extinction, which differs from previous events in its predominantly anthropogenic origin and its much shorter time frame. Based on these observations, the current event is defined as that of the Anthropocene (Laurance 2019).

Although it is difficult to predict the exact consequences of anthropogenic impacts on species communities, their potential to irrevocably affect and change population structures and even entire ecosystems is considerable. Alarming trends have been addressed in recent work, examining shifts and declines in biodiversity patterns (Blowes *et al.* 2019; Dornelas *et al.* 2014; Johnson *et al.* 2017; Shaw *et al.* 2025; Turvey & Crees 2019; Wagner *et al.* 2021). Among the key drivers of these developments are land- and sea-use change, the overexploitation of natural resources and climate change (Jaureguiberry *et al.* 2022). In terrestrial ecosystems, the increasing establishment of monocultures and the excessive application of biocides, such as pesticides and insecticides, and fertilisers are decisive factors, impacting various groups of organisms (Raven & Wagner 2021). This extensive restructuring of the environment poses the risk of altering population dynamics and thus disrupting entire interaction networks to an unexpected extent (Newbold *et al.* 2015). Pollinators are most negatively affected by anthropogenic activity in form of monoculture cultivation. In these systems, nutritional and physiological requirements of pollinators are often not met, as the cultivated plants do not serve as a viable food source (Nicholls & Altieri 2013; St. Clair *et al.* 2020). On top of this, the increased application of biocides in agricultural landscapes poses a major threat for numerous pollinator species. While neonicotinoids, a more recent type of biocide, offer advantages, such as lower required application doses compared to other pesticides and being an effective pest control, ecological implications arise (Kaur & Kaur 2024). For instance, a specific set of pollinator species is commonly used for tests with chemical compounds. Biotic communities are however highly diverse, with numerous interactions between species and different functional groups (Basu *et al.* 2024). Moreover, there is increasing evidence that non-target organisms, like pollinating insects, birds and bats, are negatively affected by neonicotinoids (Ben Amor *et al.* 2024). Reproductive success, pollinator condition and survival have also been negatively associated with increased neonicotinoid applications (Main *et al.* 2018). The changes in arthropod communities suggest that bacterial and fungal communities are similarly affected by these anthropogenic interventions. These impacts are significantly altering terrestrial ecosystems with outcomes difficult to predict.

Besides terrestrial ecosystems, human activities are also clearly visible in the aquatic realm. Within marine habitats, factors such as sea-use change, habitat loss and the overfishing of the sea are causing fundamental biodiversity losses (Dulvy *et al.* 2021; Yan *et al.* 2021). The trends are alarming, with long-term analyses showing significant declines and extinctions of ecological and economic important species in coastal and estuarine ecosystems. As a result, critical ecosystem services, such as the provision of nursery habitats and filtering services, are directly affected by this diversity loss (Worm *et al.* 2006).

One major driver of the current biodiversity crisis, alongside habitat loss and the overexploitation of natural resources, remains climate change (Jaureguiberry *et al.* 2022). With rising global temperatures and potentially more severe and frequent extreme weather events, profound impacts on all ecosystems, terrestrial and aquatic alike, are expected in the future (Häder & Barnes 2019). These alterations threaten ecosystem stability by disrupting biotic interactions and trophic networks. One potential outcome is the shift in habitat structures and the creation of new ecological niches (Henson *et al.* 2017). The combination of available niches and favourable climatic conditions can promote the immigration of invasive species. In the absence of host-specific parasites and natural predators, these non-native species can spread rapidly, potentially outcompeting and replacing native species (Peh 2010; Pyšek & Richardson 2010; Skočajić & Nešić 2020). The natural shift in community composition is thus accelerated by the immigration of invasive species and the loss of natives through direct competition. Together, these factors can result in significant ecological and economic implications and cause harm to ecosystem stability (Peller & Altermatt 2024; White *et al.* 2020). A prominent example of the negative impact of invasive species on European native fauna is seen in the Western honey bee (*Apis mellifera*) and the varroa mite (*Varroa destructor*), an ectoparasite naturally associated with the Asian honey bee (*Apis cerana*). This host switch occurred around 70 years ago and resulted from several introductions of *A. mellifera* into regions inhabited by *A. cerana* (Roberts *et al.* 2020). While the natural host *A. cerana* was able to develop defence mechanisms through a long period of coexistence with the varroa mite, *A. mellifera* has not been able to do so due to the relative novelty of the interaction (Grindrod & Martin 2023). Global trade facilitated the introduction and subsequent establishment of the mite in European habitats. Infections with the parasite reduce the fitness of *A. mellifera* and facilitate secondary infections and the transmission of pathogens. The combination of parasitism and secondary pathogen infection can lead to the collapse of entire bee colonies, with severe negative ecological and economic consequences (Sumpter & Martin 2004; Vanbergen *et al.* 2018). This example highlights the threats native faunal communities face when exposed to novel and harmful biotic interactions.

The introduction of invasive species can however be less evident than in the previous example. Population growth of invasive species often follows a logistic model, characterised by a sigmoidal curve (Figure 1). The issue arises as these species are frequently detected in the exponential growth phase, while their abundance is too low to be detected effectively in earlier stages. During the growth phase, not only does the population size increase rapidly, but the ecological and economic damage intensifies. Once established in a new habitat, invasive species are typically irreversibly integrated, making the control far more difficult and resource-intensive (Moodley *et al.* 2022). The increased abundance of the species leads to far-reaching changes in ecological processes. For example, by displacing native species and thus disrupting existing trophic networks. It is already visible that invasive species are having a significant impact, particularly in economically sensitive sectors, such as agriculture and forestry (Brockerhoff & Liebhold 2017; Paini *et al.* 2016). With the ongoing climate change, these effects are likely to intensify in the future.

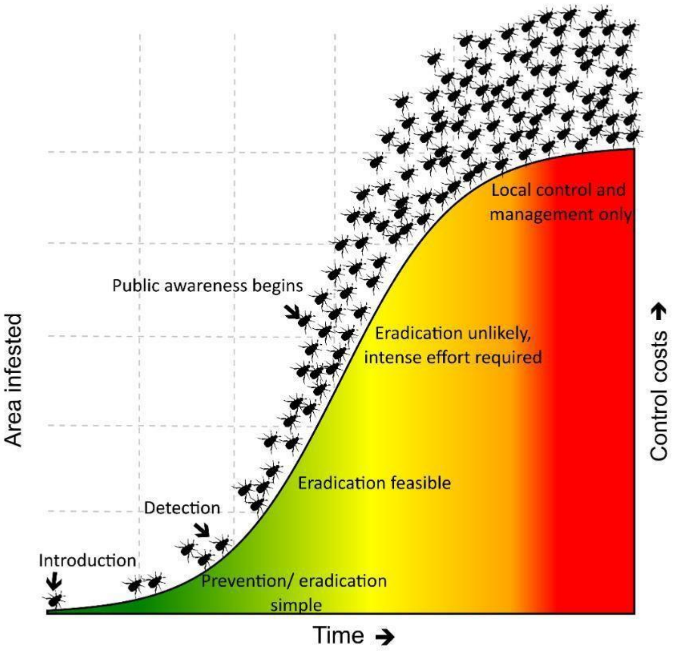

Figure 1: Modified invasion curve based on Hitchcox (2015). Black species icon was obtained from https://www.phylopic.org/.

Biodiversity loss in the Anthropocene is largely driven by human activities, including habitat destruction, overexploitation of natural resources and factors such as the ongoing climate change, and the associated introduction of invasive species. These influences pose major challenges for ecosystems worldwide. A particularly prominent example of a well-studied group exhibiting significant declines in the Anthropocene are arthropods. This issue has been addressed in numerous publications, which revealed serious declines in abundance, biomass, and species richness (Dirzo *et al.* 2014; Forister *et al.* 2019; Wagner *et al.* 2021). The results provide an insight into the development of less well-studied groups of organisms, such as microbes. To detect changes at an early stage and enable effective conservation measures, continuous monitoring of the communities is necessary.

Techniques and tools for monitoring and assessing biodiversity

In order to fully understand the dynamics within communities, detailed information on species composition and population structure is needed. Only by systematically monitoring the diversity of species and their interactions can long-term changes and dynamic processes in ecosystems be uncovered. This data serves for understanding ecological relationships and developing targeted strategies for the protection of species.

The monitoring of biodiversity can be achieved through a variety of methods, each of which possesses distinct benefits and drawbacks. One of the earliest and most straightforward methods is the use of footprints, faeces, or fur remains. These were already used by prehistoric hunters to successfully track animals (Lenssen-Erz *et al.* 2023). Besides hunting, it is a simple yet effective non-invasive monitoring approach for specific species. In Finland, for example, wolf population sizes have been successfully estimated on the basis of snow tracks (Kojola *et al.* 2014). Apart from the simple and straightforward design, this method has several downsides. For instance, it is best suited for studying larger animals, as the size of the footprint or the amount of fur remains will determine the detectability of the species. It is inappropriate for smaller organisms and microbes, or specific groups like endoparasites.

To monitor groups like arthropods, the utilisation of Malaise and pitfall traps is a prominent approach. Both monitoring methods can complement each other, as they capture different groups of arthropods. While pitfall traps are designed as containers embedded in the ground to capture epigean species, malaise traps are used to catch flying insects. Both methods possess the advantage of being relatively straightforward and require minimal labour for the collection of individuals. A prominent example of the application of Malaise traps revealed a substantial decline in the biomass of flying insects in Germany within the last three decades (Hallmann *et al.* 2017). Despite their practicality, the major challenge lies in sample processing. Sorting and taxonomic identification of thousands of individuals requires time and expert knowledge. Furthermore, biases caused by taxonomic chauvinism can lead to underrepresentation of less charismatic groups such as Diptera, although fulfilling important ecosystem services (Bonnet *et al.* 2002; Khan *et al.* 2023; Pilotto *et al.* 2020; Troudet *et al.* 2017). Moreover, the seasonal fluctuation within arthropod communities adds another layer of complexity to correctly map these organisms (Macgregor *et al.* 2019). Short-term surveys or studies with only a few repetitions may miss important species, as life stages change during the year. This is also a critique of the Hallmann *et al*. (2017) study (Thomas *et al.* 2019).

To assess the diversity of microbial communities, different approaches are required. Traditionally, microbes are cultivated on selective media, where they grow and are subsequently identified based on morphological, biochemical, physiological, and/or genetic characteristics (Ferone *et al.* 2020; Halme *et al.* 2012). As simple as the design is, there are some limitations. The selectivity of culture media restricts the growth to certain microbial taxa, making it impossible to capture the full diversity of a microbial community in a sample. Recent work has shown that a considerable amount of soil microbial species cannot be cultivated by traditional techniques (Manfredini *et al.* 2021). Additionally, this method is time-consuming, as cultivation often takes several days to weeks, and samples must be plated on multiple types of media throughout the process (Neyaz *et al.* 2024).

The methods mentioned above are only a small subset of the many available for biodiversity monitoring. While effective in specific contexts, traditional approaches share several major drawbacks, including their invasiveness, time-consuming nature, and dependence on expert knowledge. To overcome these limitations, careful study design and, where appropriate, complementary methods are required.

Metabarcoding represents a powerful tool to overcome these issues. For metabarcoding analysis, unique DNA sequences, similar to genetic barcodes, are used to identify specific species. Within the DNA sequence, conserved regions flank highly variable regions, enabling the use of universal primers. This approach allows the amplification of short diagnostic DNA sequences of multiple species while maintaining the specificity of the target group (Ruppert *et al.* 2019). During data processing, the generated sequences are clustered, which can be performed in different ways. Frequently, zero-radius operational taxonomic units (zOTUs) or haplotypes are analysed, which are often treated as species equivalent. Here, sequences with only one different base at one position are regarded as unique zOTUs. To give the sequences biological meaning, the addition of taxonomic annotation is necessary. Such data are available in reference databases (Gold *et al.* 2022). Numerous databases of metazoan, bacterial, and fungal sequences exist, allowing the analysis of specific groups in a biological context (Abarenkov *et al.* 2024; Quast *et al.* 2012; Stoeckle & Hebert 2008). Alternatively, sequence clustering using Poisson tree processes models can be conducted to generate OTUs (Zhang *et al.* 2013). Both options are applicable, depending on the study design. Recently, bulk sample metabarcoding has become a valuable tool for biodiversity research. Here, multiple organisms, like insects from traps, are homogenized and DNA is extracted and sequenced (Steigerwald *et al.* 2025). A great advantage of bulk sample metabarcoding is the simultaneous analysis of numerous sequences from a single sample, reducing time and costs. Moreover, robust datasets can be generated rapidly, and extensive taxonomic expertise is not necessarily needed. Still, it shares a key drawback with the previously mentioned approaches. The use of hundreds to thousands of individuals for each sample makes it highly invasive for biological communities. Therefore, a monitoring approach that also meets the criterion of minimal invasiveness is urgently needed.

A promising complement is the application of environmental DNA (eDNA) in monitoring studies. This relatively new method is based on sampling and analysing environmental samples, which have an imprint of the surrounding communities. Plant tissue and water are frequently taken due to the simplicity of sampling and the relatively high DNA concentrations they contain. DNA extracted from environmental samples can be analysed using modern molecular techniques, with eDNA metabarcoding approaches combined with high-throughput sequencing being of particular importance. The first applications of eDNA technology to reconstruct diversity patterns in larger organisms, such as mammals, plants and birds, were used with sediment samples in the early 2000s (Sahu *et al.* 2023). Since then, eDNA-based biodiversity research has developed dynamically and established itself as an extremely reliable and versatile tool. Its efficacy and accuracy have been proven in numerous scientific studies for terrestrial and aquatic ecosystems (Bista *et al.* 2017; Deiner *et al.* 2017; Krah & March-Salas 2022; Ladin *et al.* 2021; Nørgaard *et al.* 2021). Over time, this method has advanced, enabling the incorporation of new sample matrices. Plants and even whole organisms can act as natural DNA samplers for biodiversity assessment (Kennedy *et al.* 2020; Krehenwinkel *et al.* 2022a; Weber *et al.* 2023). Examples include filter-feeders such as mussels, which filter more than one litre of water per hour, or leaves, which serve as food supply for many arthropod species (Beyer *et al.* 2017; Krehenwinkel *et al.* 2022b). By capturing genetic imprints of surrounding taxa, these samplers offer high-resolution biodiversity data (Junk *et al.* 2023). Whether in the form of feeding traces, faeces, skin cells, secretions, or traces of organisms like fungi and bacteria, the genetic material can be extracted and analysed (Bohmann *et al.* 2014; Zizka *et al.* 2022). All samples are then processed in the laboratory often following standard metabarcoding protocols. Besides its straightforward design, a significant advantage of eDNA metabarcoding is its minimal invasive nature, a major drawback of traditional and even bulk sample metabarcoding approaches. Moreover, using natural DNA samplers, invasive species can be detected in an early stage (Klymus *et al.* 2017; Westfall *et al.* 2020). As previously described, these species pose a serious threat to ecosystems, as they often have highly adaptable strategies to changing environmental conditions. With climate change further favouring the immigration of invasive species, their relevance is expected to increase in the future (Early *et al.* 2016; Gallardo *et al.* 2017; Hellmann *et al.* 2008). The combination of the minimally invasive nature and the potential for early detection of invasive species makes eDNA metabarcoding a highly effective monitoring concept.

Despite the promising advantages of bulk sample and eDNA metabarcoding, these methods face various challenges. One source of errors when analysing natural samplers is the high risk of contamination. Since DNA is continuously released by organisms in the environment, contaminations can occur easily right at the start during sample collection. Moreover, eDNA naturally degrades in the environment, which is accelerated by UV radiation, temperature and microbial activity (Strickler *et al.* 2015). Weather conditions play further important roles, as rainfall can significantly wash off DNA remains and prevent detection (Johnson *et al.* 2023). Bulk sample metabarcoding is less affected by this, as all specimens in a sample are analysed and not their DNA remains. Further issues arise during laboratory processing. When extracting eDNA from certain environmental samples, for example soil or leaves, substances such as humic and tannic acids significantly inhibit the activity of DNA polymerase enzymes during the amplification of the target sequence (Schrader *et al.* 2012). It is therefore necessary to remove these inhibitors during the laboratory workflow. Another potential source of error arises from the amplification of nuclear mitochondrial DNA sequences (NUMTs). An issue which applies for both methods. NUMTs are copies of mitochondrial genes that have been integrated into the nuclear genome and are rarely subject to selection. This makes them more susceptible to the accumulation of mutations. Due to their high similarity to the original mitochondrial sequence, NUMTs can be unintentionally co-amplified, leading to possible inflation of biodiversity (Hazkani-Covo *et al.* 2010). Due to their rapid evolutionary adaptation rate, these sequences show characteristic differences. However, NUMTS cannot be entirely detected and removed, which is a major limitation for eDNA and bulk sample metabarcoding analyses

(Graham *et al.* 2021). During sequence processing, the availability and quality of reference databases is another key factor for reliable eDNA and bulk sample metabarcoding studies (Schenekar *et al.* 2020). It is essential that the sequences listed in these databases are complete, accurate and the database is well curated for reliable conclusions.

Overall, despite certain limitations, eDNA is a promising tool for efficiently recording biodiversity patterns while minimising disturbance to natural habitats. Its ability to detect species from different environmental samples makes it a versatile approach for ecological studies. With the further development of sequencing technologies and the application of standardised protocols, eDNA metabarcoding has the potential to revolutionise biodiversity monitoring by providing high-resolution data on species and community dynamics. In the future, its integration into long-term monitoring programmes could significantly enhance efforts to protect endangered species, track invasives and assess ecosystem health. The application of eDNA metabarcoding in combination with traditional monitoring approaches could generate comprehensive information on a variety of groups as well as new investigative approaches, such as analysing interaction networks.

Time series analysis and Environmental Specimen Banks

To comprehensively understand biodiversity patterns and dynamics requires not only momentary snapshots, but rather the analysis of long-term trends to draw robust conclusions (Macgregor *et al.* 2019). Since environmental conditions constantly shape populations, their spatial and temporal composition is undergoing steady change. Effective biodiversity monitoring in the Anthropocene therefore demands for tailored approaches to understand past changes and predict future trends. One potential method is the space-for-time substitution, a procedure that analyses the relationships between ecological variables at sites in different stages of development (Walker *et al.* 2010). By creating a chronosequence, potential future changes at sites in earlier stages can be predicted. However, this approach also has some limitations. For example, sites in different stages of succession or large-scale habitats are required for this method (Damgaard 2019). To evaluate habitat changes at comparable developmental stages, alternative approaches must be employed.

The analysis of time series, which allows for the examination of past trends within communities and enables the prediction of potential future developments, is a potential method. The key elements to drawing reliable conclusions from time series analyses lies in the availability of robust and representative datasets covering long time periods. In a prominent study, the temporal changes in communities of different biomes were analysed (Dornelas *et al.* 2014). For this approach, 100 time series from terrestrial and aquatic ecosystems with time spans over 80 years were analysed. The survey focussed on two important components of diversity: the temporal trend in species richness (temporal α-diversity), measured by the number of species, and the temporal change in species composition (temporal β-diversity), measured by the Jaccard similarity over the years. The study provides important insights into long-term trends in key biodiversity indices. The results on temporal α-diversity show that the trends vary greatly depending on the ecosystem and the time series analysed. However, an overarching analysis revealed no significant changes in temporal α-diversity. In contrast, temporal β-diversity showed a significant turnover across all analysed biomes throughout the study period. This turnover indicates a continuous change of community composition over time, affecting ecosystems worldwide. Various drivers are likely to contribute to this turnover, with climate change and the spread of invasive and anthrophilic species named as potentially important factors. Key drivers yet need to be identified. The immigration of non-native species compensates for the extinction of natives, resulting in stable α-diversity values. However, the combination of changes in community composition and the loss of site-specific species promotes spatial homogenization, which refers to the gradual reduction of ecological diversity across different habitats and regions. This results in the loss of genetic, taxonomic and functional diversity, as communities tend to increase similarity over larger areas (Olden *et al.* 2004). The homogenization across space has profound consequences on the multifunctionality of ecosystems and global biodiversity (Hautier *et al.* 2018; van der Plas *et al.* 2016; Wang *et al.* 2021b). Recent studies confirm this alarming trend (Blowes *et al.* 2019; Magurran *et al.* 2010; Magurran *et al.* 2015).

Although robust long-term time series are urgently needed to understand biodiversity change in the Anthropocene, these are currently lacking for many taxa and ecosystems (Bálint *et al.* 2018; Magurran *et al.* 2010). Environmental Specimen Banks (ESBs) represent a promising tool for this issue. Currently, 28 ESBs serve as archives for biological samples worldwide (Zhao *et al.* 2015). Their primary objective is to archive environmental samples for future analyses. For this purpose, indicator species, i.e. species that meet specific criteria such as biological relevance and high abundance in their respective ecosystems, are sampled. ESBs are well-designed monitoring projects that apply highly standardised sampling protocols with sampling conducted at the same time of the year. For sampling, the use of sterile equipment minimises the carry-over of even trace amounts of contaminants. This consistency, together with a well-thought-out study design, enables the analysis of temporal and spatial changes both within and between study sites. ESB samples are large, in most cases including hundreds to thousands of specimens or tissue compartments per sample. The storage at ultra-low temperatures ensures rapid and stable preservation of the samples and associated molecular information.

The German ESB is among the longest-acting monitoring programs, routinely collecting and archiving environmental samples from across Germany since 1985. This archive provides a comprehensive representation of terrestrial, marine and limnic ecosystems. For this program, 15 indicator species are currently sampled and analysed throughout Germany (Figure 2). In terrestrial ecosystems, leaves/ needles from trees (*Fagus sylvatica, Populus nigra* 'Italica', *Picea abies, Pinus sylvestris*), earthworms (*Lumbricus terrestris, Aporectodea longa*) and deer livers (*Capreolus capreolus*) are analysed. In freshwater ecosystems, samples include freshwater fish (*Abramis brama*, *Barbus barbus*) and mussels (*Dreissena polymorpha*, *Dreissena rostriformis bugensis*) and in marine ecosystems, eel pout (*Zoarces viviparus*), European herring gull eggs (*Larus larus*), bladderwrack (*Fucus vesiculosus*) and blue mussels (*Mytilus edulis*) are surveyed. This extensive sampling is conducted at more than 70 sites across Germany, covering a broad range of species and ecosystem types. Whenever possible, samples are immediately stored in the gas phase over liquid nitrogen in the field after collection. To obtain the final sample, all tissues/ specimens, depending on the sampling species, from a specific sampling location are mixed. Without interrupting the cold chain, this raw material is ground several times using a cryogenic mill to a final particle size of 200 µm. By this, all traces of chemicals and nucleic acids are evenly distributed in the sample. A unique feature of the German ESB, compared to other traditional monitoring methods, is the use of highly standardised protocols for sample collection and processing (Klein *et al.* 2018a; Klein *et al.* 2018b; Klein *et al.* 2018c; Paulus *et al.* 2010; Paulus *et al.* 2018a; Paulus *et al.* 2018b; Rüdel *et al.* 2008; Tarricone *et al.* 2018a; Tarricone *et al.* 2018b; Tarricone *et al.* 2018c; Teubner *et al.* 2018a; Teubner *et al.* 2018b; Teubner *et al.* 2019). Due to this high level of standardisation, the German ESB can serve as a model for international monitoring efforts. The close cooperation between different EBSs further offers the great opportunity to systematically monitor and compare global biodiversity patterns across organism groups. For instance, the Korean ESB closely aligns with the German ESB in terms of sample collection and sample processing, enabling direct comparisons of biodiversity trends in regions far apart.

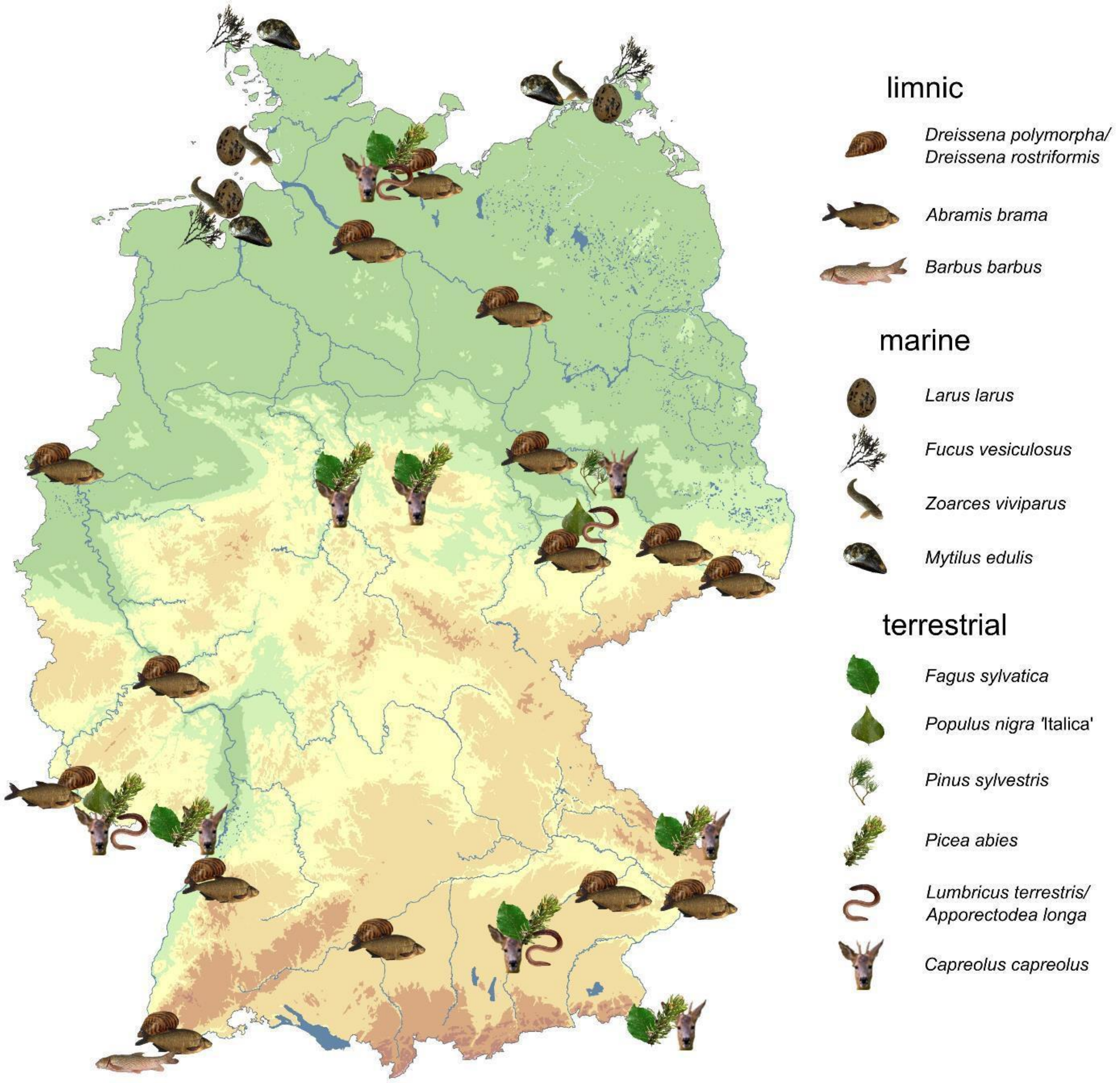

Figure 2: Sampling species and sampling locations by the German Environmental Specimen Bank. Map of Germany and species icons are provided by the ESB project group Trier.

So far, numerous studies have used samples from the German ESB to analyse trends of relevant environmental pollutants. Since 1994, more than 170 scientific publications addressed this topic (Umweltbundesamt 2025). Many species sampled by the ESB act as natural samplers, like leaves from trees and mussels as filter-feeders. With the application of new methods, such as eDNA metabarcoding and high-throughput sequencing, it is now possible to analyse retrospective trends in the sample-associated communities of these natural samplers. This enables an additional dimension of biodiversity analysis with these samples that was previously impossible. First studies have already shown the great potential of ESB samples for retrospective biodiversity analysis (Junk *et al.* 2023; Krehenwinkel *et al.* 2022a; Weber *et al.*

2023). In the future, these highly valuable samples can be used to analyse biodiversity trends across the tree of life, helping to track changes of communities in the Anthropocene.

Research aims of this thesis

While biodiversity trends of many groups of organisms in the Anthropocene have not yet been adequately studied, samples from the German ESB provide a powerful tool to close this gap. For this thesis, samples from the German ESB were used to analyse temporal and spatial changes in sample-associated communities across the tree of life over the last three decades. The perfect preservation of the samples, combined with the use of state-of-the-art molecular techniques made it possible to reveal retrospective trends of numerous sample-associated groups of organisms in German habitats for the first time. Moreover, trends in arthropod communities of South Korean ESB samples were compared to German ESB samples, thus enabling a wider perspective of diversity trends. Finally, the potential of analysing changes in functional fungal groups associated with German ESB samples were investigated to assess tree health. Based on recent work, in the subsequent chapters the following hypotheses were investigated:

- Using eDNA from natural samplers of a long-term monitoring archive in Germany reveal significant declines of species richness across the tree of life.

- Biotic communities experience significant compositional changes in the last decades.

- Arthropod communities around the globe exhibit similar pattern in terms of α- and β-diversity trends.

- Changes within fungal communities and functional fungal groups are a suitable proxy to assess alterations in tree health.

Chapter 1

DNA metabarcoding is valuable for biodiversity assessment. In the first chapter, the methodological workflow of metabarcoding is described in greater detail. Essential steps to extract and analyse genetic information of associated communities from environmental samples are addressed. Emphasis is placed on challenges throughout laboratory procedures and bioinformatic data processing as well as common pitfalls and how to address them. Furthermore, examples illustrating the wide range of ecological applications for eDNA metabarcoding approaches in biodiversity research are presented. Finally, emerging research questions, including the potential to yield quantitative insights into community composition, are considered. This chapter provides the basis of the methodological framework for the following chapters and has been published as a book chapter in the *Encyclopedia of Biodiversity*:

Krehenwinkel H., **Hans J**., Junk I., Mahla L., Melcher A., Stothut M. & Kennedy S. (2024) Metabarcoding for Biodiversity Estimation. In: *Encyclopedia of Biodiversity (Third Edition)*. (ed. Scheiner SM), pp. 388–407. Academic Press, Oxford.

Chapter 2

After the introduction of eDNA metabarcoding, Chapter two presents the first results of trends in arthropod communities associated with leaves from the German ESB archive over the last three decades. To begin with, a protocol was developed to obtain arthropod community information from the ESB samples. Subsequently, trends in species richness (α-diversity) over the study period were examined. Contrary to recent work (Hallmann *et al.* 2017), the results indicate relatively stable richness values. Since ESB samples cover landscapes with varying degrees of anthropogenic impact, a comparison of richness trends in low and high impact areas was examined. The results revealed a decline in richness in agricultural sites, while natural sites remained stable. Land use intensification is considered to be a major driver for the decline in canopy-associated arthropod richness in agricultural sites. Finally, trends in community composition over space and time (temporal and spatial β-diversity) were examined based on recent work (Dornelas *et al.* 2014). A universal trend was observed for both indices across the dataset. Using German ESB samples provided for the first time insights into long-term trends of arthropod communities, revealing a general turnover in community composition and homogenization affecting terrestrial arthropod communities. This article was published in the journal *eLife*:

Krehenwinkel H., Weber S., Broekmann R., Melcher A., **Hans J**., Wolf R., Hochkirch A., Kennedy S. R., Koschorreck J., Künzel S., Müller C., Retzlaff R., Teubner D., Schanzer S., Klein R., Paulus M., Udelhoven T., Veith M., Creer S., Weigel D., Valentin R. & Gilbert T.

(2022) Environmental DNA from archived leaves reveals widespread temporal turnover and biotic homogenization in forest arthropod communities. *eLife*, doi: 10.7554/eLife.78521.

Chapter 3

To gain deeper understanding of trends in German communities, samples from marine, limnic and terrestrial ecosystems from the German ESB were analysed in Chapter three. This chapter is the centrepiece of this thesis. The samples cover a period from 1985 to 2022 and offer the opportunity to analyse trends in numerous sample-associated taxonomic groups in German ecosystems. A novel null-model was developed for this study, enabling the comparison of spatial and temporal changes in biodiversity under natural conditions, i.e. in the absence of human influence. The dynamic model is based on the equilibrium theory of island biogeography (MacArthur & Wilson 1963) and generates null expectations for diversity trends. This framework enables the comparison of the observed changes with those expected to occur without anthropogenic influence.

The samples exhibited highly diverse communities with hundreds to thousands of different zOTUs for each associated community, representing the respective sampler organism. As massive losses of biodiversity are expected (Ceballos *et al.* 2017; Dirzo *et al.* 2014; Seibold *et al.* 2019), these changes are assumed to be reflected in the taxonomic composition. However, no significant change within the higher taxonomic make-up was revealed. The subsequent analysis of α-diversity revealed differing trends for the communities and ecosystems. Results from the previous study for terrestrial arthropod communities were able to be reproduced. Moreover, the species richness of leaf associated microbial communities remained relatively stable. The same holds true for aquatic communities, with slight increase or decreases, however, not statistically significant. When compared to the null model, equal results arise, with no universal trends for the respective communities. In contrast, the analysis of temporal and spatial β-diversity changes provided more conclusive results. Here, the temporal component showed a general trend for all communities, revealing a continuous change of the species composition over the time resulting in a turnover. In addition, all temporal changes deviated significantly from the model-based expectations, indicating that biodiversity shifts were stronger than expected. The spatial analysis showed increasing homogenization for many terrestrial communities, while limnic communities tended to diversify. These developments are expected to be closely linked to the spread of generalist or invasive species and, especially in terrestrial ecosystems, to the displacement of site-specific species. Finally, regional diversity (γ-diversity) was assessed, which exhibited inconsistent trends across the systems. Overall, the study shows that German communities undergo significant temporal changes in the last decades which are stronger than expected. The main drivers for the result yet need to be identified. This chapter was published in the journal *Nature Ecology & Evolution*:

Junk I., **Hans J**., Perez-Lamarque B., Weber S., Stothut M., Gold E., Schubert C., Schumacher A., Schmitt N., Melcher A., Paulus M., Klein R., Teubner D., Koschorreck J., Kennedy S., Morlon H. & Krehenwinkel H. (2024) Archived natural DNA samplers reveal four decades of biodiversity change across the tree of life. *Nature Ecology & Evolution*, https://doi.org/10.1038/s41559-025-02812-6.

Chapter 4

The South Korean ESB closely resembles the German ESB in terms of sample collection and sample processing, offering the great opportunity to conduct biodiversity trend analyses on a global scale. In Chapter four, the trends of terrestrial arthropod communities in South Korea and Germany within the last decade were analysed. Based on the results of Krehenwinkel *et al.* (2022a) the analysis focused on the temporal changes of species richness. As already provided in the previous studies, German communities remained stable in terms of α-diversity, while South Korean communities experienced significant declines over the study period. This decline is irrespective of land use type, i.e. low and high human impacted areas. Arthropod communities in both areas experienced significant decreases. German communities however did not show a significant change in the different land use types. This result in German communities contrasts to previous findings and is mainly driven by the length and completeness of the data set. This becomes apparent, when the trends are compared to the whole time series, where substantial declines in agricultural sites occurred. Following, temporal changes of community composition were investigated. Despite the relatively short period investigated, Korean arthropod communities already exhibit significant changes. The objective should be to expand the time series in the future to confirm these changes. The study shows for the first time how arthropod communities in South Korean terrestrial ecosystems are changing over the last ten years and offers the possibility to conduct biodiversity analysis on a global scale. This chapter was published in the journal *Insect Conservation and Diversity*.

Hans, J., Stothut, M., Schubert, C., Gold, E., Chung, D., Lee, J., Koschorreck, J., Kennedy, S., Oh, J., Udelhoven, T. and Krehenwinkel, H. (2025) eDNA metabarcoding of archived leaf samples reveals arthropod diversity decline in South Korean but not in German forest ecosystems. Insect Conservation and Diversity, 1–13. Available from: https://doi.org/10.1111/icad.12860.

Chapter 5

Fungal communities are a highly diverse and complex group of organisms, fulfilling essential functions in the environment. Despite their ecological importance, functional groups within the fungal kingdom remain largely understudied. Chapter five focuses on trends in fungal communities associated with tree canopies in Germany, with particular emphasis to functional groups. Changes within important groups could be a first hint for declining tree health. For this approach, the two sampling species beech (*Fagus sylvatica*) and spruce (*Picea abies*) were analysed. While the beech has its native range within Central Europe, the spruce is commonly located in colder regions, like Scandinavia or Eastern Europe but has been recently cultivated in Central Europe in forestry plantations (San-Miguel-Ayanz *et al.* 2022). Microclimatic conditions in Germany are less favourable for spruce. Therefore, pronounced changes will be expected to be seen in spruce-associated communities compared to those in beech. The study first investigates general biodiversity patterns, including trends in α- and β-diversity. While beech-associated communities showed no significant change in species richness, spruce communities exhibited slightly yet statistically significant increases. A more general trend is the significant compositional change over the time for the associated communities of both tree species. These initial findings already suggest more substantial ecological shifts in spruce than in beech. This assumption is reinforced by the analysis of temporal trends within specific functional groups. For this approach, relevant fungal groups, namely epiphytes, phytopathogens, saprotrophs and mycoparasite, were analysed. While no clear trends emerged in the functional groups associated with beech, spruce communities showed a significant increase in abundance in many of these groups. Phytopathogens are among the most rapidly increasing groups in spruce-associated communities. It is hypothesised that the climatic conditions cause stresses, which facilitate pathogen infestation in spruce and thus contribute to a decline in tree health, a self-reinforcing mechanism. This study has not yet been completed and will include further analysis in changes in interaction networks. The manuscript of this chapter will be submitted to the journal *Forest Ecosystems*.

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Author’s note

The following chapters differ in formatting and citation style, reflecting the guidelines of the journal in which the papers were published in or submitted to. Published chapters and supplements are presented in the printed version available in the respective journal.

Chapter 1

Metabarcoding for Biodiversity Estimation

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Highlights:

- Metabarcoding as valuable tool for eDNA analyses
- Generation of informative datasets using high-throughput sequencing
- Identification of biases related to laboratory work and data processing of metabarcoding methods
- Benefits of integrating metabarcoding with complementary monitoring approaches
- Potential application methods of metabarcoding to assess biodiversity indices

Metabarcoding for Biodiversity Estimation

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Abstract

Our planet harbors a biodiversity of millions of species, of which only a small proportion is yet known to science. For yet a smaller proportion do we know the ecological needs, relatedness and interactions and how the species assemble to form biological communities. This knowledge, however, is critical to ultimately understand how species respond to human alterations of their environment. In recent years, metabarcoding has revolutionized our ability to characterize biodiversity. By combining the method of DNA barcoding with high-throughput sequencing, the taxonomic diversity of entire communities can be rapidly and exhaustively recovered by metabarcoding. Besides simple taxonomic characterization, metabarcoding also enables the community-wide detection of interactions and relatedness between taxa, and even genetic variation within species. The resulting wealth of data enables unprecedented insights into biodiversity assembly and closes critical gaps in our knowledge on biological communities. This new knowledge will significantly facilitate the critical task of protecting biodiversity in the Anthropocene. In this chapter, we will (1) provide a methodological overview of the approach of DNA metabarcoding, (2) highlight pitfalls of the approach and how to overcome them and (3) show applications of metabarcoding for characterizing different levels of community diversity, from community composition and assembly processes to interactions and relatedness.

doi:10.1016/B978-0-12-822562-2.00380-7

Glossary

ASV Amplicon sequence variant, a cluster of identical DNA barcode sequences. In contrast to OTUs, no clustering threshold is used. Instead, all unique sequences in a community are retained. ASVs thus allow for the assessment of intraspecific genetic diversity.

Amplicon The product of a PCR, primarily consisting of identical copies of a massively enriched DNA fragment.

Barcode marker A DNA fragment which is variable enough to distinguish species and analyzed in a metabarcoding experiment to taxonomically classify the studied community.

Blocking primer A PCR primer that is specific to a certain species and blocks the amplification of that species in a PCR with degenerate primers. The blocking primer either competes with the degenerate primers for binding sites or prevents polymerase extension of a PCR fragment in its target lineage. This way, a rare DNA can be enriched from a complex DNA mixture, for example in dietary metabarcoding.

Chimeric sequences Chimeric sequences can arise when incomplete PCR products of different species serve as PCR primers to each other in a metabarcoding PCR. The result is a chimeric sequence which consist of parts of both species and inflates the recovered taxonomic diversity.

eDNA Environmental DNA (eDNA) comprises DNA traces that are left by an organism in its environment. Enriching these traces makes it possible to characterize taxonomic diversity, without the need for catching or observing the target organisms.

High-throughout sequencing Also referred to as second-generation sequencing or next-generation sequencing. Distinguished from the previously used Sanger sequencing by its lower cost and higher sequencing throughput and by the possibility to sequence mixed samples of different DNA fragments.

Indexes Unique identifier sequences added to samples in barcoding/metabarcoding experiments so that individual samples can be distinguished from one another.

Lineage-specific primer A primer that prevents the amplification of a certain species or higher taxon via SNPs that are specific to that lineage. These SNPs will mismatch with that taxon and prevent efficient polymerase amplification during PCR.

Malaise trap A passive trapping device used to catch flying insects. The trap is based on a barrier that intercepts the insects in flight and then guides them into a collection vial.

Metabarcoding Analysis of DNA barcodes from a bulk community sample via high-throughput sequencing with the aim to characterize the species composition of the community. The DNA barcode sequences are selectively enriched from a larger community DNA mixture by PCR amplification.

Metagenomics The analysis of the entire genomic DNA of a community sample by high-throughput sequencing. The sequenced DNA can be used to taxonomically and functionally characterize the community based on its gene content. In contrast to metabarcoding, PCR is avoided in metagenomics. Metagenomics thus shows no amplification bias and can be used for more accurate quantitative assessments of biodiversity than metabarcoding.

NUMTs Nuclear mitochondrial DNA. NUMTs are mitochondrial DNA sequences that have been integrated into the nuclear genome. NUMTs are usually under no evolutionary constraint and randomly accumulate mutations. They are readily amplified with degenerate primers and can significantly inflate the recovered taxonomic diversity in a metabarcoding sample.

OTU Operational taxonomic unit, a cluster of similar DNA barcode sequences, which are merged to reduce the complexity of a metabarcoding sample. A clustering threshold is usually defined (often 3% distance). All sequences falling under this threshold are merged into a single OTU sequence. OTUs should approximate actual species in the community sample.

PCR Polymerase chain reaction, or PCR, is the selective enrichment of a short region of DNA from a larger DNA mixture by amplification with a polymerase and specific DNA primer sequences. PCR successively doubles the target sequence in repeating cycles, leading to an exponential amplification of the target DNA.

PCR amplification bias The preferential amplification of the DNA of a certain taxon during PCR. Due to the exponential nature of PCR, amplification bias can lead to strongly skewed assessments of the true abundance of a taxon in a community sample.

Primer A short DNA fragment (usually ~20 bases) that matches to a DNA stretch to be amplified by PCR. The primer sequences form a double strand with the target DNA, which serves as initiation or priming sites for the DNA polymerase in PCR.

rDNA Ribosomal DNA. The RNA product of the rDNA genes is an essential component of the ribosome. rDNA is a popular marker for DNA metabarcoding of prokaryotes and eukaryotes.

Reverse transcription The process of reverse transcribing RNA into DNA. The reaction is based on reverse transcriptase, an enzyme retroviruses use to integrate their RNA genome into their host's DNA.

SNP Single nucleotide polymorphism, a variation of the four DNA bases Adenine, Thymine, Cytosine and Guanine in a specific position of a DNA sequence between different species or individuals of the same species. SNPs are used to distinguish species in DNA barcoding.

Third-generation sequencing The latest technological developments in high-throughput sequencing technology, distinguished from second-generation sequencing by the considerably longer length of analyzed sequences.

Key Points

The chapter will:

- Provide a methodological overview of the approach of DNA metabarcoding.
- Highlight pitfalls of the approach and how to overcome them.
- Show applications of metabarcoding for characterizing different levels of community diversity, from community composition to interactions to relatedness.
- Highlight future novel developments of the technology.

Introduction

The 21st century is a time of unprecedented global change, dramatically reshaping the taxonomic composition and function of ecosystems worldwide (Barnosky *et al.*, 2012; Thomas *et al.*, 2004). The monitoring of biological communities to understand these changes poses a major challenge for several reasons (Hortal *et al.*, 2015; Emerson *et al.*, 2022). First and foremost, the sheer diversity of biological communities and ever-decreasing taxonomic expertise make the identification of entire community samples of diverse organisms very difficult. Moreover, to accurately predict anthropogenic impacts on global ecosystems, information far beyond taxonomic diversity is required. Current shortfalls in knowledge of biodiversity include the distribution and abundance of species, abiotic parameters of species' niches, genetic variation within species, and biotic interactions and phylogenetic relationships within communities as well as the processes underlying community assembly. Considering the rapid pace of global environmental change, fast and efficient ways to overcome these shortfalls are urgently needed.

A possible solution to this problem is found in the methodology of DNA metabarcoding (Taberlet *et al.*, 2012; Ji *et al.*, 2013), an extension of the discipline of DNA barcoding (see Infobox DNA barcoding). Similar to the latter, metabarcoding uses diagnostic DNA sequences, so-called DNA barcodes, to taxonomically identify species. The key difference is that, while DNA barcoding is limited to single specimens, metabarcoding enables the characterization of entire community samples consisting of multiple taxa, by analyzing mixtures of DNA barcode sequences. Metabarcoding massively speeds up the taxonomic identification of biological samples from large biomonitoring projects. This enables the recovery of patterns of biodiversity at an unprecedented scale and resolution. The method is now routinely used to characterize diversity in communities across the tree of life, from microbes to multicellular animals and plants (Yu *et al.*, 2012; Tedersoo *et al.*, 2022; Johnson *et al.*, 2019). By circumventing the need for morphological identification, metabarcoding is also a powerful approach to discover cryptic species, i.e., taxa that are not distinguishable by their morphology (Hebert *et al.*, 2004). Recent developments in environmental DNA metabarcoding now even allow the characterization of communities from DNA traces left in their environment, so-called environmental DNA (Deiner *et al.*, 2017). This essentially obviates the need to collect specimens for large-scale biodiversity monitoring efforts. Besides its value for basic research, metabarcoding has also developed into an essential tool for applied biodiversity monitoring, for example of agricultural pests or invasive species (Piper *et al.*, 2019; Westfall *et al.*, 2020).

Besides providing a rapid means for taxonomic identification of community samples, developments in metabarcoding promise to overcome further shortfalls in biodiversity analysis. For example, DNA barcodes can serve as phylogenetic markers, recovering community-level metrics of relatedness (Hao *et al.*, 2020). This application is particularly promising with the use of long-read, third-generation sequencing technology, potentially enabling metabarcoding with high phylogenetic resolution (Benítez-Páez *et al.*, 2016; Curry *et al.*, 2022; Krehenwinkel *et al.*, 2019b). Further, intraspecific variation in DNA metabarcodes can recover community-level data on genetic diversity (Elbrecht *et al.*, 2018). And last, metabarcoding can be used to understand interactions and food web structure within a community, for example host-microbe or prey-predator relationships (Kennedy *et al.*, 2020; Alberdi *et al.*, 2019). Recent developments in statistical modeling now also promise to leverage metabarcoding analysis to provide a mechanistic understanding of community assembly processes (Overcast *et al.*, 2023). Hence, DNA metabarcoding has the potential to bridge critical gaps in biodiversity science, bringing within our grasp the enormous task of quantifying and countering the human imprint on global ecosystems.

DNA Barcoding – The Predecessor of Metabarcoding

Our planet is home to a vast biodiversity of millions of species, of which only a small proportion has yet been discovered and scientifically described (Stork, 2018). At the same time, global ecosystems are exposed to unprecedented environmental change, causing mass extinctions across the tree of life (Barnosky *et al.*, 2012). The rate of species loss is currently outpacing the discovery and description of species (Costello *et al.*, 2013). At the same time, morphotaxonomic expertise is steadily declining for many groups of organisms (Engel *et al.*, 2021). Inventorying and describing global biodiversity across the tree of life is therefore a major challenge.

In response to the urgent need to discover and characterize biodiversity, the method of DNA barcoding was developed (Hebert *et al.*, 2003; Tautz *et al.*, 2003). Different species are traditionally distinguished by phenotypic differences, especially morphological characters. But evolutionary divergence between species also translates into genetic differences. These differences can be characterized by DNA sequencing, and then used to distinguish evolutionary lineages. DNA barcoding uses short diagnostic DNA sequences as markers, so-called DNA barcodes, which unambiguously identify taxonomic lineages (Hajibabaei *et al.*, 2007). DNA is isolated from the specimen of interest, then the barcode sequences are amplified via polymerase chain reaction (PCR), sequenced, and compared to available reference databases to assign them to a species or identify them as a possibly novel evolutionary lineage (Hebert *et al.*, 2003). In the latter case, the specimen can then be morphologically analyzed and formally described. DNA barcoding is thus not a replacement for morphotaxonomy, but a complement that can speed up species discovery.

DNA barcoding is methodologically simple, does not require any taxonomic expertise and hence significantly speeds up the discovery and characterization of global biodiversity. However, traditional DNA barcoding reaches its limits in large biodiversity monitoring projects, which aim to characterize the taxonomic composition of communities of thousands of specimens across multiple sites. With every single specimen requiring a separate DNA isolation, PCR amplification and sequencing reaction, barcoding of entire community samples can be prohibitively expensive and laborious. As an advancement of DNA barcoding, community metabarcoding promises to close this gap (Ji *et al.*, 2013).

DNA Metabarcoding - How Does it Work?

A typical metabarcoding workflow entails (1) the collection of a biological community sample, (2) DNA isolation from the sample of interest, (3) the amplification of a specific barcode sequence from the DNA extract via PCR, (4) sequencing of the barcode amplicons and (5) the comparison of the resulting sequences to databases for taxonomic identification. In the following, we will give an overview of these different work steps (see **Fig. 1**).

Before the process of metabarcoding can begin, a community sample first has to be collected. This is for example achieved by sampling soil for microbial communities or using passive trapping devices to collect insects. An important consideration here is to assure the integrity of the sample and its nucleic acids. Good preservation of DNA can be achieved in collection media like specific buffers, ethanol or propylene glycol or by freezing the sample right after collection (Robinson *et al.*, 2021; Liu *et al.*, 2020; Taberlet *et al.*, 2018). After collection of the community sample, DNA has to be isolated by either dissolving tissue or cells in a buffer, which breaks open cell membranes. After removing cellular debris, a pure DNA solution is isolated for downstream analysis. To support efficient lysis of tissues and cells, a mechanical homogenization step, in which tissues or cells are broken up, is often included (Hestetun *et al.*, 2021; Yeates *et al.*, 1998). In many cases, this homogenization means destroying the specimens' morphological integrity, which can be important for future taxonomic assessments. Thus, non-destructive protocols have been developed to efficiently isolate DNA while keeping specimens intact (Kirse *et al.*, 2023).

The resulting DNA extract is a mixture of the entire genomic DNA of all specimens in the original community sample. For metabarcoding, only a short marker of a few 100 bases has to be isolated from the vast genomic diversity (Blaalid *et al.*, 2013; Leray *et al.*, 2013; Walters *et al.*, 2016). The enrichment of the DNA of the barcode marker in relation to the background DNA is done by PCR amplification. The target region is amplified using a thermostable DNA polymerase, which retains its function at the high melting temperature required during PCR. In every PCR cycle, the amount of barcode marker DNA is doubled; after the typical 30–35 cycles, an enrichment of 10^9 to 3×10^{10} is achieved. An important consideration is the choice of PCR primers, as biological community samples often contain highly diverse mixtures of evolutionarily divergent taxa. A bacterial biofilm or leaf litter animal community will comprise members of various phyla that have been evolutionarily isolated for hundreds of millions of years. Suitable metabarcoding primers thus need to efficiently amplify such a diverse range of taxa (see Infobox Metabarcoding Marker Genes), i.e., be universally applicable, for example across all bacteria or all animals (Leray *et al.*, 2013; Walters *et al.*, 2016). Such primers are often referred to as universal primers.

After PCR, a metabarcoding DNA sample will be dominated by amplicons of the barcode sequence. These amplicons are then sequenced on a high-throughput sequencer. Before this can happen, specific adapter sequences need to be attached to both ends of the DNA barcodes (Bohmann *et al.*, 2022). These adapters serve two purposes: First, they contain unique identifier sequences (so-called indexes) on each side of the barcode amplicons of each sample. This way, hundreds of separate community metabarcoding samples can be pooled into one tube and later separated by index combination after sequencing. Second, the adapters contain specific sequences needed by the sequencing platform to carry out the sequencing reaction. The prepared sample with adapters attached to both sides is referred to as a metabarcode library. Adapters can be incorporated into a metabarcode sample in different ways. One approach is the ligation of the adapter to the sample after PCR. Alternatively, very long primers can be used during PCR, which already contain the necessary adapter sequences at their 5′-end. Yet another possibility is the addition of adapter sequences through a second PCR after the initial amplification of the DNA barcode.

The finished metabarcode library is sequenced on a high-throughput sequencer and then separated into individual community samples based on their unique index combination. As in a natural community, barcode sequences in a metabarcoding sample have varying levels of abundance. Abundant barcodes often dominate the sample and make up a considerable proportion of the sequences. For downstream analysis, the complexity of the sample is reduced by clustering individual barcodes into groups of similarity. Different software tools for clustering the sequenced libraries are available, which either rely on simple sequence similarity cut-offs (Callahan *et al.*, 2016; Edgar, 2010, 2016a; Rognes *et al.*, 2016) or on phylogenetic distance between sequences (Zhang *et al.*, 2013). After clustering, each species in the dataset should ideally be represented by one true biological sequence. There are different options for the clustering process. A commonly applied clustering threshold is 97%, meaning all sequences with a dissimilarity of less than 3% are grouped into one cluster. The 3% divergence is assumed to approximate actual species diversity

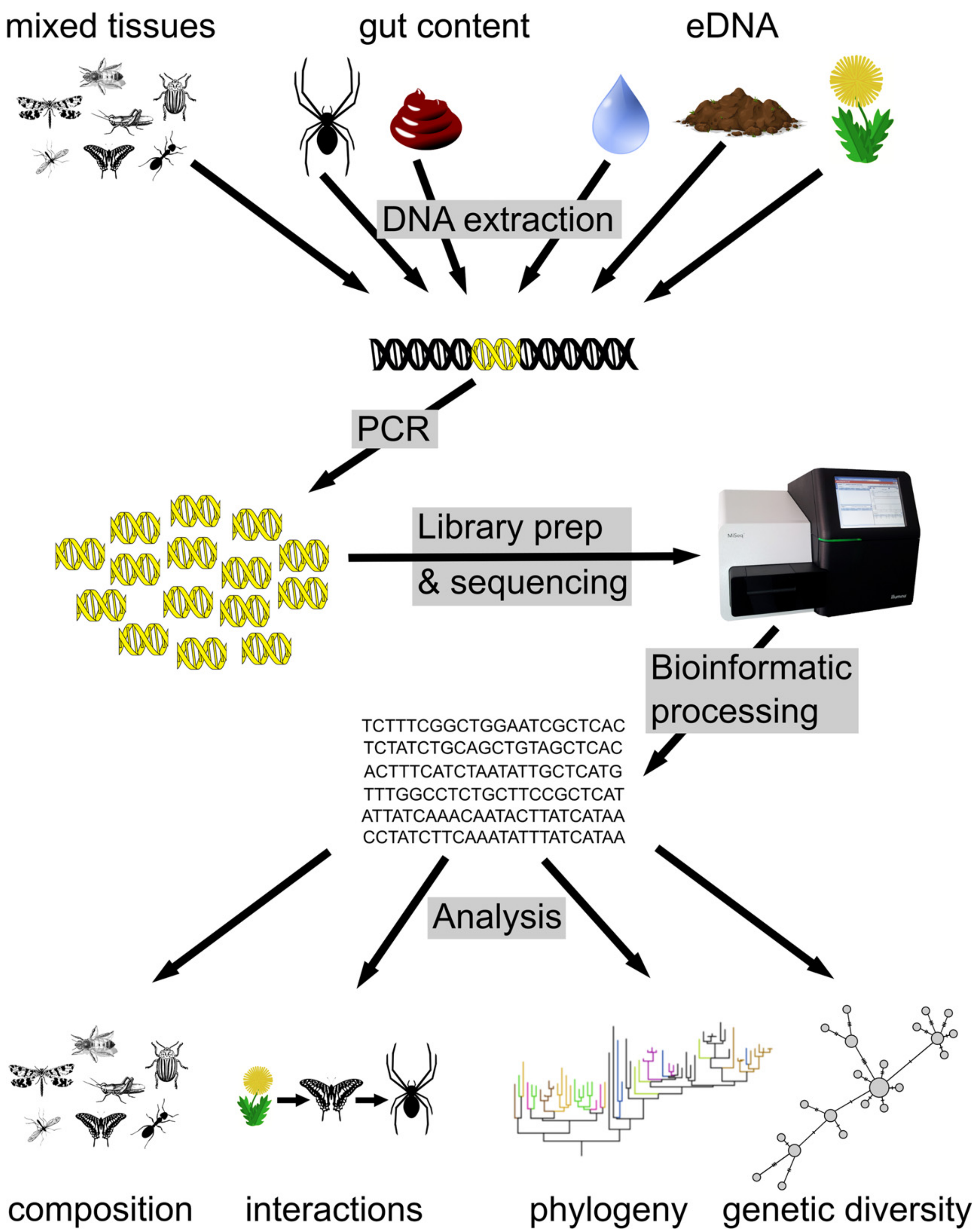

Fig. 1 Overview of a common DNA metabarcoding workflow from sample collection to DNA isolation, PCR amplification, sequencing, data analysis and biological interpretation. The figure shows different types of samples that can be used for metabarcoding, from bulk communities to single specimen samples for gut content analysis to environmental DNA matrices. The method allows various biological analyses. Although the most widespread use is the reconstruction of the taxonomic diversity and species composition of a community, metabarcoding also holds great promise to enable reconstructions of biotic interactions, phylogenetic relatedness and genetic diversity within communities.

in the sample (Edgar, 2010). The resulting sequences are used for the analysis of community diversity and referred to as operational taxonomic units, or OTUs. However, a 3% radius of OTUs may lead to the loss of biological variation. In particular, recently diverged species or ecologically different strains of microbes may only be distinguished by a lower sequence dissimilarity (Mysara *et al.*, 2017). To recover all biological diversity, clustering can also be performed in such a way that every unique sequence variant in the community sample is retained. In this case, a single nucleotide difference between barcodes warrants a new cluster. The resulting sequences are referred to as amplicon sequence variants or ASVs (Callahan *et al.*, 2016). ASVs not only provide a more complete image of the taxonomic diversity of a community, but also capture genetic variation within species.

After OTU clustering, the next very important step is the taxonomic annotation of sequence clusters. This is commonly achieved by comparing them to reference databases of identified species. Different software solutions allow this annotation (Wang *et al.*, 2007)Altschul *et al.*, 1990; Huson *et al.*, 2007; Edgar, 2016b). The more complete and well annotated the reference database, the more accurate the taxonomic annotation will be (Edgar, 2018). Different databases have been developed for different marker types and taxonomic groups and are publicly accessible for annotation. Examples include the GenBank database as a diverse collection of sequences from taxa across the tree of life (Benson *et al.*, 2012), the BOLD database for mitochondrial COI (Ratnasingham and Hebert, 2007), the Unite database for fungal ITS (Nilsson *et al.*, 2019) and the SILVA (Quast *et al.*, 2012), Greengenes (DeSantis *et al.*, 2006) and RDP databases (Cole *et al.*, 2014) for ribosomal RNA genes. After assigning taxonomy to the OTUs, the original sequences are mapped on the taxonomically identified OTUs to generate a so-called OTU table. This table contains each individual community sample and the abundance of sequences for each recovered OTU in the metabarcoding dataset. The annotated OTU table can then be used for downstream analysis of biodiversity (Edgar, 2010).

Metabarcoding Marker Genes

In metabarcoding, short diagnostic DNA fragments, so-called DNA barcodes, are amplified from entire biological communities. These communities are composed of taxa that can range in their evolutionary relationships from very old and divergent phyla to young sister species pairs. A suitable DNA metabarcoding marker needs to contain enough genetic variation to distinguish even recently diverged species. At the same time, the fragment needs to be flanked by sequences sufficiently conserved to allow the design of primers that amplify diverse taxonomic groups (universal primers). Moreover, due to inherent limitations in high-throughput sequencing technology, the length of the amplified fragment cannot exceed 500 base pairs (bp) (Fadrosh *et al.*, 2014). Commonly used metabarcoding markers are usually based on gene regions already established as DNA barcode markers for the targeted group of organisms. For these markers, comprehensive barcode reference libraries are already available, significantly improving taxonomic assignment.

Particularly popular markers for metabarcoding of prokaryotes and various groups of eukaryotes are genes that encode ribosomal RNA (rDNA genes). rRNA is folded in a secondary structure of double-stranded stems and single-stranded loops in the ribosome. In the rDNA gene sequence, evolutionarily highly conserved stems are interspersed with highly variable loop sequences. While the stem conservation is so extensive that these sequences can be identical across all eukaryote or prokaryote taxa, the variable loop sequences distinguish closely related species. This means that universal primers for all prokaryotes or eukaryotes can be designed in rDNA stems, while amplifying variable loops. For prokaryotes, the 16S rDNA gene is the most commonly used marker for metabarcoding (Janda and Abbott, 2007). In eukaryotes, popular metabarcoding markers are spread across the entire nuclear ribosomal gene cluster (18S, 5.8S and 28S rDNA), especially in the 18S and 28S rDNA genes (Latz *et al.*, 2022; Krehenwinkel *et al.*, 2018; Machida and Knowlton, 2012). The three rDNA genes of eukaryotes are present as arrays of multifold copies in the nuclear genome, with each cluster spanning several thousand bp. The entire gene cluster is transcribed and then spliced into the separate genes for the ribosomal assembly. Within these long clusters are two gene spacers, which are also transcribed, and are hence called internal transcribed spacers (ITS) 1 & 2. The spacers are highly variable and also widespread metabarcoding markers, for example for fungi (Tedersoo *et al.*, 2022; op de Beeck *et al.*, 2014). Another advantage of rDNA genes is their high copy number in the genome, which makes them more accessible for PCR amplification compared to single-copy markers, especially in samples that have undergone DNA degradation.

Other popular multi-copy markers for metabarcoding are found in the genomes of plastids, e.g., the chloroplast and the mitochondrion, both of which are present in hundreds or thousands of copies per cell. The relatively faster rate of evolution of plastids in relation to the nuclear genome provides good taxonomic resolution even for young species pairs. The relatively conserved nuclear rDNA genes, in contrast, have been shown to underestimate eukaryote species diversity in some cases (Tang *et al.*, 2012). Common plant metabarcoding markers are found in the chloroplast rbcL and matK genes (Hollingsworth *et al.*, 2009). The most widespread metabarcoding marker for animals is the mitochondrial cytochrome oxidase subunit I (COI) gene (Hebert *et al.*, 2003).

Pitfalls of Metabarcoding and How to Avoid Them for Accurate Recovery of Community Diversity

The metabarcoding workflow outlined above comprises steps from sample collection and DNA isolation to amplification and library preparation, sequencing and computational analysis (**Fig. 1**). A properly tested metabarcoding protocol can be highly reliable and accurately recover taxonomic diversity. Each step of the protocol, however, has the potential to introduce biases into the analysis (van der Loos and Nijland, 2021; Alberdi *et al.*, 2018). In the following section, we will highlight potential sources of error that can inflate or decrease recovered diversity, and then we will describe measures that can be taken to avoid these errors.

Controlling DNA Extraction Bias

A bias in the taxonomic composition of recovered species can already arise at the DNA isolation step. Different species in the community often release DNA with different efficiency, leading to dropout of taxa from the analysis. For example, bacteria with thick cell walls may barely release any DNA in comparison to species surrounded by only a lipid membrane. The same may hold true for heavily sclerotized vs. soft-bodied insects (Marquina *et al.*, 2019). A solution to this problem is to thoroughly homogenize the sample mechanically, which breaks open cells and tissue (Hestetun *et al.*, 2021; Yeates *et al.*, 1998). Another common bias in DNA extraction is the varying biomass of specimens in a community sample. A malaise trap, for example, contains insect species differing by several orders of magnitude in biomass and hence DNA content. The DNA of small specimens is thus highly underrepresented in relation to that of large ones. Very small taxa may then drop out in the PCR amplification step. Size-sorting the sample before DNA isolation is a promising avenue to mitigate this problem (Elbrecht *et al.*, 2021). Species recovery may also be more efficient when DNA is isolated from a single leg only (Braukmann *et al.*, 2019). However, single-leg treatment and every sorted size category will increase the processing effort and cost.

Avoiding PCR Inhibitors in DNA Extractions

Another important consideration for DNA isolation is the purity of the extracted nucleic acid. Many species release secondary metabolites during DNA extraction, which can remain in the extract. The metabolites may interfere with the polymerase molecule and prevent efficient amplification during PCR (Wang *et al.*, 2017; Schrader *et al.*, 2012). Examples include phenolic compounds from plant material or defense chemicals from arthropod community samples. Additional purification or dilution of the DNA extract can help remedy this problem.

Use of Controls in the Workflow

Perhaps the most important consideration to assure accurate metabarcoding results is the use of proper controls throughout the experimental workflow (Ficetola *et al.*, 2016; Sepulveda *et al.*, 2020; van der Loos and Nijland, 2021). The use of PCR makes it possible to enrich even trace amounts of a barcode sequence from a sample, but this also means that trace amounts of contaminants can bias the recovered community diversity. Contamination can affect the results at all steps of a protocol, from sample collection to DNA extraction and PCR amplification. For example, the collection bottle of a malaise trap will contain DNA traces of the arthropod community it contained. If the bottle is not properly sterilized, these traces will cross-contaminate the next sampled community. Many microbes occur ubiquitously in the environment, and so does their DNA. Recent work shows that even common lab reagents can contain traces of microbial DNA, which will inflate diversity in metabarcoding (de Goffau *et al.*, 2018). Reagent contamination can also happen due to negligence, for example when a pipet tip is not changed between samples. To control for all these sources of contamination, negative controls must be included in the metabarcoding workflow. A minimum standard is the use of non-template PCR controls, which are processed and sequenced alongside all other samples. But ideally, blank DNA extractions and field control samples should also be sequenced in metabarcoding experiments (Furlan *et al.*, 2020). Sequences recovered from the negative controls should then be treated as possible contaminants.

Proper PCR Primer Choice to Maximize Recovered Diversity

Another very important consideration for a successful metabarcoding protocol is the choice of PCR primers. Community metabarcoding targets highly diverse mixtures of DNA. Primer binding is of critical importance to recover the entire diversity (**Fig. 2**). Besides other factors, mismatches between primer and template DNA explained about 75% of the variation in amplification efficiency in a COI library (Piñol *et al.*, 2015). Even a single mismatch close to the 3′ end of the primer can cause a complete failure of PCR for a taxonomic group, which will then be excluded from the community data (Krehenwinkel *et al.*, 2019a). A well-suited metabarcoding primer pair should thus match sequences of a very broad taxonomic community. This can be achieved by either choosing evolutionarily extremely conserved gene regions as primer-binding sites (for example in rDNA, see Infobox Metabarcoding Marker Genes), or using degenerate primers, a mixture of primers that represent sequence diversity (Krehenwinkel *et al.*, 2017b; Elbrecht *et al.*, 2019; Elbrecht and Leese, 2017). There is often a trade-off between efficient amplification of a taxonomically broad sample and species differentiation. Very conserved barcode markers allow the design of universal primers, but can lack taxonomic resolution. This has been shown in some nuclear rDNA genes, which are commonly used in metabarcoding (Tang *et al.*, 2012).

Co-Amplification of Undesired Taxa by Universal Primers

Sometimes, the advantage of degenerate or conserved primers amplifying an evolutionarily broad range of taxa can turn into a disadvantage. Frequently, a highly degenerate primer pair will not only amplify the target lineage, but also other taxa. For example, degenerate mitochondrial COI primers for arthropods will frequently also amplify the COI gene of bacteria or fungi associated with the animals. In some cases, the undesired amplification can make up a significant proportion of the sequenced barcodes, swamping the recovered diversity (Collins *et al.*, 2019). The problem can be so prevalent that metabarcoding primers must sometimes be chosen as a trade-off between maximizing the amplification of target taxa and avoiding undesired co-amplification.

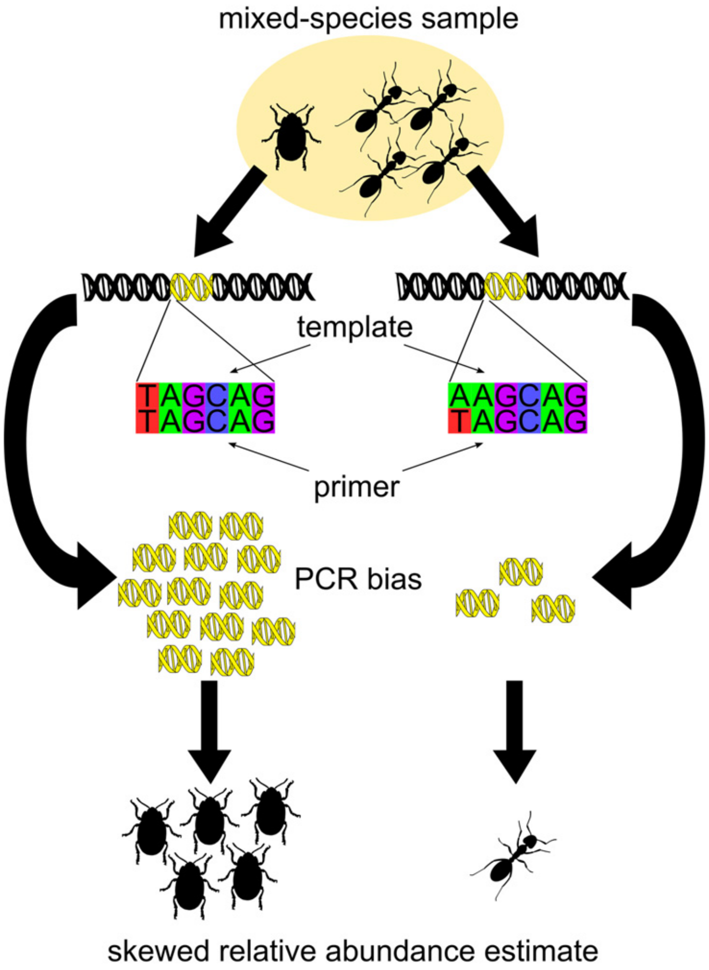

Fig. 2 PCR amplification bias is one of the most common problems in DNA metabarcoding, which can skew qualitative and quantitative assessments of biodiversity. Due to the exponential amplification of barcode sequences in PCR, the sequence abundances between taxa in a community can be greatly skewed in relation to their initial abundance in a bulk community sample.

PCR Replication to Overcome Amplification Stochasticity

Even with perfectly matching primers and an optimized PCR protocol, a very rare taxon may be missed due to PCR stochasticity. The DNA of a very rare species may not be amplified in the initial cycles of a PCR. Due to the exponential nature of PCR, this can cause the rare sequence to become almost absent in the final PCR product and consequently lead to a dropout of the rare species from the analysis. The number of recovered rare species, and hence the completeness of the community, can be increased by replicating PCRs for each metabarcoded sample. With each independent PCR, rare species have a chance to be picked up in early cycles. For samples with many rare species, multiple replicates may thus need to be run (Ficetola *et al.*, 2015; Alberdi *et al.*, 2018; Shirazi *et al.*, 2021).

Adjusting the Sequencing Depth

Besides PCR replication, another important consideration for the exhaustive recovery of taxonomic diversity from a metabarcoding sample is the sequencing depth, i.e., the number of DNA barcode sequences generated per sample. Like a natural community, a metabarcode sample will contain abundant and rare species, with the latter being underrepresented in the sequences. If the sample is not sequenced to a sufficient coverage, rare barcodes may be lost, leading to an underestimation of community diversity (Shirazi *et al.*, 2021). The necessary sequencing coverage to saturate diversity can be estimated in a rarefaction analysis and varies with the complexity of the community.

Accounting for Mito-Nuclear Discordance

Mitochondrial markers are commonly used for metabarcoding animal communities, with the assumption that the resulting OTUs approximate species status. However, the evolutionary trajectory of the mitochondrion often does not mirror speciation events (Papakostas *et al.*, 2016). Factors like male-biased gene flow (Mao *et al.*, 2010) or infections with reproductive parasites like *Wolbachia* bacteria (Jiggins, 2003) can lead to greatly increased mitochondrial divergence in relation to the nuclear genome. On the other hand, introgression of mitochondrial DNA between species can mask actual species differentiation at the nuclear level (Wilson and Bernatchez, 1998). Hence, mitochondrial divergence can over- or underestimate the species diversity derived from metabarcoding. This problem can be solved by relying on well-developed barcode reference libraries. Alternatively, a nuclear marker can be run alongside mitochondrial markers for metabarcoding to explore the magnitude of the problem (de Kerdrel *et al.*, 2020).

Controlling for Sequence Error

A metabarcode sample represents the entire barcode diversity of the sequenced community. An important goal for downstream analysis is to remove various sources of sequence error, which can spuriously inflate the recovered biological diversity of the community (Schirmer *et al.*, 2015; Bokulich *et al.*, 2013). Sequence errors can be introduced during PCR due to the polymerase enzyme incorporating incorrect nucleotides. Commercially available polymerases have different error rates. Depending on the enzyme, the rate of incorporated sequence errors can vary from $10/10^6$–$0.5/10^6$ bases (McInerney *et al.*, 2014). Enzymes with very high error rates may be especially unsuitable for metabarcoding. Another source of sequence error is the actual sequencing process, where up to another 0.5% of error is introduced (Stoler and Nekrutenko, 2021). As PCR and sequencing errors are incorporated randomly in the barcode sequence, they will appear as very rare in the final metabarcode dataset. By removing very rare sequences (for example singletons) and sequences of low quality (with likely high error) during data analysis, the rate of error can be significantly reduced. Various software tools are available for cleaning and clustering the sequenced libraries (Callahan *et al.*, 2016; Edgar, 2016a).

Removal of Chimeric Sequences

Yet another source of error, which is incorporated during PCR, are chimeric sequences. These chimerae emerge when incomplete PCR products of different species act as PCR primers for each other (Haas *et al.*, 2011). The resulting sequence is a chimera of two different species. In diverse community samples, the emergence of chimerae can significantly inflate diversity. Commonly used sequence clustering algorithms contain a chimera detection step, which removes a large proportion of chimerae (Edgar, 2010).

Inflation of Biodiversity by NUMTs

Yet another cause of inflated diversity in metabarcoding can be the amplification of NUMTs or other paralogous sequences during PCR (**Fig. 3**). NUMTs are copies of mitochondrial genes that are integrated into the nuclear genome (Hazkani-Covo *et al.*, 2010). Many lineages of eukaryotes carry hundreds of mitochondrial copies scattered throughout their nuclear genome. NUMTs are often under no selective constraint and hence accumulate mutations quickly, yet they will still remain similar to their mitochondrial sequence of origin. Due to their high prevalence, NUMTs can massively inflate sequence diversity in a metabarcoding experiment (Graham *et al.*, 2021). Early metabarcoding studies frequently reported considerably higher diversity in metabarcoding datasets than in datasets based on morphological identification. While the recovery of cryptic species is one possible explanation, another could be the amplification of NUMTs during PCR. Fortunately, the fast evolutionary rate of NUMTs may allow their identification and filtering from the dataset (Graham *et al.*, 2021). For example, NUMTs often show mutations that break the reading frame in protein-coding genes. These characteristics allow at least the reduction of NUMTs to a certain proportion (Schultz and Herbert, 2022). However, if a NUMT is integrated into the nuclear genome close to a gene under strong purifying selection or if it is simply evolutionarily young, it may be extremely similar to the true barcode (Song *et al.*, 2008). For such young or conserved NUMTs, identification is exceedingly difficult. Recent software solutions aim to clean metabarcoding data from such conserved or young NUMTs by using experimentally informed sequence coverage thresholds (Andújar *et al.*, 2021). However, this may lead to the exclusion of rare species along with the NUMTs and hence also bias the recovered community diversity. The most straightforward way to remove likely NUMTs is to work with community samples for which complete reference databases are available. Sequences not matching the expected species in the database can then be flagged as likely NUMTs. Another solution is to use RNA metabarcoding, which excludes NUMTs because NUMTs are not transcribed and hence not present in the RNA population of a community (see Section "Extracting Active Communities and Avoiding NUMTs and Secondary Predation by RNA Metabarcoding" below).

False Positives in Barcode Databases

Metabarcoding experiments depend on the completeness of reference databases. However, complete reference databases are still more often the exception than the rule. To identify undesired taxa in a metabarcoding sample, a proper taxonomic annotation of the recovered sequences is also essential. Yet, a common issue in the taxonomic annotation process is the presence of false positives in the database (Edgar, 2018). For example, a bacterial COI sequence that co-amplifies with an insect specimen may be deposited and incorrectly labeled as an insect DNA barcode in a database. Sequence databases are often not curated. A wrong annotation in the

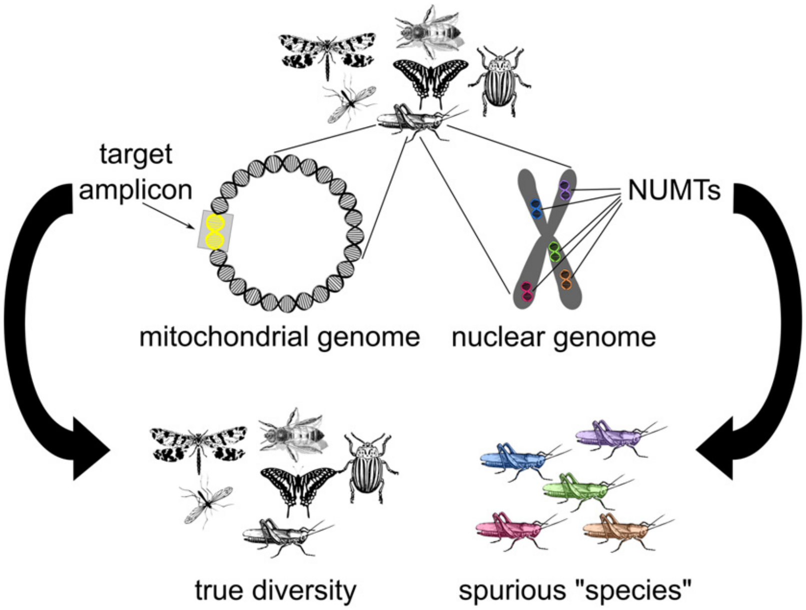

Fig. 3 Metabarcoding with mitochondrial markers can be strongly biased by the presence of NUMTs, copies of mitochondrial DNA in the nuclear genome. NUMTs are co-amplified along with the true mitochondrial barcode sequence during PCR with universal primers. This can significantly inflate the recovered species diversity for groups with a large number of NUMTs in their genome. Filtering metabarcoding datasets for the presence of NUMTs is thus of great importance to recover accurate diversity estimates.

reference may then affect the accuracy of the annotation of the recovered barcode. Curation and high quality of the database are therefore essential. The taxonomic annotation of a metabarcoding dataset should always be double-checked for plausibility. For example, an assignment to a taxon that does not occur in the geographic study region may be flagged as an incorrect annotation. Despite the risk of incorrect taxonomic assignment, recent work has shown that the GenBank database has an overall low rate of erroneously assigned sequences ($<1\%$ at the genus level; Leray *et al.*, 2019).

Applications of DNA Metabarcoding to Characterize our Dwindling Global Biodiversity – From DNA Barcodes to Ecosystems

Bulk Community Metabarcoding – Accelerating Biodiversity Monitoring

The most common application of metabarcoding is to characterize bulk community samples, for example flying insects caught in a malaise trap, marine meiofauna or microbes (Gibson *et al.*, 2014; Leray and Knowlton, 2015; Emerson *et al.*, 2022). DNA is extracted from the bulk community and barcode markers for specific taxonomic groups amplified from the DNA sample. A great advantage over traditional identification is the speed and simplicity with which communities can be characterized, even for hyperdiverse taxa like microbes or insects. The resulting data provide a detailed image of diversity patterns within a community and turnover between communities. For example, metabarcoding can help identify environmental drivers of community diversity and composition and barriers to dispersal (Lim *et al.*, 2022). Biomonitoring with metabarcoding allows detection of invasive species, as well as rare and beneficial taxa, and can also be used to assess conservation status (Westfall *et al.*, 2020). It should be noted that community metabarcoding is primarily limited to qualitative assessments of biodiversity (Lamb *et al.*, 2019). This means the presence of a species can be readily detected, but not its abundance. Quantitative assessments are hampered by various biases inherent to the method (see Section "How Quantitative is DNA Metabarcoding? Recovering Abundances and Biomass From Community Samples" for quantitative capabilities of the method). With proper taxonomic annotation, functional biodiversity can also be recovered from metabarcoding data (Hörren *et al.*, 2022; Douglas *et al.*, 2018). The manifold applications of community metabarcoding make it a highly valuable tool for basic research, but also for legislative monitoring of ecosystem health.

Environmental DNA Metabarcoding – Monitoring Ecosystems Without Collecting Organisms

Every organism leaves traces of its DNA in its environment, so-called environmental DNA or eDNA. For example, eDNA can be deposited in the form of feces, skin particles, gametes or saliva. These eDNA traces can be enriched from environmental samples and analyzed by metabarcoding (Taberlet *et al.*, 2018; Thomsen and Willerslev, 2015). Metabarcoding the eDNA extract enables the reconstruction of entire biological communities, without the need for collecting actual specimens. In comparison to many traditional monitoring approaches, eDNA metabarcoding is minimally invasive and thus provides a revolutionary approach to monitor species. The non-invasiveness and relative simplicity of the method also make it ideally suited for community science projects to study biodiversity (Johnston *et al.*, 2023; Meyer *et al.*, 2021). Water appears to be a particularly well-suited carrier of community eDNA (Deiner *et al.*, 2017; Taberlet *et al.*, 2018; West *et al.*, 2020; Gold *et al.*, 2021). Entire communities of aquatic organisms can be reconstructed using eDNA metabarcoding from a small volume of water. Examples include fish, amphibians, arthropods and plants. However, terrestrial ecosystems can also be sampled for eDNA. DNA of arthropods can be enriched from plant material, which allows reconstruction of plant-pollinator or plant-herbivore interactions (Thomsen and Sigsgaard, 2019; Banerjee *et al.*, 2022) (see more details on plant-arthropod interactions below). Soil samples are a suitable matrix to recover the eDNA of soil communities (Ariza *et al.*, 2023). Recent work suggests that even air is a suitable carrier of eDNA (Lynggaard *et al.*, 2022; Roger *et al.*, 2022). For example, the biota of a zoo could be reconstructed by metabarcoding filtered air. To analyze an eDNA sample, the eDNA first has to be isolated from its matrix, e.g., water, air, soil or leaf material. Aquatic samples are simply filtered through a fine-pored membrane using a pump (Spens *et al.*, 2017). eDNA-containing particles are retained on the filter. A positively charged membrane like nylon retains the negatively charged free DNA molecules particularly well (Bessey *et al.*, 2021). eDNA from terrestrial samples is commonly washed from the substrate by water or buffers and then also filtered (Valentin *et al.*, 2020). Filtration of aerial eDNA occurs in a similar fashion, but without liquid, basically functioning like a vacuum cleaner (Lynggaard *et al.*, 2022).

As rare environmental traces of organisms, eDNA molecules of individuals are usually present in very low abundances. Also, eDNA is not very temporally stable. It is quickly degraded by UV light, acidity in water or microbial activity, and it can be washed off of surfaces by rainfall (Strickler *et al.*, 2015; Valentin *et al.*, 2021). The common half-life of eDNA molecules is only a few hours (Harrison *et al.*, 2019). Its quick degradation additionally increases the rarity of eDNA in an environmental sample. Due to its degraded state, it is advisable to use primers targeting very short barcode fragments for eDNA metabarcoding (Krehenwinkel *et al.*, 2022b). With too-long PCR fragments, the degraded eDNA of many taxa may be missed. The rarity of eDNA makes its analysis extremely sensitive to external contamination. Appropriate controls should be run alongside all experimental steps in eDNA metabarcoding, to make sure the amplified community DNA is not biased by false positives. Due to the massive enrichment of even trace amounts of DNA in PCR, it is very important to separate work steps before and after PCR, ideally to perform them in separate laboratories. Moreover, due to the rarity of eDNA, the replication of PCRs is especially important to recover all taxa in a community sample (Ficetola *et al.*, 2016).

However, eDNA is not always unstable and short-lived. Under optimal storage conditions, it can be quite persistent. Long-term stability of eDNA can be achieved by cold temperatures or dry conditions. For example, eDNA from archived leaf material stored at subzero temperature allows the reconstruction of temporal changes of arthropod communities in forests over several decades (Krehenwinkel et al., 2022a), and tea bags contain dried plant material from which eDNA of arthropods that interacted with the tea plant can be enriched (Krehenwinkel et al., 2022b). eDNA from arctic permafrost soil or lake sediment can even survive for hundreds of years to millennia (Ibrahim *et al.*, 2021; Balint *et al.*, 2018). A study from Greenland shows that in exceptional cases, eDNA can survive more than a million years (Kjær *et al.*, 2022). eDNA analysis is thus a promising tool to understand retrospective patterns of biological community assembly, throughout the Anthropocene and even into prehistoric times. However, long-term preservation is the exception rather than the rule for eDNA molecules.

Metabarcoding to Recover Biotic Interactions – From Microbiomes to Food Webs to Natural eDNA Samplers

Besides providing information on the taxonomic composition of biological communities, metabarcoding can also uncover highly accurate and detailed information on the interactions of different species, making it possible to reconstruct interaction networks of entire biological assemblages (Graham *et al.*, 2022; Hrcek *et al.*, 2011; de Sousa *et al.*, 2019). Such information is essential to fully understand community responses to environmental change. Below, we highlight different applications of metabarcoding to recover biotic interactions.

Metabarcoding for host-microbiome interactions

Metabarcoding is commonly used to characterize biological communities consisting of numerous specimens, for example an insect community from a malaise trap. However, each insect in that trap also makes up a whole community of its own. Animals and plants host diverse microbial communities of bacteria, fungi and protozoans (McFall-Ngai *et al.*, 2013; Laforest-Lapointe and Arrieta, 2018). These organisms can fulfill various roles, from mutualist to commensal to parasite or pathogen. Characterizing the immense global microbial diversity has long proven difficult due to the majority of microbial taxa being unculturable in the laboratory (Epstein, 2013). Metabarcoding provides a rapid and simple means to identify the host-associated microbiome without the need for cultivation (Fadrosh *et al.*, 2014). The gut microbiota of animals can for example be assessed by sequencing fecal samples and the skin microbiome by analyzing swab samples (Ogai *et al.*, 2018; Zierer *et al.*, 2018). Metabarcoding has provided unprecedented insights into the drivers of the assembly of host-associated microbiota and the often critical importance of host-microbiome interactions for the fitness and evolution of host organisms (Vandenkoornhuyse *et al.*, 2015). For example, symbiotic microbiota have been shown to contribute to digestion or detoxication of food (Boone *et al.*, 2013) and can provide their host with essential nutrients (Douglas, 1998) or resistance against pathogens (Kwong and Moran, 2016). Metabarcoding of human microbiota has significantly contributed to our understanding of the role of these microbes in human disease and well-being (Byrd *et al.*, 2018; Shreiner *et al.*, 2015), with many human diseases now known to be associated with changes in microbial community composition (Cho and Blaser, 2012).

Gut content metabarcoding for dietary interactions

Metabarcoding of gut content or feces can not only recover host-associated microbiota, but also reveal trophic interactions. The gut content usually contains DNA traces of the dietary organisms. For example, feces of herbivorous mammals can be used to reconstruct the community composition of their plant diet (Kartzinel *et al.*, 2015). The gut content of predatory animals reveals their prey preferences and level of specialization. Examples of metabarcoding to trace dietary composition can be found across the metazoan tree of life, from small invertebrates to large vertebrates (Alberdi *et al.*, 2020; Ford *et al.*, 2016; Guenay-Greunke *et al.*, 2023; Srivathsan *et al.*, 2015; Krehenwinkel *et al.*, 2017a). For small animals like arthropods, feces are often not readily available to screen for interactions. In such animals, metabarcoding of whole-body extracts or dissected guts can also yield information on dietary interactions (Piñol *et al.*, 2014).

In relation to the DNA of the predator, the DNA residues of prey organisms are very rare in the gut (Cuff *et al.*, 2023). Hence, as in eDNA metabarcoding, the approach is sensitive to contamination and appropriate controls need to be included. It is also advisable to target short DNA barcode fragments of only 100–200 bp, as the DNA of the ingested organism is usually heavily degraded in the gut content of the predator (Krehenwinkel *et al.*, 2017a). Another issue in gut content metabarcoding of predatory taxa is secondary predation. Due to the sensitivity of the method, not only DNA of the prey can be detected, but possibly also that of the diet of the prey (Cuff *et al.*, 2022). However, sequences from secondary predation are usually much rarer than those of the actual prey. Thus, read abundance thresholds may help to counter this issue. Another recently suggested option is the use of RNA for gut content metabarcoding (Neidel *et al.*, 2022). The temporally unstable RNA will degrade very quickly in the gut and only RNA of recently consumed prey items will be present, likely precluding the detection of secondary prey.

Gut content metabarcoding is often performed with universal primers to recover a broad taxonomic spectrum of prey organisms. However, this can lead to problems when analyzing predatory interactions between closely related organisms. For example, spiders primarily prey on insects. A universal primer pair for arthropods will amplify the insect prey and spider predator equally well. The rare, degraded DNA of the prey will then be hugely underrepresented in the metabarcoding dataset. Various solutions to this problem have been proposed. First, the sample could be sequenced to a very high depth and unused predator reads simply discarded (Piñol *et al.*, 2014). Alternatively, blocking oligos that specifically bind to the predator's DNA and prevent its amplification during PCR can be used (Vestheim and Jarman, 2008). This approach has been applied with varying success for different predator and prey taxa. Last, lineage-specific SNPs at the 3′-end of a primer can be used to distinguish prey and predator, while still retaining a broad specificity of the primer (Krehenwinkel *et al.*, 2019a). Yet another option to improve prey DNA yield is to enrich degraded DNA from a DNA extract of a predator (Krehenwinkel *et al.*, 2017a). The DNA of the predator should be mostly intact, while that of the prey has been broken down to small pieces in the digestive tract. Thus, by targeting the degraded DNA, a higher proportion of prey DNA can be recovered.

Monitoring plant-arthropod interactions with eDNA metabarcoding - from herbivores to pollinators

Arthropod-plant interactions are yet another important type of biotic interaction whose study has been greatly simplified by DNA metabarcoding. Herbivorous arthropods are major consumers of plants and comprise many of the most important global pest species in forestry and agriculture. At the same time, arthropod pollination is an essential element of plant reproduction and is therefore critical for global food security. A proper understanding of arthropod-plant interactions is thus essential for conservation and economic purposes alike. However, arthropod-plant interactions are often cryptic and difficult to observe. Hence, the complex interaction networks of plant and arthropod communities are often badly understood and poorly characterized (Graham et al., 2022; Weber et al., 2023b).

A traditional method of characterizing interactions between plants and arthropods is direct observation. This is achieved by visually observing pollinator visits to flowers or the presence of, for example, galls or mines on leaves (Bosch *et al.*, 2009). Alternatively, bulk arthropod samples are collected by methods such as branch clipping or beat sampling, and the presence of those arthropods on the plant is taken as evidence for a probable interaction (Graham *et al.*, 2022). The problem with this approach is that the mere presence of an arthropod on a plant does not necessarily indicate a specific biotic interaction. Recent work suggests that many vagrant species, which show no specialization towards the plant from which they were sampled, will be recovered with beat sampling approaches (Weber et al., 2023b).

A powerful new approach to recover plant-associated arthropod communities is offered by eDNA metabarcoding. Insects leave traces of their DNA when interacting with plant material, for example from feces and chew marks on leaves or after visiting flowers (Krehenwinkel *et al.*, 2022b; Harper *et al.*, 2023; Thomsen and Sigsgaard, 2019). This eDNA can be enriched and sequenced. Interestingly, the detectability of arthropod eDNA positively correlates with interaction intensity, with specialist herbivores leaving more eDNA than vagrant taxa (Weber *et al.*, 2023b). This suggests that eDNA may even provide a more accurate approach to detect biotic interactions than specimen collection. eDNA collection from plant material is also fast and efficient. The eDNA is recovered either from surface washes of the plant or by powdering plant material and directly extracting DNA from the powder. One difficulty of this approach, as in the gut content analysis described above, is that arthropod eDNA is extremely rare in relation to DNA of the plant and its associated fungi. Thus, typical degenerate metabarcoding primers may primarily amplify this non-target DNA. To overcome this problem, specific primers can be used that omit the non-target taxa while enriching arthropod DNA (Krehenwinkel *et al.*, 2022b).

Another option, rather than extracting arthropod DNA traces from plant material, is to extract plant DNA traces from arthropod bodies. For example, pollen isolated from the bodies of insects reveals the plant preference of pollinators (Arstingstall *et al.*, 2021; Suchan *et al.*, 2019; Tommasi *et al.*, 2022). Even the analysis of honey can recover pollen profiles of the plant community utilized by a bee colony (Hawkins *et al.*, 2015).

Using dietary metabarcoding to monitor biodiversity

Besides recovering biotic interactions and community-level food webs, the reconstruction of diets by metabarcoding can also serve another important purpose. Metabarcoding the gut content of consumers holds the potential to monitor entire communities of their prey. Generalist consumers in particular will feed on diverse assemblages of prey organisms. Their gut content can thus provide a good approximation of local diversity of their prey taxa. Metabarcoding the blood meals of terrestrial leeches or mosquitos has enabled the monitoring of rare mammal species (Drinkwater *et al.*, 2019; Estrada-Franco *et al.*, 2020). The gut content of detritus-feeding marine shrimps has revealed the diversity of fish communities in their environment (Siegenthaler *et al.*, 2019). Dietary metabarcoding of aquatic filter-feeding organisms like mussels and sponges makes it possible to recover the diversity of aquatic organisms such as eukaryotic phytoplankton or zooplankton (Mariani et al., 2019; Weber et al., 2023a). Moreover, filter feeders will feed on organic particles left by macroorganisms in their environment, for example fish. Sequencing the tissues of these natural eDNA samplers thus holds the potential to monitor entire aquatic communities across the tree of life. Metabarcoding of archived filter-feeding organisms has shown that aquatic community diversity can be analyzed across multiple decades (Junk *et al.*, 2023). Recent work shows that spider webs can also serve as natural eDNA samplers (Gregorič *et al.*, 2022). They not only contain DNA of the spider's insect prey, but also sample various other particles from the air, for example fungal spores, bacteria and plant pollen.

Metabarcoding Beyond Species Diversity – Community-Wide Meta-Phylogeography and Population Genetic Analysis

The most common goal of metabarcoding studies is the recovery of species-level diversity. This is achieved by either clustering the recovered barcode sequences into OTUs or identifying them as actual species by database comparison. However, a typical community sample not only consists of individual species, but also contains numerous individual specimens. Especially abundant species are thus usually present as large populations in a community. Each specimen may harbor unique genetic variation contributing to the population genetic diversity of the species at a given site. By merging different sequences into hypothetical species clusters, this population genetic variation is lost. For this reason, recent work suggests that clustering approaches should be avoided in metabarcoding and every unique biological sequence retained for analysis (Edgar, 2016a). These unique sequences are commonly called amplicon sequence variants (ASVs). ASV-level community analysis makes it possible to sample not only species diversity, but also population genetic variation within individual species in a community. The approach of ASV barcoding opens up an entirely new application of metabarcoding: the community-wide analysis of population genetic diversity and population structure (Shum and Palumbi, 2021). Metabarcoding analysis using ASVs can indicate community-level barriers to gene flow, or losses of genetic diversity within species. ASV analysis also holds the potential to disentangle large-scale phylogeographic patterns across multiple taxa (Turon *et al.*, 2020; Elbrecht *et al.*, 2018). However, it should be kept in mind that only species represented in the community sample in actual population sizes can be used for population-level analysis. Many barcodes in a metabarcoding sample will stem from only a single specimen of a rare species, which will result in highly skewed estimates of population genetic diversity.

New Frontiers in Metabarcoding

Extracting Active Communities and Avoiding NUMTs and Secondary Predation by RNA Metabarcoding

DNA is a relatively stable molecule; under optimal conditions it can survive for extensive time periods. This is impressively shown by the research field of ancient DNA, which has recovered the genetic legacy of ecosystems and humans for thousands of years (Hofreiter *et al.*, 2001). Under ideal conditions, even eDNA can survive for many years. For example, the eDNA of an extinct fish could survive in the sediment of a lake or river. This DNA can be released when the sediment is disturbed and thereby inflate the measured species diversity in a contemporary aquatic eDNA sample. eDNA released by an aquatic organism upstream of a river can also be carried downstream for extended times, contributing to false positives in biodiversity assessments (Roussel *et al.*, 2015). Inflated diversity can also result from sequencing bulk community samples containing predatory animals. The gut content of these specimens will be included in the metabarcoding analysis and prey specimens co-amplified. False positive amplification by DNA metabarcoding is also a significant issue in microbial community characterization. A considerable proportion of free, extracellular eDNA of long-dead bacteria is present in most ecosystems, and can be co-amplified in metabarcoding. Moreover, large numbers of dormant and physiologically inactive cells reside in the environment. DNA metabarcoding thus often recovers a much higher diversity than that of the actual active microbial community (Geisen *et al.*, 2015; Gkarmiri *et al.*, 2017), but in many cases, only this active component is of interest to researchers. Separating the inactive from the active component is not possible with DNA metabarcoding.

A possible solution to avoid such inflation of diversity by historical or remotely transported eDNA, and only recover the active component of a community, is the use of RNA metabarcoding (Geisen *et al.*, 2015). RNA is considerably less stable than DNA and consequently has a much shorter half-life. An RNA molecule will degrade swiftly after it is released in the environment. And only physiologically active organisms will produce RNA of barcode markers in significant amounts. By isolating the RNA from a community or the eRNA from an environmental sample, the active community present at the site of interest can be analyzed (Pochon *et al.*, 2017; Cristescu, 2019). This way, the detection of RNA can also verify the presence of a species already detected by DNA (Marshall *et al.*, 2021). Furthermore, RNA seems to have a higher positive predictivity than DNA and is considered a good tool for identifying and excluding false positives (Miyata *et al.*, 2022).

Another factor well known to inflate biodiversity estimates from metabarcoding are NUMTs, nuclear copies of mitochondrial DNA (see Section "Pitfalls of Metabarcoding and how to Avoid Them for Accurate Recovery of Community Diversity": Inflation of biodiversity by NUMTs, and **Fig. 3**). NUMTs are usually not transcribed and hence are only present in the DNA population of a community. By analyzing a community with RNA metabarcoding, NUMTs can be selectively excluded from metabarcoding datasets (Graham *et al.*, 2021).

RNA metabarcoding is performed in an almost identical fashion to DNA metabarcoding. The only difference is that RNA is isolated from a sample instead of DNA. The RNA is then reverse transcribed into DNA, which is then used for regular metabarcoding. The fast degradation of RNA needs to be considered, and samples must be stored appropriately or processed as soon as possible after collection.

How Quantitative is DNA Metabarcoding? Recovering Abundances and Biomass From Community Samples

Metabarcoding reliably recovers the qualitative taxonomic composition of a biological community. That means that this method reveals which species are present at an analyzed site, but not how many individuals of which species. To fully understand the assembly of biological communities, quantitative information on community diversity is required, i.e., the abundance distribution of individual species. In theory, the read abundance of a taxon in a metabarcoding sample should correlate with its abundance in the actual community. However, generating reliable abundance estimates for species in a community by metabarcoding has been notoriously difficult. This is primarily due to the inherent quantitative bias of PCR. With strong amplification bias, a rare species can become the most common one in a community sample after PCR (Lamb *et al.*, 2019) (**Fig. 2**).

Amplification bias can be reduced to a certain extent by using highly degenerate PCR primers, which amplify a broad range of taxa equally well. But even a degenerate primer will not necessarily amplify all taxa in the community with the same efficiency (Krehenwinkel et al., 2017b; Fonseca, 2018). Factors like length differences in the amplified fragment between taxa, with shorter fragments being preferentially amplified, can additionally skew recovered abundances (Krehenwinkel et al., 2019b). The same holds true for variation in GC content of the amplified sequence, with some DNA polymerases preferentially amplifying fragments with lower GC content (Nichols *et al.*, 2018). The exponential nature of PCR will magnify even small biases, leading to the dropout of taxa from the analysis.

A common suggestion to overcome the limitations of PCR-based metabarcoding and enable quantitative assessments of community composition is the use of PCR-free metagenomics (Nayfach and Pollard, 2016). By directly sequencing genomic DNA of the target community and avoiding a PCR step, amplification bias is entirely excluded. While this approach enables more accurate quantitative assessments of community composition, it is limited by its considerably increased cost in relation to PCR-based metabarcoding. PCR enriches the targeted barcode marker several billion-fold in relation to the background DNA. In a PCR-free metagenomic sample, the barcode DNA will only comprise a very small proportion of the total sequences. Metagenomic samples thus have to be sequenced to a considerably higher coverage than metabarcoding ones.

Moreover, while metagenomic sequencing circumvents the issue of PCR amplification bias, other factors independent of PCR can lead to skewed abundance estimates (Krehenwinkel et al., 2017b). The copy number per cell of a barcode marker can vary between taxa by several orders of magnitude. Different organisms may also release DNA with different efficiency during DNA extraction due to their morphological traits. And differently sized taxa in a community may contribute vastly different amounts of DNA to the community extract. Hence, even without amplification bias, a taxon can be over- or underrepresented in relation to its true biomass in the community.

But even though these various factors are known to skew abundance estimates from metabarcoding, recent work has also shown that the inherent biases of the method can at least be limited by optimizing the protocol (Krehenwinkel et al., 2017b; Piñol et al., 2019). As mentioned above, degenerate primers can significantly reduce amplification bias. Copy number variation can be controlled for in microbial datasets by using available reference databases (Kembel *et al.*, 2012). And optimized DNA extraction protocols maximize the amount of recovered DNA irrespective of a taxon's morphology. Size-sorting of a community before DNA isolation can also even out different biomass contributions of taxa (Lim *et al.*, 2022). The number of reads per taxon often positively correlates with its biomass (Elbrecht and Leese, 2015). In fact, the association of recovered sequence numbers and biomass is often quite strong within a species of interest. This means that the relative abundance of a taxon between sites can be recovered by metabarcoding, while the remaining biases primarily concern abundances between different taxa within a site. A relative quantification of individual species thus works even in complex communities. This can for example be used to monitor the relative abundance of invasive or threatened species across sites. For communities that are not too complex, correction factors can be derived for individual taxa, making it possible to quantify community-level diversity (Krehenwinkel et al., 2017b; Thomas et al., 2016). However, in complex communities, this will be considerably more difficult and may only be worthwhile if the community will be studied for the long term. It should also be noted that the quantitative estimates recovered by metabarcoding and metagenomics are relative and not absolute. Only when the number of individuals or the biomass of a community is known can the relative read abundances within that community be compared between sites or samples. This can be achieved by counting individuals or weighing samples before isolating DNA (Lim *et al.*, 2022; Graham *et al.*, 2022). While the counting can be performed by hand for macroorganisms, flow cytometry has been successfully used for quantitative metabarcoding of microbial communities (Vandeputte *et al.*, 2017).

Another possibility to recover information on abundances from metabarcoding data is to classify taxa into categories of probability of occurrence. If multiple subsamples are collected at a site, rare species will appear in only one or few subsamples, while common ones will be omnipresent. In this way, a semiquantitative assessment of community diversity is possible.

Metabarcoding With Third-generation Sequencing – From Taxonomic Diversity to Community Phylogenies

Metabarcoding is usually performed with high-throughput sequencers with limited read length. In order to generate high-quality barcode sequences, a metabarcode cannot be much longer than 500 bp. Such short fragments, however, contain only a limited number of mutations and consequently a limited taxonomic resolution. Especially young evolutionary lineages may not be properly distinguishable with short barcodes. Moreover, the limited number of bases limits the phylogenetic resolution of short barcodes, i.e., they do not allow reliable reconstruction of the relationships of taxa (Krehenwinkel *et al.*, 2019b).

Recent developments in third-generation sequencing technology now allow the sequencing of fragments of considerably increased length. This enables the recovery of very long DNA barcodes, covering entire genes or gene clusters. Several-kilobase-long barcode fragments can be amplified, enabling analysis of complete microbial 16SrDNA (Benítez-Páez *et al.*, 2016; Matsuo *et al.*, 2021), entire gene clusters of nuclear ribosomal RNA in eukaryotes (18S, ITS1, 5.8S, ITS2 and 28S) (Lu *et al.*, 2022; Krehenwinkel *et al.*, 2019b), or long stretches of mitochondrial genomes (Zascavage *et al.*, 2019). Using such long barcode amplicons in metabarcoding will allow community characterization at considerably improved taxonomic and phylogenetic resolution. This means the taxonomic composition of a community can be resolved to a finer scale, while at the same time, the phylogenetic relatedness of community members can be reliably inferred. This will enable inference of phylogenetic diversity from entire communities, an important parameter of community diversity.

However, third-generation sequencers still suffer from a considerably increased error rate in relation to previous high-throughput sequencers (Loit *et al.*, 2019). This makes a reliable differentiation of true biological sequences from sequencing error even harder. Long-range metabarcoding data thus need to be carefully evaluated. Developments in sequencing technology will hopefully overcome the issue of sequencing error in the near future. However, long-range metabarcoding faces another challenge: PCR tends to become much less predictable for very long templates, possibly increasing taxonomic biases and false negatives in the recovered communities (Krehenwinkel et al., 2019b). Long-range PCR is also dependent on a high integrity of the template DNA. Many community samples have been stored in passive sampling devices for extensive times and already show signs of degradation, precluding the use of long-range metabarcoding.

Despite these drawbacks, long-range metabarcoding is a highly promising future application, holding the potential for unprecedented understanding of the assembly of biological communities in the future.

Metabarcoding Anywhere and Anytime With Miniaturized, Portable Laboratory Equipment

The emergence of high-throughput sequencing technology with its ever-increasing throughput and read length has revolutionized biological research. Yet another technological advance that promises to revolutionize biology is the miniaturization of laboratory equipment, including DNA sequencers. The latest generation of nanopore DNA sequencers comes in the size of a USB stick and allows portable high-throughput sequencing. Not only sequencing technology can be miniaturized: an entire molecular laboratory, including PCR cycler, centrifuge and gel electrophoresis equipment, can be miniaturized and combined into a portable “lab in a backpack” (Pomerantz *et al.*, 2018). Hence, portable DNA barcoding and metabarcoding can be conducted anywhere in the world, without the need for a well-equipped laboratory. This offers exciting new perspectives for the application of metabarcoding for immediate biodiversity characterization, for example when time is of the essence after an environmental disaster. Another highly important step forward for miniaturized portable metabarcoding is the opportunity to establish metabarcoding research in global biodiversity hotspots, where it is most direly needed (Pomerantz *et al.*, 2018). Metabarcoding is an indispensable tool to tackle the important task of characterizing global biodiversity. The majority of biodiversity is found in the tropics, where laboratory and research capacities are often least developed. With simple and inexpensive miniaturized laboratory equipment and sequencers, metabarcoding will become an affordable technology in underfunded countries with high local biodiversity. The simplicity of mobile lab equipment also makes miniaturized metabarcoding a highly attractive option for capacity building, e.g., teaching classes to establish the necessary knowledge and expertise for community metabarcoding in developing countries (Watsa *et al.*, 2020; Salazar *et al.*, 2020). Making the technology available in places where it is most needed and establishing the necessary expertise to use it is of very high priority for the future of biodiversity monitoring.

Conclusions and Outlook

The emergence of high-throughput sequencing technology has revolutionized molecular biology. The methodology is still rapidly developing, and constantly improving in terms of sequence quality, sequence length and sequencing cost. This will significantly speed up the development of the field of metabarcoding. Recent developments promise community characterization away from the simple descriptions of taxonomic diversity for which the method is currently mostly used. Future metabarcoding work will contribute to an unprecedented understanding of biological communities, from quantitative biodiversity assessments to highly resolved phylogenetic diversity measures to community-level analysis of biotic interactions and genetic variation. Hence, metabarcoding will help to overcome major shortfalls in our understanding of biodiversity, providing critical information to mitigate the human imprint on global ecosystems.

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Chapter 2

Environmental DNA from archived leaves reveals widespread temporal turnover and biotic homogenization in forest arthropod communities

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Highlights:

- Application of new developed eDNA metabarcoding assays to obtain arthropod community information from leaf samples of a long-term monitoring archive in Germany
- Successful application of quantitative PCR to obtain information, equivalent to biomass
- Retrospective biodiversity trends of terrestrial arthropod communities over the last three decades
- Stable α-diversity in many study sites
- Wide-spread cryptic biodiversity loss within the last three decades

RESEARCH ARTICLE

Environmental DNA from archived leaves reveals widespread temporal turnover and biotic homogenization in forest arthropod communities

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Abstract A major limitation of current reports on insect declines is the lack of standardized, long-term, and taxonomically broad time series. Here, we demonstrate the utility of environmental DNA from archived leaf material to characterize plant-associated arthropod communities. We base our work on several multi-decadal leaf time series from tree canopies in four land use types, which were sampled as part of a long-term environmental monitoring program across Germany. Using these highly standardized and well-preserved samples, we analyze temporal changes in communities of several thousand arthropod species belonging to 23 orders using metabarcoding and quantitative PCR. Our data do not support widespread declines of α-diversity or genetic variation within sites. Instead, we find a gradual community turnover, which results in temporal and spatial biotic homogenization, across all land use types and all arthropod orders. Our results suggest that insect decline is more complex than mere α-diversity loss, but can be driven by β-diversity decay across space and time.

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Competing interest: The authors declare that no competing interests exist.

Funding: See page 16

Preprinted: 29 April 2022
Received: 10 March 2022
Accepted: 06 November 2022
Published: 10 November 2022

Reviewing Editor: Simon Creer, Bangor University, Bangor, United Kingdom

Editor's evaluation

This landmark study reveals novel temporal arthropod biodiversity insights that can be leveraged from environmental DNA traces, that have been cryopreserved on leaf tissue as part of a long-term monitoring scheme. The strength of the evidence underlying the major conclusions is convincing and limitations in the quantitative aspects of the data synthesis are acknowledged appropriately. The work will be of interest to a breadth of ecological practitioners.

Introduction

Dramatic declines of terrestrial insects have been reported in recent years, particularly in areas of intensified land use (***Hallmann et al., 2017***; ***van Klink et al., 2020***; ***Seibold et al., 2019***; ***Sánchez-Bayo and Wyckhuys, 2019***). However, some authors have urged caution in generalizing these results (***Didham et al., 2020***; ***Thomas et al., 2019***; ***Cardoso et al., 2019***), suggesting that reported patterns of decline may be more localized than currently assumed or reflect long-term natural abundance fluctuations (***Macgregor et al., 2019***; ***Crossley et al., 2020***). Most studies on insect decline suffer from a

eLife digest Insects are a barometer of environmental health. Ecosystems around the world are being subjected to unprecedented man-made stresses, ranging from climate change to pollution and intensive land use. These stresses have been associated with several recent, dramatic declines in insect populations, particularly in areas with heavily industrialised farming practices.

Despite this, the links between insect decline, environmental stress, and ecosystem health are still poorly-understood. A decline in one area might look catastrophic, but could simply be part of normal, longer-term variations. Often, we do not know whether insect decline is a local phenomenon or reflects wider environmental trends. Additionally, most studies do not go far back enough in time or cover a wide enough geographical range to make these distinctions.

To understand and combat insect decline, we therefore need reliable methods to monitor insect populations over long periods of time. To solve this problem, Krehenwinkel, Weber et al. gathered data on insect communities from a new source: tree leaves. Originally, these samples were collected to study air pollution, but they also happen to contain the DNA of insects that interacted with them before they were collected – for example, DNA deposited in chew marks where the insects had nibbled on the leaves. This is called environmental DNA, or eDNA for short.

To survey the insect communities that lived in these trees, Krehenwinkel, Weber et al. first extracted eDNA from the leaves and sequenced it. Analysis of the different DNA sequences from the leaf samples revealed not only the number of insect species, but also the abundance (or rarity) of each species within each community. Importantly, the leaves had been collected and stored in stable conditions over several decades, allowing changes in these insect populations to be tracked over time.

eDNA analysis revealed subtle changes in the make-up of forest insect communities. In the forests where the leaves were collected, the total number of insect species remained much the same over time. However, many individual species still declined, only to be replaced by newcomer species. These ‘colonisers’ are also widespread, which will likely lead to an overall pattern of fewer species that are more widely distributed – in other words, more homogeneity.

The approach of Krehenwinkel, Weber et al. provides a reliable method to study insect populations in detail, over multiple decades, using archived samples from environmental studies. The information gained from this has real-world significance for environmental issues with enormous social impact, ranging from conservation, to agriculture and even public health.

lack of long-term time series data and are limited in geographic and taxonomic breadth, often using biomass as a proxy for diversity estimates (***Hallmann et al., 2017***; ***Daskalova et al., 2021***). Hence, what is needed are methods and sample types that yield standardized long-term time series data for the diversity of arthropod communities across broad taxonomic and geographic scales (***Forister et al., 2021***).

In recent years, environmental DNA metabarcoding has offered a promising new approach to monitor biological communities (***Taberlet et al., 2018***; ***Tautz et al., 2002***; ***Thomsen and Sigsgaard, 2019***). This includes terrestrial arthropods, whose eDNA can be recovered from various substrates, for example plant material (***Nakamura et al., 2017***). Here, we develop a DNA metabarcoding and quantitative PCR (qPCR) protocol to simultaneously recover diversity and relative DNA copy number of arthropod community DNA from powdered leaf samples. We then analyze 30-year time series data of arthropod communities from canopy leaf material of four tree species from 24 sites across Germany. These sites represent four land use types with different degrees of anthropogenic disturbance: urban parks, agricultural areas, timber forests, and national parks (***Figure 1***). The samples were collected using a highly standardized protocol by the German Environmental Specimen Bank (ESB), a large biomonitoring effort for Germany’s ecosystems, and stored at below −150°C. By basing our analyses on DNA sequences, we can measure diversity from haplotype variation within species to taxonomic diversity of the whole community.

Current studies on insect decline primarily focus on site-based assessments of α-diversity and biomass (***Hallmann et al., 2017***; ***Seibold et al., 2019***). Yet, these metrics alone are insufficient for characterizing ongoing biodiversity change (***Marta et al., 2021***; ***Kortz and Magurran, 2019***; ***Magurran and Henderson, 2010***). Significant temporal community change and declines can also

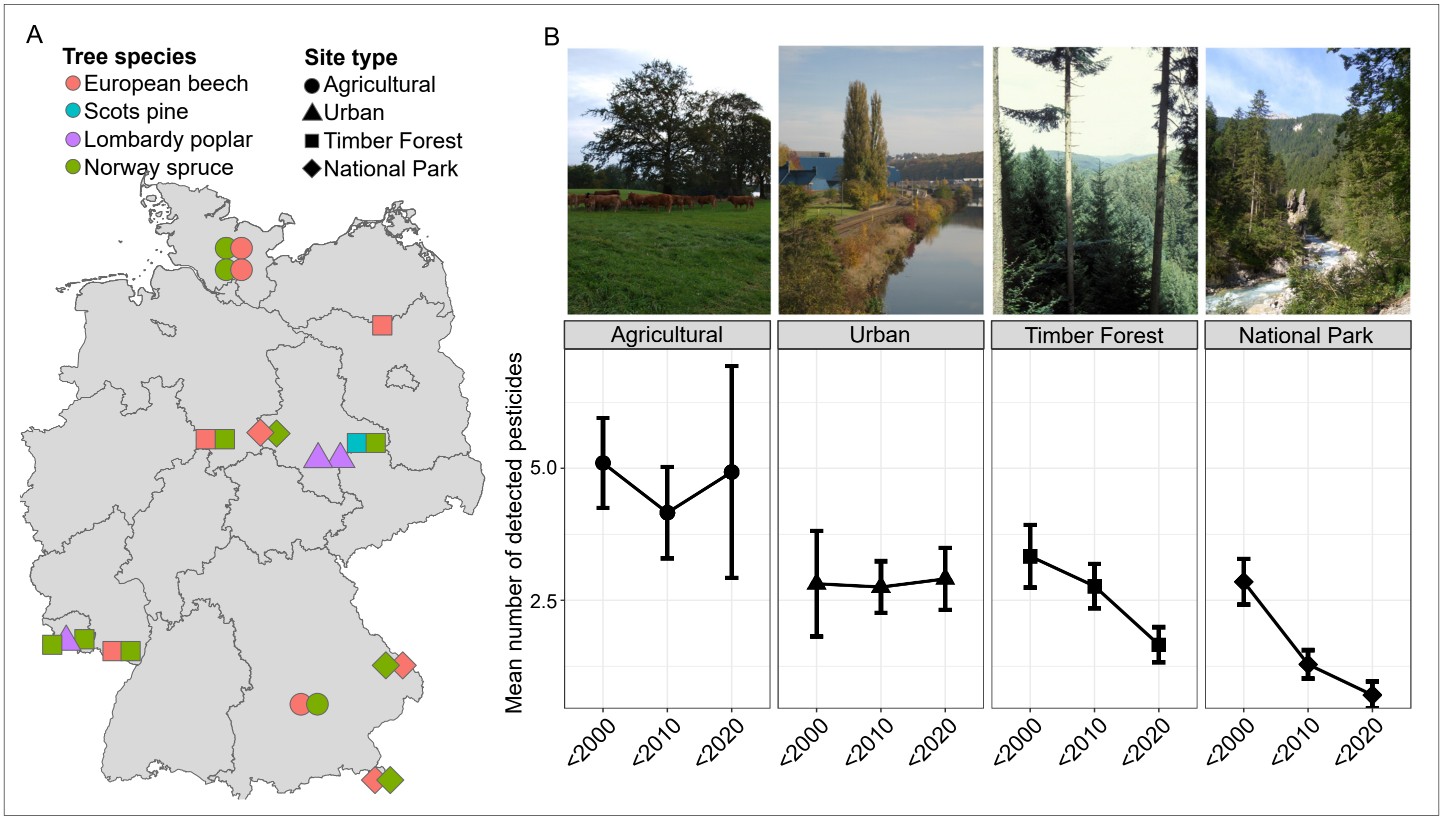

Figure 1. Overview of different sampling sites and samples in this study. (**A**) Sampling sites, land use types, and the four tree species (European Beech *Fagus sylvatica* (N = 98), Lombardy Poplar *Populus nigra* '*italica*' (N = 65), Norway Spruce *Picea abies* (N = 123), and Scots Pine *Pinus sylvestris* (N = 26)) sampled by the ESB in Germany. (**B**) Representative images of sampling sites for the four land use types and average number of detected pesticides (and 95 % confidence interval) across these land use types in three time periods (before 2000, 2000–2009, and 2010–2018). Gas chromatography–tandem mass spectrometry (GC–MS/MS) analysis (***Löbbert et al., 2021***) shows that the detected pesticide load distinguishes the different land use types, with agricultural sites continuously showing the highest number of pesticides.

occur at the scale of β- or γ-diversity, without affecting local richness. This may be driven by community turnover and spatial biotic homogenization (***Karp et al., 2012***). But diversity may also vary temporally. Fluctuating occurrences of transient species considerably increase diversity within single sites over time (***D'Souza and Hebert, 2018***). The loss of such transient species in favor of taxa with a temporally stable occurrence results in an increasingly predictable community and hence a temporal diversity decline. Biotic turnover may occur gradually following changing environmental conditions, but can also occur abruptly, when ecosystems reach tipping points (***Barnosky et al., 2012***). In the latter case, a rapid and considerable biotic remodeling of the ecosystem may be found. The relevance of these spatial and temporal factors in insect decline remains largely elusive.

Here, we use our high-resolution data on arthropods from canopy leaf samples to test the hypotheses that (1) α-diversity and biomass of canopy-associated arthropod communities have declined in the last 30 years (***Hallmann et al., 2017***; ***Seibold et al., 2019***), or (2) community change has occurred in the form of turnover and possibly homogenization of communities across space and time (***Kortz and Magurran, 2019***; ***Dornelas et al., 2014***; ***Thomsen et al., 2016***). Last, we hypothesize that (3) biodiversity declines will be particularly pronounced in areas of intensified land use (***Outhwaite et al., 2022***).

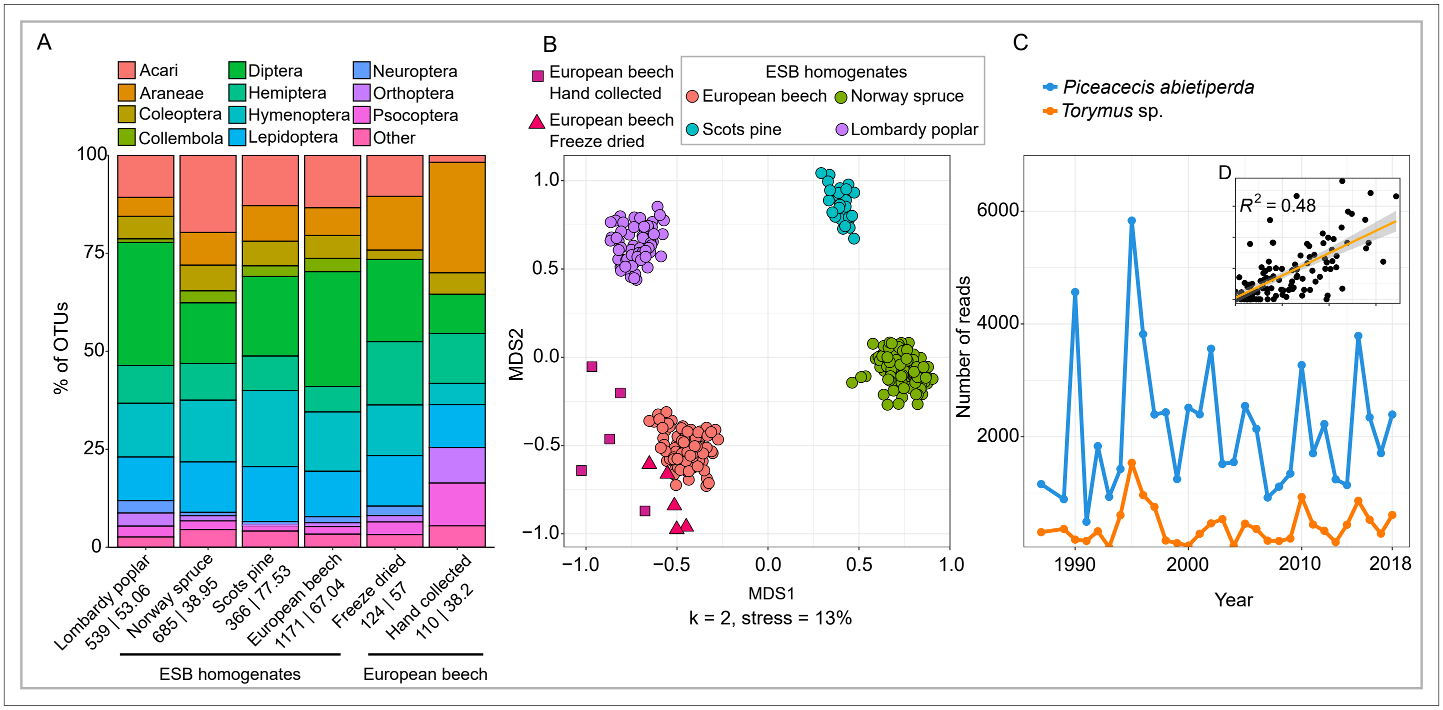

Figure 2. Recovery of diversity and interactions in canopy-associated arthropod communities from leaf material. (**A**) Barplot showing recovered order composition of OTUs across the four tree species (N = 312). In addition to ESB samples, results from freeze-dried leaves stored at room temperature for 6–8 years (N = 5) and hand-collected bulk insect samples (N = 5) from European Beech are shown. Orders amounting to less than 1% of the total OTU number are merged as 'Other'. Numbers below each barplot show the total number of arthropod OTUs followed by the mean OTU number per sample. (**B**) Non-metric multidimensional scaling (NMDS) plot showing tree-specific composition of arthropod communities for the same samples. (**C**) Temporal changes in abundance of the spruce gall midge *Piceacecis abietiperda* and its parasitoid *Torymus* sp. between 1987 and 2018 in a spruce forest in the Saarland. Inset (D) shows correlation of relative abundance between the two species across all ESB spruce samples.

The online version of this article includes the following figure supplement(s) for figure 2:

Figure supplement 1. Effect of replication and sample input on recovered arthropod diversity.

Figure supplement 2. Effect of weight of plant material and precipitation events before sampling on the recovered arthropod diversity.

Figure supplement 3. Contamination check in the cryomill.

Figure supplement 4. Rarefaction curves for all analyzed samples.

Figure supplement 5. Comparison of recovered taxonomic composition and diversity patterns for the two COI markers (ZBJ-ArtF1c/ZBJ-ArtR2c vs. NoPlantF_270/mlCOIintR_W) used in this study.

Figure supplement 6. Ecological diversity of arthropod species recovered from the four tree species.

Figure supplement 7. NMDS showing arthropod community differentiation by site, separated by tree species.

Results

A standardized protocol to characterize plant-associated arthropod communities

We developed a standardized and robust protocol to reproducibly recover plant-associated arthropod communities from powdered leaf material. We controlled for effects of the amount of leaf material per sample, rainfall before sampling, amount of leaf homogenate used for DNA extraction, extraction replication, and primer choice on the recovered diversity (see Methods and ***Figure 2—figure supplements 1–5*** for details on standardization).

Using our optimized protocol, we analyzed 312 ESB leaf samples. We recovered 2054 OTUs from our samples, with different tree species having significantly different OTU numbers on average (***Figure 2A***, linear mixed model [LMM], $p < 0.05$). Nevertheless, all tree species showed a balanced and relatively similar taxonomic composition at the order level (***Figure 2A***, ***Figure 2—figure supplement 5E***). We identified 23 orders, 218 families, and 413 genera. The richest order was Diptera (600 OTUs in 48 families), followed by Hymenoptera (369 OTUs in 21 families), Acari (293 OTUs in 21

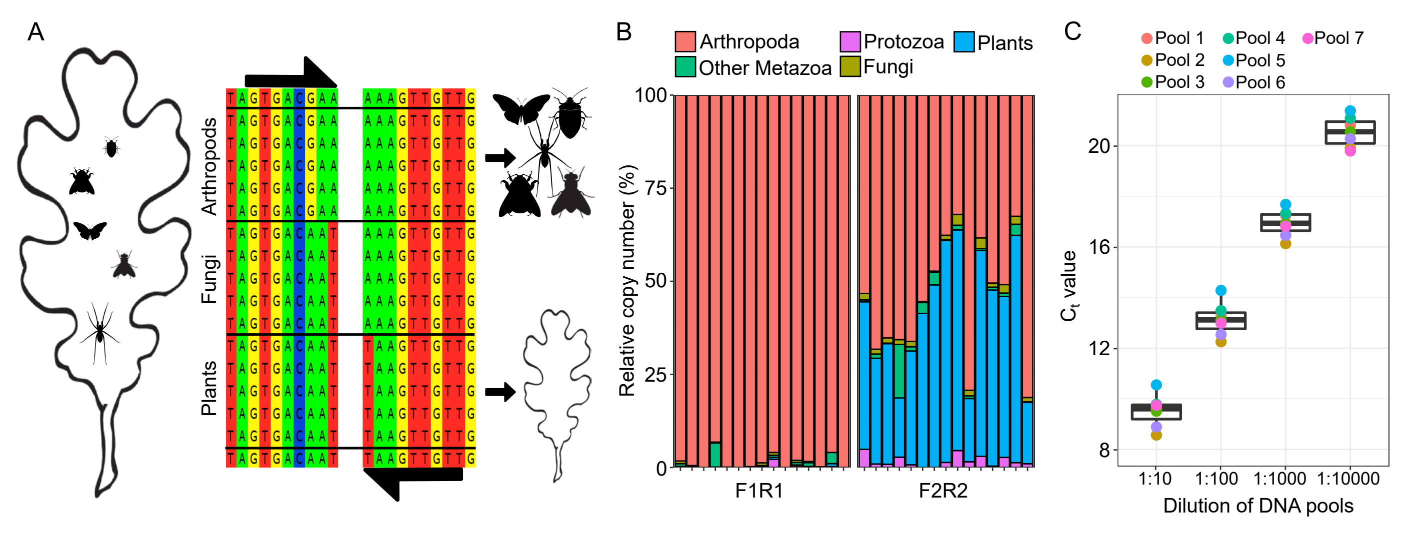

Figure 3. Quantitative PCR (qPCR) experiment to detect relative rDNA copy number of arthropods in plant homogenates. (**A**) Schematic overview of the blocking approach to amplify homologous 18SrDNA fragments for either arthropod or plant DNA, based on lineage-specific priming mismatches. (**B**) Effect of primer mismatches on the recovery of arthropod sequences. Barplots show recovered read proportion of different higher taxa from 15 ESB leaf samples. The left plot shows the effect of a diagnostic mismatch in forward and reverse primers, while the right plot shows the effect of only a forward primer mismatch. (**C**) Boxplot showing CT values recovered from the seven mock communities of arthropod species from 13 different orders (see ***Figure 3—figure supplement 1*** for community composition) and across a 1.10000 dilution series. Separate CT values for each community are indicated by the dots (***Figure 3—figure supplement 1***).

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. Taxonomic composition of the mock communities used to test the qPCR assay.

families), Lepidoptera (233 OTUs in 32 families), Hemiptera (152 OTUs in 19 families), Coleoptera (133 OTUs in 29 families), and Araneae (99 OTUs in 15 families). The recovered species assemblages were ecologically diverse, including herbivores, detritivores, predators, parasites, and parasitoids (***Figure 2—figure supplement 6***). Each tree species harbored a unique arthropod community (***Figure 2B***, ***Figure 2—figure supplement 5C, D***), with typical monophagous taxa exclusively recovered from their respective host trees. The arthropod communities from different sites and land use types were also differentiated within tree species (***Figure 2—figure supplement 7***, PERMANOVA, $p < 0.05$). In addition to arthropod–host plant associations, we were able to detect interactions between arthropods. For example, abundances of the spruce gall midge *Piceacecis abietiperda* and its parasitoid, the chalcid wasp *Torymus* sp., were well correlated across all analyzed spruce sites (LM, $p < 0.05$). Both underwent coupled abundance cycles, with similar maxima every 6–8 years (***Figure 2C***).

The recovered community composition from ESB leaf homogenate samples was similar to hand-collected branch clipping samples (***Figure 2A, B***). Branch clipping recovered a larger diversity of spiders, a taxon which is exclusively found on leaf surfaces. In contrast, about 25% of the recovered taxa from ESB leaf powder likely inhabited the insides of leaves, e.g., gallers and miners (***Figure 2—figure supplement 6B***). Overall, arthropod DNA in leaf homogenates appears temporally very stable: Even freeze-dried leaf material that had been stored at room temperature for 8 years yielded surprisingly similar arthropod communities to ESB samples (***Figure 2A, B***). Besides analyzing diversity, we generated information on relative arthropod 18S rDNA copy number in relation to the corresponding plant 18S rDNA copy number by qPCR. Relative eDNA copy number should be a predictor for relative biomass. We designed a standardized qPCR assay based on lineage-specific blocking SNPs in the 18SrDNA gene (***Figure 3A***). We tested two primer combinations with 3′-blocking SNPs in (1) only the forward or (2) both forward and reverse primer sequences. The primer combination with mismatches in both forward and reverse primers led to a near complete suppression of non-arthropod amplification in all tested samples (5.41% vs. 44.49% on average) and was hence chosen for the qPCR experiment (***Figure 3A, B***). The qPCR assay accurately predicted changes in relative copy number of arthropod DNA on plant material across a 10,000-fold dilution series. Even when comparing taxonomically

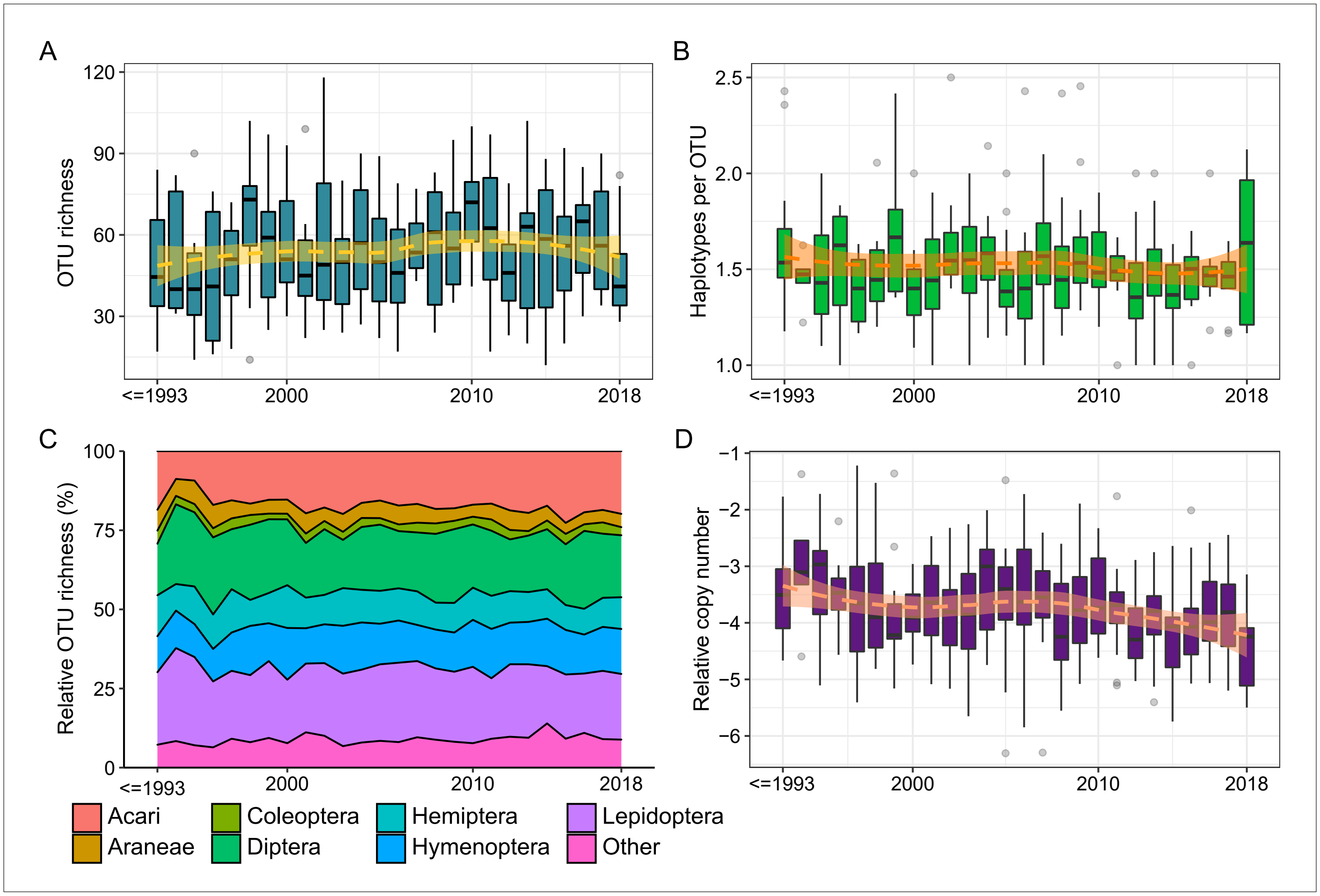

Figure 4. Temporal changes of diversity and copy number across all sites and samples (N = 312). (**A**) Arthropod OTU richness (representing α-diversity). (**B**) Haplotype richness within OTUs (representing genetic variation). (**C**) Relative OTU richness per order. (**D**) Relative copy number of arthropod DNA (representing biomass).

The online version of this article includes the following figure supplement(s) for figure 4:

Figure supplement 1. Metabarcoding-based diversity indices and quantitative PCR (qPCR)-based relative copy number of arthropod rDNA per land use type over three decades.

Figure supplement 2. Boxplots of OTU richness by decade and land use type for the six most speciose arthropod orders in our dataset.

Figure supplement 3. Arthropod diversity and relative copy number over time for all sites with time series longer than 10 years.

heterogeneous mock communities, very similar CT values were recovered (***Figure 3C***, ***Figure 3—figure supplement 1***), highlighting the accuracy and wide applicability of our approach. Overall, we found a significant positive correlation of OTU richness and relative arthropod copy number in the ESB samples (LMM, $p < 0.05$; see Methods: '*Statistical analysis*'), supporting recent work suggesting a biomass–diversity relationship (***Hallmann et al., 2021***).

Temporal changes of diversity, copy number, and species composition in canopy arthropod communities

Based on our time series data of archived ESB leaf samples, we tested the hypothesis that α-diversity (including intraspecific genetic diversity) and biomass (relative rDNA copy number) have undergone widespread temporal declines, particularly in areas of intensive land use. Our statistical analysis does not support previously reported widespread temporal α-diversity declines (***Figure 4A***, ***Figure 2—figure supplement 5H, I***, LMM, $p > 0.05$), even when different land use types are analyzed separately (***Figure 4—figure supplement 1***, LMM, $p > 0.05$). Instead, warm summers and cold winters were negatively associated with richness (LMM, $p < 0.05$). The temporal pattern of diversity was also largely independent of taxonomy: most orders did not show temporal trends when analyzed separately (**Figure 4C**; **Figure 4—figure supplement 2**). Exceptions include a significant loss of lepidopteran diversity, which is primarily driven by OTU loss at urban sites, and an overall increasing diversity of mites (LMM, $p < 0.05$). The overall temporally stable diversity is also visible at separate sites; a diversity decline across all orders was observed at only a single site (**Figure 4—figure supplement 3**). Similar to α-diversity, we did not find widespread temporal declines of genetic diversity. Neither community-level zero radius OTU (zOTU) richness (which was well correlated to OTU richness, $R^2 = 0.89$) nor within-OTU haplotype richness declined significantly over time (**Figure 4B**; **Figure 4—figure supplement 1C, D**).

In contrast to the stable α-diversity, relative copy number showed an overall decrease over time (**Figure 4D**, LMM, $p < 0.05$), suggesting that arthropod biomass may indeed be declining in woodlands (**Seibold et al., 2019**). The effect appears to be particularly driven by urban sites, coinciding with a loss of lepidopteran diversity (**Figure 4—figure supplements 1–3**). However, declines of arthropod DNA copy number are also visible in several agricultural and timber forest sites, particularly in the last 10 years of our time series (**Figure 4—figure supplement 1**, **Figure 4—figure supplement 3**).

We next explored temporal changes in abundance for 413 separate OTUs from a total of 19 sites. In line with our hypothesis (1), we predicted a majority of declining species. However, we found no significant difference between the average number of declining (6.94%) and increasing (10.04%) OTUs (*t*-test, $p > 0.05$, **Figure 5A**). With the exception of Acari, which showed an overrepresentation of increasing OTUs, declines and increases in OTU read abundance were independent of arthropod order and land use type (**Figure 5A, B**, Fisher's exact test $p > 0.05$). The observed replacement of about 15% of OTUs within sites translates into a significant temporal change of taxonomic β-diversity (**Figure 5C**, **Figure 2—figure supplement 5J and K**). We found a strong positive correlation of temporal distance and Jaccard dissimilarity for most sites (PERMANOVA, $p < 0.05$). Thus, species are continuously replaced in all land use types (**Figure 4—figure supplement 1F**). In the majority of analyzed sites, β-diversity did not show a correlation with differences in copy number (**Figure 5—figure supplement 2**).

While the community turnover did not affect local α-diversity, we still observed associated losses of overall diversity. The first noteworthy pattern concerns a loss of temporal β-diversity within sites. β-Diversity between consecutive sampling years dropped significantly in many sites, particularly in beech forests (**Figure 5D**). Thus, diversity within sites is increasingly homogenized over time. We also found a significant decrease of β-diversity between sites for beech forests (**Figure 5E**). Our data suggest a loss of site-specific species and a gain of more widespread generalists, irrespective of land use. This pattern also emerges at the level of individual OTUs. Several novel colonizers spread rapidly in woodlands and showed similar abundance trends across various sites in parallel (**Figure 5—figure supplement 1**). The spatial and temporal change of β-diversity is illustrated by an NMDS plot of arthropod communities from two beech forests in National Parks, the Harz and the Bavarian Forest (**Figure 5F**). While the two sites are well separated by the first NMDS axis, the second axis shows a pronounced temporal turnover of communities. In the past decades, this turnover has led to temporally more predictable communities within sites and increasingly similar communities between the two national parks.

Discussion

Here, we show that DNA from archived leaf material provides a robust source of data to reconstruct temporal community change across the arthropod tree of life. Leaf samples should also cover a broad phenological window: Adults of many insect species are only active during a short time period of the year, but their larvae spend the whole year on their host plant (**Gagné and Graney, 2014**). A Malaise trap will miss these taxa most times of the year, while an eDNA approach should detect the larvae throughout. As sampled leaves make up the habitat and often food source of arthropods, it is also possible to infer the exposure of arthropod communities to chemical pollution by analyzing chemicals in the leaf material. This is of critical importance, as pesticide use has often been invoked as a driver of insect decline (**Goulson et al., 2015**; **Siviter et al., 2021**). The high temporal stability of arthropod DNA in 8-year-old dried leaf samples also suggests the utility of other plant archives, for example, herbaria, for arthropod eDNA. However, for such less standardized and less well-preserved sample

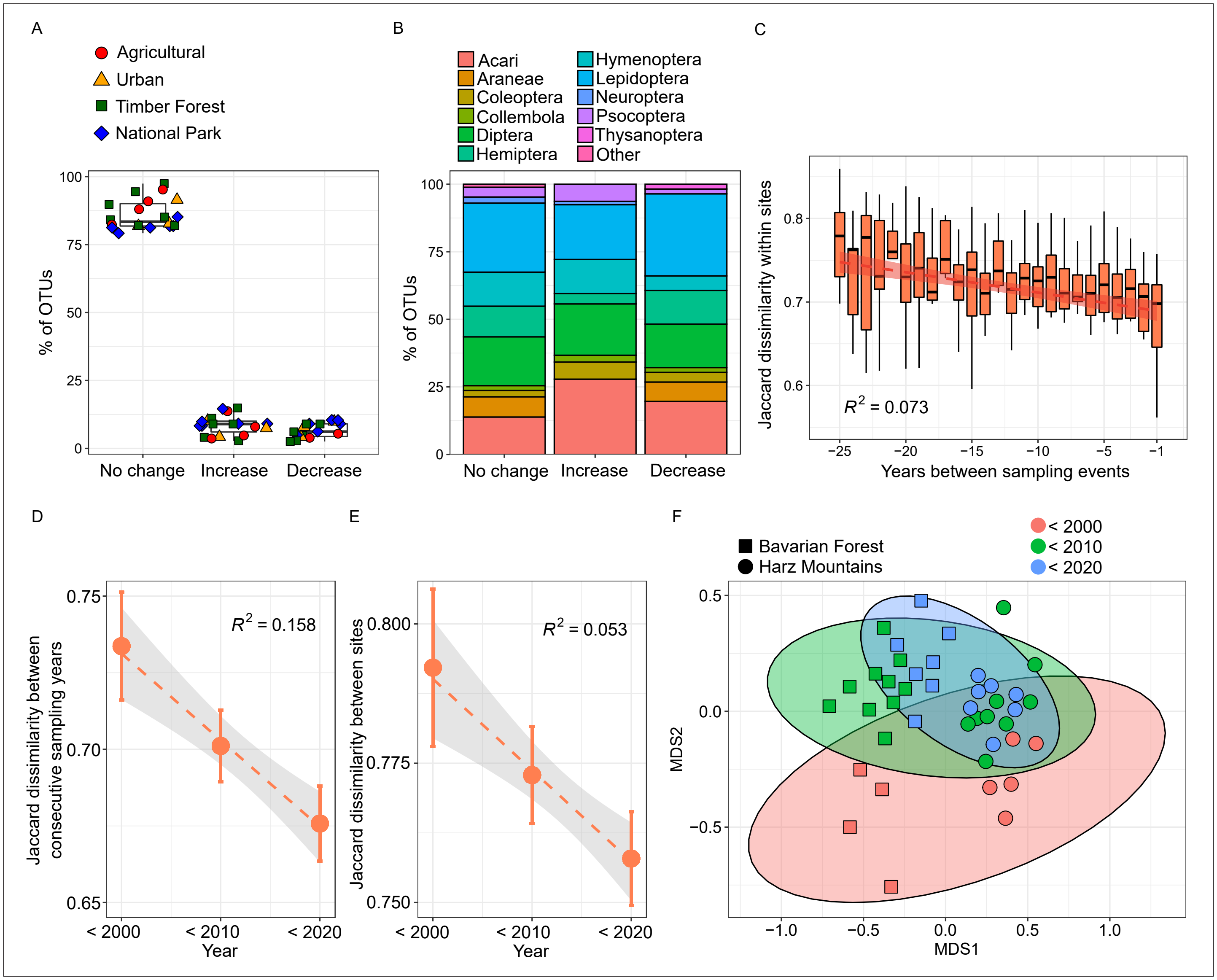

Figure 5. Temporal changes of species composition and β-diversity within and between sampling sites. (**A**) Boxplots of the proportion of 413 separate OTUs that significantly increased, decreased, or did not show temporal abundance changes. The colored symbols overlaying the boxplots represent land use type for each site. (**B**) Stacked barplot showing the recovered order composition of the same three categories of OTUs. Orders amounting to less than 1% of the total OTU number are merged as 'Other'. (**C**) Within-site Jaccard dissimilarity as a function of number of years between sampling events, showing a pronounced taxonomic turnover (N = 312). (**D**) Jaccard dissimilarity between consecutive sampling years in each decade of sampling (N = 15, 44 & 39), calculated within sites for beech forests. We find a loss of temporal β-diversity over time. Points indicate means and error bars show 95% confidence interval. (**E**) Jaccard dissimilarity between sites for the same samples as in D. We find a loss of average between-site β-diversity, that is spatial homogenization. (**F**) NMDS plot showing community dissimilarity within and between Bavarian Forest and Harz Mountains national parks over three decades (<2000: sampled before 2000; <2010: sampled between 2000 and 2009; <2020: sampled between 2010 and 2018).

The online version of this article includes the following figure supplement(s) for figure 5:

Figure supplement 1. Temporal change in relative read abundance for five exemplary arthropod OTUs at all beech and poplar sites and abundance change for three OTUs at two sites each in Germany.

Figure supplement 2. Correlation of dissimilarity in 18S rDNA copy number and β-diversity for all sites with time series longer than 10 years.

types, careful evaluation of cross-contamination or chemical DNA modification (***Orlando et al., 2021***), which could inflate the recovered diversity, may be warranted.

We here analyze a unique leaf archive and provide unprecedented insights into arthropod community change in the tree canopy, an ecosystem known for its high and often cryptic diversity (***Nakamura***

et al., 2017). Our results do not confirm the hypothesis of widespread losses of α-diversity. Initial reports of insect declines originate mainly from grassland ecosystems that have undergone massive changes in land use (**Hallmann et al., 2017**). Central European canopy communities may be less affected by such change. Interestingly, the only sites that showed declines of richness were agricultural and urban sites, suggesting that land use may at least locally affect neighboring canopy communities (**Hallmann et al., 2014**). Our data also suggest negative effects of warm summers on richness. Climate warming has recently been suggested to act in conjunction with land use change to drive insect declines (**Outhwaite et al., 2022**).

Instead of declining richness, we detected DNA copy number declines in all land use types, suggesting that overall biomass may indeed be in decline. Extinction is the endpoint of a long trajectory of decline and increasingly affects common species (**Collen et al., 2011**), hence biomass decline could foreshadow future biodiversity loss. Alternatively, the dropping copy number may reflect a taxonomic turnover of species with either different eDNA shedding rates or different rDNA copy numbers in the different communities. However, if the latter were true, an association of copy number changes and turnover would be expected, which we did not find in our data.

Instead of widespread losses of α-diversity, we indeed found pronounced taxonomic turnover in nearly all communities (**Dornelas et al., 2014**), supporting our second hypothesis. While resident species are continuously lost, they are mostly replaced by novel colonizers. This turnover can result in biotic homogenization across space and time. Less interannual variation of the occurrence of taxa within sites and reduced spatial variation of their occurrence between sites both cause a decline in overall β-diversity. Biotic homogenization is often associated with intensification of land use (**Karp et al., 2012**; **Gossner et al., 2016**) and landscape simplification (**Holland et al., 2005**). However, the pattern we observed affected all land use types equally. The universality of these changes suggests that neither site- nor taxon-specific factors are responsible. Possible explanations include factors that act at a larger scale, such as climate change-induced range shifts (**Marta et al., 2021**), nitrogen deposition (**Gámez-Virués et al., 2015**), and the introduction of invasive species (**Soroye et al., 2020**; **Lister and Garcia, 2018**). Given that our leaf samples recover a fairly broad phenological window, the alternative explanation that the observed community-wide turnover pattern may have resulted from shifting phenologies (**Cohen et al., 2018**) is unlikely. The gradual replacement of species also suggests that we are not yet observing ecosystems reaching tipping points (**Barnosky et al., 2012**).

In summary, our work shows the great importance of standardized time series data to accurately reveal biodiversity change in the Anthropocene (**Thomsen et al., 2016**) across space and time, beyond the decline of α-diversity and biomass (**Dornelas et al., 2014**). Taxonomic replacement and biotic homogenization, even in seemingly pristine habitats such as national parks, signify an important and hitherto insufficiently recognized facet of the current insect crisis.

Materials and methods

Samples and metadata used in this study

Tree samples of the German Environmental Specimen Bank – standardized time series samples stored at ultra-low temperatures

We used a total of 312 leaf samples of four common German tree species: the European Beech *Fagus sylvatica* (98 samples), the Lombardy Poplar *Populus nigra* '*italica*' (65 samples), the Norway Spruce *Picea abies* (123 samples), and the Scots Pine *Pinus sylvestris* (26 samples). The samples have been collected annually or biannually by the German Environmental Specimen Bank (ESB) since the 1980s and serve as indicators for aerial pollutants (**Schulze et al., 2007**). A total of 24 sampling sites were included, covering sampling periods of up to 31 years and representing four land use types of varying degrees of anthropogenic disturbance (**Figure 1**). These include natural climax forest ecosystems in core zones of national parks (six sites, National Park), forests commercially used for timber (six sites, Timber Forest), tree stands in close proximity to agricultural fields (six sites, Agricultural), and trees in urban parks (three sites, Urban). The sites were initially chosen to represent their land use type permanently for long-term monitoring, and the corresponding land use categories have mostly remained temporally stable.

ESB samples are collected and processed according to a highly standardized protocol at the same time every year. Sampling events between different years of the time series usually do not differ by more than 2 weeks. All used equipment is sterilized before field work by several washes and heat treatments (**Tarricone et al., 2018a**; **Tarricone et al., 2018b**; **Klein et al., 2018**). A defined amount of leaf material (>1.100 g) is collected from a defined number of trees (15 at most sites) from each site and from 4 branches from each tree. The branches are distributed equally spaced in the outer crown area of the tree. The amount sampled translates to several thousand leaves from each site, which should suffice to saturate the recovered arthropod diversity. For a subset of samples, biometric analysis is performed, for example the weight of individual leaves and general condition of the tree are noted. Leaf weight has not changed over the time series at most sites. The sampled leaves are intended to represent the exact natural state of the tree. They are not washed or altered in any way before processing, and eDNA traces, and small arthropods on the leaves' surfaces, as well as from galls and leaf mines, are included in the sample. Each sample is stored on liquid nitrogen immediately after collection and ground to a powder with an average diameter of 200 μm using a cryomill. The cryomill is thoroughly cleaned between samples to prevent cross-contamination. The resulting homogenates are then stored for long term on liquid nitrogen (**Rüdel et al., 2009**; **Rüdel et al., 2015**). The cold chain is not interrupted after collection and during processing, ensuring optimal preservation of nucleic acids in the samples. The homogenization of the sample also guarantees a thorough mixing, resulting in equal distribution of environmental chemicals and probably eDNA in the sample. Previous work suggests that very small subsamples of the homogenate suffice to detect even trace amounts of environmental chemicals in the sample (**Gámez-Virués et al., 2015**). eDNA from leaf surfaces may be affected by weather conditions before sampling, for example, washed away by heavy rain or damaged by strong UV exposure (**Valentin et al., 2020**). However, leaves are only collected dry by the ESB, that is, not immediately after rain. The date of the most recent rainfall is noted for each ESB sampling event, allowing us to explore the effects of recent weather conditions on the recovered arthropod communities.

The utility of plant material stored at room temperature to recover arthropod DNA

ESB samples are stored under optimal conditions for nucleic acid preservation. By contrast, most archived leaf samples are stored at room temperature, for example dried leaf material in herbaria. To test the general suitability of archived leaf material for arthropod community analysis, we included 25 additional beech leaf samples, each consisting of 100 leaves from a total of 5 sites. The samples were freeze-dried and then ground to a fine powder by bead beating. The resulting powder was comparable to our ESB homogenates, but unlike them, it was stored at room temperature for 6–8 years.

Hand-collected branch clipping samples to explore the accuracy of leaf-derived arthropod DNA

To evaluate the performance of our leaf DNA-based protocol in comparison to commonly used sampling methods, we generated a branch clipping dataset from five beech stands close to Trier University. Branch clipping is a widely used method to collect arthropods residing on leaf surfaces in trees (**Delvare, 1997**) and thus the best comparable traditional methodology to our protocol. We sampled five trees per site and collected ten branches of about 40 cm length from each tree. The branches were clipped off, stored in plastic bags and then brought to the laboratory. Here, arthropods were manually collected from each sample. All collected arthropod specimens were pooled by tree and stored for later DNA extraction in 99% ethanol.

Climate data

We downloaded monthly climate data for all study sites from the German Climate Center distributed as a raster dataset interpolated from the surrounding weather stations by the German Meteorological Service (Deutscher Wetterdienst – DWD). We collected data for average annual temperature and rainfall as well as summer and winter temperature and rainfall.

Measurement of pesticide content from archived leaf material

The ESB sampling was historically set up as a tool for pollution assessment (**Schulze et al., 2007**), and the samples are therefore stored to preserve any possible pollutant. Because these leaves serve as a habitat for the associated arthropods, such well-preserved material allows us to explore levels of chemical pollution occurring directly within the arthropods' environment. Our samples were screened for pesticides and persistent organic pollutants with a modified QuEChERS approach (**Löbbert et al., 2021**). 2.0 g of sample material were extracted with acetonitrile (10 ml) and ultrapure water (10 ml), followed by a salting-out step using magnesium sulfate, sodium chloride and a citrate buffer (6.5 g; 8:2:3 [wt/wt]). After a dispersive solid-phase extraction cleanup step with magnesium sulfate, PSA (primary-secondary amine), and GCB (graphitized carbon black) (182.5 mg; 300:50:15 [wt/wt]), the supernatant was analyzed with a sensitive gas chromatography–tandem mass spectrometry (GC–MS/MS) instrument. All samples were analyzed for 208 GC-amenable compounds of different pesticide and pollutant classes, including pyrethroids, organochlorine and organophosphate pesticides, and polychlorinated biphenyls.

Molecular processing

DNA isolation

We developed a highly standardized protocol for the analysis of leaf-associated arthropod community DNA. We optimized various protocol steps to ensure the reliability and reproducibility of our data. We first explored the effect of DNA extraction on recovered diversity.

As mentioned above, the cryo-homogenization of ESB samples ensures a very homogeneous distribution of even trace amounts of chemicals in the sample. This should also hold true for DNA. Hence small subsamples of large homogenate samples should suffice for analysis. To test this hypothesis, we first performed a weight series extraction from 50, 100, 200, 400, 800, and 1600 mg of homogenate with several replicates for each weight. Additional extraction replicates of 16 beech samples at 200 mg were also included. This analysis confirms the pronounced homogenization of the samples, with 200 mg of homogenate sufficing to accurately recover α- and β-diversity (***Figure 2—figure supplement 1A, B***).

A single DNA extract was made from each ESB and freeze-dried sample, using the Puregene Tissue Kit according to the manufacturer's protocols (Qiagen, Hilden, Germany). All samples were processed under a clean bench and kept over liquid nitrogen during processing to prevent thawing. Samples were transferred using a 1000-µl pipette with cutoff tips. The resulting wide bore tips were used to drill out cores of defined sizes from the leaf powder. To remove undesired coprecipitates, we performed another round of purification for each sample, using the Puregene kit following the manufacturer's protocol. The hand-collected arthropod specimens from our branch clipping were pulverized in a Qiagen Tissuelyzer at 200 Hz for 2 min using new 5-mm stainless steel beads, and DNA was extracted from the pulverized samples using the Puregene kit as described in **de Kerdrel et al., 2020**. Branch clipping and leaf samples were processed in separate batches and using separate reagents to avoid possible carryover between sample types.

Primer choice, PCR amplification, and sequencing

As the standard DNA barcode marker for arthropods (**Andújar et al., 2018**), the mitochondrial COI gene offers the best taxonomic identification of German arthropod species. We thus selected COI for our metabarcoding analysis. We tested several primer pairs to optimize recovery of arthropod DNA from the leaf homogenates (**Gibson et al., 2014**; **Leray et al., 2013**; **Jusino et al., 2019**). Leaf homogenates are dominated by plant DNA, with arthropod eDNA only present in trace amounts. The majority of commonly used arthropod metabarcoding primers are very degenerate and will readily amplify mitochondrial COI of plants. Thus, for such degenerate primers, the vast majority of recovered reads will belong to the plant. We found an ideal tradeoff between suppressing plant amplification while still recovering a taxonomically broad arthropod community in the primer pair ZBJ-ArtF1c/ZBJ-ArtR2c (**Zeale et al., 2011**). While this primer pair is known to have taxonomic biases for certain arthropod groups (**Piñol et al., 2015**), it is still widely used as an efficient and reliable marker for community analysis (**Thomsen and Sigsgaard, 2019**; **Eitzinger et al., 2021**). Recently, we designed a novel and highly degenerate primer pair by modifying two degenerate metabarcoding primers (**Gibson et al., 2014**; **Leray et al., 2013**), which allows the suppression of plant amplification (*NoPlantF_270/mICOIintR_W*; ***Supplementary file 1***; **Krehenwinkel et al., 2022**). To ensure the reproducibility of the diversity patterns recovered from our original ZBJ-ArtF1c/ZBJ-ArtR2c dataset, we additionally processed eleven complete ESB time series (174 samples) for this novel primer pair and compared results for species composition, α- and β-diversity. This analysis supports very similar patterns for temporal species abundance trends, as well as α- or β-diversity for both primer pairs (***Figure 2—figure supplement 5***).

All PCRs were run with 1 µl of DNA in 10 µl volumes, using the Qiagen Multiplex PCR kit according to the manufacturer's protocol and with 35 cycles and an annealing temperature of 46°C. A subsequent indexing PCR of five cycles at an annealing temperature of 55°C served to attach sequencing adapters and 8-bp dual indexes (all with a minimum 2 bp difference) to each sample (using the layout described in ***Lange et al., 2014***). We had previously tested the effect of DNA extraction and PCR replicates, which showed well correlated and reproducible OTU composition ($R^2_{\text{extract1vs2}} = 0.90$, $R^2_{\text{PCR1vs2}} = 0.97$, LM $p < 0.05$), as well as α-diversity patterns ($R^2_{\text{extract1vs2}} = 0.93$, $R^2_{\text{PCR1vs2}} = 0.90$, LM $p < 0.05$). PCR and extraction replicates also recovered a significantly lower β-diversity than within- and between-site comparisons ($\beta_{\text{PCR1vs2}} = 0.14$; $\beta_{\text{extract1vs2}} = 0.19$; $\beta_{\text{within site}} = 0.62$; Pairwise Wilcoxon Test, $p < 0.05$) (***Figure 2—figure supplement 1***). Considering the similar recovered diversity patterns for PCR and extraction replicates and the observed saturation of diversity with single 200 mg homogenate extractions, we did not perform extraction replicates, but ran all PCRs in duplicate as technical replicates. To assure that dual replicates suffice to recover diversity patterns, we also added a PCR triplicate for two time series of beech samples (42 samples in total) and sequenced each of these samples to 78,000 reads on average. Patterns of diversity were highly correlated between duplicate and triplicate datasets ($R^2 = 0.95$). The final libraries were quantified on a 1.5% agarose gel and pooled in approximately equal abundances based on gel band intensity. The final pooled sample was cleaned using 1× Ampure Beads XP (Beckmann-Coulter, Brea, CA, USA) and then sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) using several V2 kits with 300 cycles at the Max Planck Institute for Evolutionary Biology in Plön, Germany. Branch clipping samples were amplified and sequenced separately using the above protocol. Negative control PCRs and blank extraction PCRs were run alongside all experiments and sequenced as well, to explore the effect of possible cross-contamination or index carryover between samples.

Test for DNA carryover in the cryomill

The sample processing pipeline of the ESB is laid out to be sterile and entirely avoid cross-contamination between samples. To test the efficiency of these protocols for eDNA sampling, we included a test on the possibility of carryover in the cryomill. Using the milling schedule of tree samples from 2015 to 2018, we compared the β-diversity between tree samples that were processed in the cryomill consecutively. Assuming an eDNA carryover takes place, the β-diversity should be significantly reduced compared to samples which are processed in different years. We did not find an effect of processing order in the cryomill on beta diversity for 18 within- and between-tree species comparisons (***Figure 2—figure supplement 3***). We also explored the effect of single species carryover in the cryomill. This was done using samples of different tree species, which were processed consecutively in the cryomill. We compared the read abundances of the 10 most abundant monophagous species with that found in the consecutively processed sample of a different tree species. The comparisons were done for one poplar and beech sample as well as one pine and spruce sample. To ensure even minor carryover would be detected, we sequenced all samples to a high depth of 78,000 reads on average. Yet, no signal of carryover was observed (***Supplementary file 3***).

Sequence processing

Reads were demultiplexed by dual indexes using CASAVA (Illumina, San Diego, CA, USA) allowing no mismatches in indexes. Demultiplexed reads were merged using PEAR (***Zhang et al., 2014***) with a minimum overlap of 50 and a minimum quality of 20. The merged reads were then quality filtered for a minimum of 90% of bases >Q30 and transformed to fasta files using FastX Toolkit (***Gordon and Hannon, 2010***). Primer sequences were trimmed off using *sed* in UNIX, with degenerate sites allowed to vary and only retaining sequences beginning with the forward and ending with the reverse primer. The reads were then dereplicated using USEARCH (***Edgar, 2010***). The dereplicated sequences were clustered into zero radius OTUs (hereafter zOTUs) using the *unoise3* command (***Edgar, 2016***) and 3% radius OTUs using the *cluster_otus* command in USEARCH with a minimum coverage of 8 and a minimum occurrence of three reads in a sample. Chimeras were removed de novo during OTU clustering. All resulting sequences were translated in MEGA (***Takahara et al., 2012***) and only those with intact reading frames were retained. To assign taxonomic identity to the zOTU sequences, we used

BLASTn (**Altschul et al., 1990**) against the complete NCBI nucleotide database (downloaded February 2021) and kept the top 10 hits. Sequences were identified to the lowest possible taxonomic level, with a minimum of 98% similarity to classify them as species. All non-arthropod sequences were removed. We then built Maximum Likelihood phylogenies from alignments of the zOTU sequences for all recovered arthropod orders separately using RaxML (**Stamatakis, 2014**). These phylogenies were used to perform another clustering analysis using ptp (**Zhang et al., 2013**) to generate OTUs from the data. Due to the well-developed German Barcode of Life database (**Geiger et al., 2017**), actual species identity can be reliably inferred by database comparisons for many arthropod groups. 3% radius OTUs often oversplit species, for example several 3% radius OTUs comprised one actual species. The ptp clustering often merged several 3% radius OTUs, but came closest to the actual species assignments by BLAST. Moreover, the recovered diversity values for 3% OTUs, zOTUs and ptp-based OTUs were well correlated ($R^2 = 0.90$). We thus proceeded to use ptp-based OTUs (hereafter referred to as OTUs) for subsequent analysis on taxonomic diversity, as it should best approximate actual species diversity. zOTUs represent individual haplotypes in the dataset and were used as an indicator of genetic diversity. Using the taxonomic assignments, we estimated which taxonomic groups were particularly well represented in our data. Each tree species likely harbors a unique arthropod community with numerous monophagous species, a majority of which should be recovered by a broadly applicable molecular method. Where possible, we performed a finer scale ecological assessment for the recovered taxa, classifying them by trophic ecology and expected position on the outside or inside of the leaf. For example, mining taxa would likely be recovered from the inside of the leaf, while other taxa likely reside on the leaf’s surface.

Detection of relative arthropod DNA copy number using qPCR

Initial reports on insect decline were entirely based on biomass (**Hallmann et al., 2017**). Biomass, however, does not necessarily predict diversity (**Gough et al., 1994**). We therefore aimed to generate information not only on diversity, but also on relative biomass of arthropods in tree canopies. Previous eDNA studies show that DNA copy number is correlated with the biomass of a target taxon (**Takahara et al., 2012**), making qPCR a possible approach for biomass estimation. We developed a qPCR protocol to detect relative abundance of arthropod DNA copy number in leaf samples, using the plant DNA copy number as an internal reference for quantification. We used the nuclear 18SrRNA gene (hereafter 18S). Although 18S can show interspecific copy number variation, it provides relatively good approximations of actual taxon abundances in amplicon assays (**Krehenwinkel et al., 2017**; **Krehenwinkel et al., 2019b**). Primer pairs targeting plants and arthropods were designed to meet the following criteria: (1) Identical PCR fragments should be amplified for plants and arthropods so that PCR for both taxa will perform similarly. (2) The arthropod-specific primer should not amplify plants, and vice versa. (3) Fungi should be excluded from amplification, as DNA of fungal endophytes is probably at least as abundant in leaf samples as arthropod eDNA. (4) The primers should target conserved regions in order to amplify a broad spectrum of plants or arthropods. We used diagnostic SNPs at each primer’s 3′-end to achieve the lineage specificity (**Krehenwinkel et al., 2019a**).

Two possible qPCR primer pairs were designed, one targeting a 172 bp and the other a 176 bp fragment of 18S (**Supplementary file 1**). The first primer pair contained a 3′-AA-mismatch discriminating arthropods from plants and fungi in the forward primer and a 3′-TT-mismatch discriminating plants from arthropods in the reverse primer, while the second pair had the same 3′-AA-mismatch in the forward primer but no reverse primer mismatch (**Figure 3A**). To test the lineage specificity of both primer pairs, we performed an amplicon sequencing experiment with the arthropod-specific primers. Four samples from each of the four tree species were amplified with both primer pairs, then indexed, pooled, sequenced, and processed as described above. All reads were clustered into 3% radius OTUs. Taxonomy was assigned to the OTUs, and an OTU table was built, as described above for the ESB sample metabarcoding experiment. The proportion of arthropod reads was then estimated for each sample and primer pair. The first primer combination (3′-mismatch in both forward and reverse primers) led to a near complete suppression of plant and fungal amplification in all tested samples and was therefore used for the qPCR (**Figure 3A, B**).

To account for the low quantity of arthropod DNA in relation to plant DNA, we used a nested qPCR assay, with a high accuracy for low DNA copy numbers (**Tran et al., 2014**). The sample was first amplified in a regular PCR with 15 cycles using the Qiagen Multiplex PCR kit. Two separate PCRs were run: one using the arthropod primers with an undiluted DNA extract as template, and the other using the plant primers with a 1:100 dilution of the DNA extract. The primers included a 33-bp forward and 34-bp reverse tail, based on Illumina TruSeq libraries, which were complementary to sequences in the qPCR primers. After being cleaned of residual primers with 1× AMPure beads XP, the products of the first PCR were used as template in the qPCR. qPCR was run with the Power SYBR Green Mastermix (Fisher Scientific, Waltham, MA, USA) on an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol, using 35 cycles and an annealing temperature of 55°C. All reactions were run in triplicate and the average of the three CT values used for analysis. CT values showed high reproducibility between triplicate PCRs ($SD_{plant} = 0.13$; $SD_{arthropod} = 0.10$). Non-template controls were run alongside all qPCRs to rule out contamination.

The qPCR efficiency was estimated for the plant and arthropod-specific marker using two ESB tree samples. A 10,000-fold dilution series was used for efficiency estimation. This assay was very stringent, as it corresponds to a dilution of the naturally occurring arthropod eDNA in a plant sample by 10,000. Both assays showed a high efficiency across the dilution series ($E_{Plant} = 94.77\%$, $E_{Arthropod} = 99.73\%$). The 1:10000 dilution ($CT_{plant\ 1.1000} = 15.4$; $CT_{insect\ 1.1000} = 27.2$) is far less than the actual amount of insect DNA in an ESB sample (average $CT_{insect} = 21.3$; average $CT_{plant} = 15.7$), supporting the reliability of our experiment.

To estimate the accuracy of relative arthropod DNA copy detection across diverse arthropod communities, we also performed a spike-in assay, in which a dilution series (10,000-fold) of arthropod mock community DNA was added to a leaf extract and analyzed using qPCR. Seven mock communities were prepared, each containing varying amounts of DNA from 13 arthropod species representing 13 different orders (***Figure 3—figure supplement 1***). The relative copy number of arthropod DNA in relation to plant DNA was estimated using the Delta CT Method (***Schmittgen and Livak, 2008***). The optimized qPCR protocol was then used to quantify the relative DNA copy number of arthropods in all 312 ESB leaf samples.

Statistical analysis

Using USEARCH, an OTU table was built including all samples with the taxonomically annotated zOTU sequences as reference. A species-level OTU table was then generated by merging the zOTUs into their respective *ptp*-based OTU clusters.

The negative control samples were mostly free of arthropod sequences. We found 1.88 arthropod reads per control on average (0–5 reads per control). The recovered reads belonged to taxa that were highly abundant in one of the analyzed tree species, suggesting minor carryover during PCR or sequencing. Based on the negative control samples, we removed all entries in the OTU table with fewer than three reads to counter this possible carryover.

We used two approaches to rarefy our OTU table. First, using rarefaction analysis in vegan (***Oksanen et al., 2013***) in R (v 4.1.0) (***Team RS, 2015***), we explored saturation of diversity. Based on this analysis, 5000 reads were randomly sampled for each of the two PCR duplicates using GUniFrac (***Chen et al., 2018***), and the duplicates were merged into a final sample of 10,000 reads (***Figure 2—figure supplement 4***) after the replicates were checked for reproducible patterns of species composition, α- and β-diversity. To ensure that undersampling did not affect our results, we performed an additional analysis with the unrarefied dataset, which yielded an average coverage of 21,676 reads per sample. A second rarefaction was informed by the relative copy number of arthropod DNA recovered from the tree samples with our qPCR assay. Assuming the copy number reflects biomass, we sampled read numbers proportional to the specific relative copy number for each sample. The copy number-informed and unrarefied datasets showed highly correlated diversity patterns with the dataset rarefied to 10,000 reads ($R^2 \geq 0.91$).

Taxonomic α- and β-diversity were calculated in vegan in R. Quantitative biodiversity assessments by metabarcoding at the community level are likely biased (***Krehenwinkel et al., 2017***). We therefore limited our assessments of α-diversity to richness and β-diversity to binary dissimilarity.

We also measured temporal abundance changes of single OTUs within sites. Within OTUs, temporal changes in read abundance at a site should reflect the relative abundance with reasonable accuracy (***Krehenwinkel et al., 2017***). Only sites spanning a minimum time series of 10 years were included, and we only used species that occurred in at least three sampling events for a particular site and for which at least 100 reads were recovered. This filtering served to exclude rare species, which imitate abundance increases or declines by randomly occurring early or late in the time series. To account for likely fluctuations in abundance, we used the log + 1 of read abundance. Significant increases or declines of abundance over time were estimated for each OTU and site using non-parametric Spearman correlation in R. Both the qPCR-informed and the rarefied datasets were used to calculate species abundance changes.

As mentioned above, zOTUs represent individual haplotypes and thus genetic variation within species. Using the zOTU data, we calculated the haplotype (zOTU) richness within each individual OTU as a complementary measure for genetic variation. A decline in biodiversity could manifest itself in an overall loss of species, which should be detectable at the OTU level. Alternatively, biodiversity decline could initially only affect genetic variation within species, for example, be the result of declining population sizes without actual extinctions. This should be detectable by losses of overall zOTU diversity and zOTU richness within single OTUs. To derive within-OTU genetic diversity, we identified OTUs that consisted of more than one zOTU. zOTU variation could be affected by low abundance sequence noise. Hence, we used the same filtering criteria as described above for the species abundance change. Moreover, we only included zOTUs in our calculations that were present in both technical replicates of a sample. The richness of the remaining zOTUs within each of these OTUs was then calculated.

Arthropods are an ecologically very heterogeneous taxon, with different groups showing very different life histories and possibly responses to ecosystem change. To account for this heterogeneity, we calculated α- and β-diversity metrics for the complete arthropod dataset, as well as the 10 most common arthropod orders in the dataset. Using NMDS ($k = 2$, 500 replications, Jaccard dissimilarity) in vegan, we visualized differentiation of the recovered arthropod communities. We then tested for effects of tree species, sampling year, sampling site, land use type, weather before sampling, amount of leaf material in a sample, climatic variables and detected pesticide load on α- and β-diversity. Also, hand-collected branch clipping samples, as well as the freeze-dried samples, were compared with the ESB samples for their recovered arthropod community composition and diversity.

Factors contributing to β-diversity were evaluated using a PERMANOVA in *vegan*. To evaluate an association of community turnover and copy number variation (e.g., biomass), we also explored patterns of association between these two variables for each site (***Figure 5—figure supplement 2***). Statistical analysis for temporal changes of α-diversity and relative copy number were performed using the *nlme* (v 3.1-159; 2022) (**Bates et al., 2014**) package in R. LMMs were applied to analyze the statistical importance of involved fixed and random effects. Temperature (annual, summer, and winter temperatures), corresponding rainfall data, year, and land use type were treated as fixed effects. Site ID was included as random effect. The Akaike information criterion was used in stepwise regression to identify the final models. A continuous-time first-order autocorrelation model term (corCAR1) was included in the *lme* function to account for serial autocorrelation. The dominant predictor variable is the tree species, which always contributes most to the marginal R^2 in all models. Arthropod DNA copy number (marginal $R^2 = 0.26$) showed negative associations with time ($p < 0.001$) and winter temperatures ($p = 0.038$). None of the other diversity metrics (OTU and zOTU richness, copy number-corrected richness, saturated richness, and genetic diversity) showed an association with time. However, all richness values were positively correlated to winter temperatures and negatively to summer temperatures (marginal $R^2 = 0.45–0.48$, $p < 0.05$). Genetic diversity (marginal $R^2 = 0.34$) was correlated to winter rainfall ($p < 0.001$).

Acknowledgements

We thank Karin Fischer for assistance with lab work. The German Environment Agency made the leaf samples available. Thanks to Diethard Tautz, Natalie Graham, and Rosemary Gillespie for critical revision of an earlier version of the manuscript. Frank Thomas, Dorothee Krieger, and Bernhard Backes provided access to the dried leaf homogenate we used here. Andrea Koerner helped in acquiring the leaf samples from the ESB. SW was funded by a PhD fellowship of the German Federal Environmental Foundation (DBU).

Additional information

Funding

Funder	Grant reference number	Author
Deutsche Bundesstiftung Umwelt		Henrik Krehenwinkel

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

Henrik Krehenwinkel, Conceptualization, Formal analysis, Supervision, Investigation, Methodology, Writing – original draft, Project administration, Writing – review and editing; Sven Weber, Formal analysis, Visualization, Methodology, Writing – original draft, Writing – review and editing; Rieke Broekmann, Anja Melcher, Investigation; Julian Hans, Investigation, Methodology, Writing – review and editing; Rüdiger Wolf, Data curation, Methodology; Axel Hochkirch, Jan Koschorreck, Investigation, Writing – review and editing; Susan Rachel Kennedy, Supervision, Investigation, Visualization, Methodology, Writing – original draft, Writing – review and editing; Sven Künzel, Diana Teubner, Resources, Methodology; Christoph Müller, Rebecca Retzlaff, Sonja Schanzer, Investigation, Methodology; Roland Klein, Conceptualization, Resources, Investigation; Martin Paulus, Conceptualization, Resources, Funding acquisition, Investigation; Thomas Udelhoven, Formal analysis, Investigation, Methodology; Michael Veith, Conceptualization, Resources, Funding acquisition, Writing – review and editing

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Decision letter and Author response

Decision letter https://doi.org/10.7554/eLife.78521.sa1
Author response https://doi.org/10.7554/eLife.78521.sa2

Additional files

Supplementary files

- Supplementary file 1. Primers used in this study.
- Supplementary file 2. OTU table with metadata and quantitative PCR (qPCR) results.
- Supplementary file 3. Contamination check in the cryomill. The table shows the taxonomic annotation for the 10 most abundant host-specific OTUs, as well as all OTUs above 1000 reads for a poplar and a pine ESB sample from 2016 (highlighted in red). The read abundance for the same OTUs in a beech and spruce sample from 2016, which were processed directly after the poplar and pine sample in the mill, are highlighted in red. The OTU abundances for beech and spruce samples from the same sites and processed in consecutive years are also shown. The total read abundance for each sample is shown below the tables (Reads).
- MDAR checklist

Data availability

All raw reads are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.x0k6djhmp). The OTU table with metadata and qPCR results has been uploaded as supplementary files.

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Krehenwinkel H	2022	eDNA from archived leaves reveals no losses of α-diversity, but widespread community turnover and biotic homogenization as drivers of forest insect decline	https://doi.org/10.5061/dryad.x0k6djhmp	Dryad Digital Repository, 10.5061/dryad.x0k6djhmp

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Chapter 3

Archived natural DNA samplers reveal four decades of biodiversity change across the tree of life

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Highlights:

- Nationwide biodiversity analysis of sample-associated communities across the tree of life over almost four decades
- Development of a new dynamic model of community assembly for comparing empirical observations with theoretical predictions
- Stable α-diversity trends for most communities with slight yet statistically insignificant changes
- Substantial compositional changes affecting all communities, occurring faster than expected and resulting in a temporal turnover
- Most terrestrial ecosystems experienced significant declines in spatial β-diversity, potentially resulting in homogenization across space

nature ecology & evolution

Article

https://doi.org/10.1038/s41559-025-02812-6

Archived natural DNA samplers reveal four decades of biodiversity change across the tree of life

Received: 9 October 2024

Accepted: 25 June 2025

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Detecting the imprints of global environmental change on biological communities is a paramount task for ecological research. But a lack of standardized long-term biomonitoring data prevents a deeper understanding of biodiversity change in the Anthropocene. Novel sources of data for analysing biodiversity change across time and space are urgently needed. By metabarcoding highly standardized biota samples from a long-term pollution monitoring archive in Germany, we here analyse four decades of community diversity for tens of thousands of species across the tree of life. The archived samples—tree leaves, marine macroalgae, and marine and limnic mussels—represent natural community DNA samplers, preserving a taxonomically diverse imprint of their associated biodiversity at the time of collection. We find no evidence for universal diversity declines at the local scale. Instead, a gradual compositional turnover emerges as a universal pattern of temporal biodiversity change in Germany's terrestrial and aquatic ecosystems. This turnover results in biotic homogenization in most terrestrial and marine communities. Limnic communities, in contrast, rather differentiate across space, probably due to the immigration of different invasive species into different sites. Our study highlights the immense promise of alternative sample sources to provide standardized time series data of biodiversity change in the Anthropocene.

Global ecosystems have experienced unprecedented human-induced change in the past decades[1–3], with serious consequences for ecosystem functioning and resilience, as well as human well-being[4]. Understanding patterns of biodiversity change in the Anthropocene is crucial for its future protection. To achieve this, standardized, long-term and taxonomically broad biomonitoring data are essential[5,6]. Such data, however, are lacking for most taxa and ecosystems[5,7]. Available time series are often short or incomplete and limited to a few target taxa and study locations[7–9].

A promising solution to this problem is offered by environmental specimen banks (ESBs)—long-term pollution monitoring archives that collect indicator organisms from various terrestrial and aquatic ecosystems[10]. The samples are collected according to highly standardized protocols and stored at low temperatures, which ensures that not only the pollutants, but also the sample-associated nucleic acids, are excellently preserved[10,11]. The indicator species collected by ESBs are associated with diverse communities of interacting taxa,

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https://doi.org/10.1038/s41559-025-02812-6

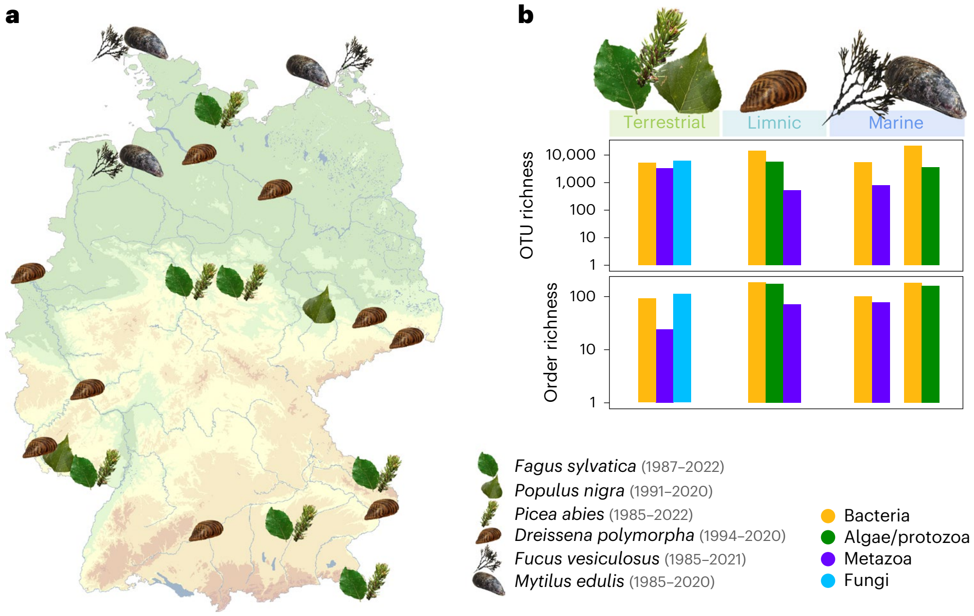

Fig. 1 | Overview of sampling sites and recovered biodiversity from ESB samples from terrestrial, limnic and marine ecosystems spanning a time window from 1985 to 2022. a, ESB sampling sites of tree leaves, zebra mussels, bladderwrack and blue mussels across Germany. **b**, Total OTU and order level richness (log scale) of different taxonomic groups across the tree of life associated with tree leaves, zebra mussels, bladderwrack and blue mussels. Taxonomic groups are represented by different colours. Icons refer to sampled species.

each of which leaves a trace of its DNA in the sample[12–14]. Recent work has shown that many ESB indicator species are excellently suited as 'natural DNA samplers'[15] for studying their surrounding biota via DNA metabarcoding[12–14]. The long-term archives of ESBs can thus provide the standardized time series data so urgently needed to understand biodiversity change[10,11].

Here we use metabarcoding of samples from the German Environmental Specimen Bank, one of the largest, most technologically advanced and longest-operating ESBs, to reconstruct biodiversity change across broad taxonomic, spatial and temporal scales. Using archived leaves from tree canopies, we characterize communities of canopy-associated fungi, bacteria and arthropods. Samples of a dominant European marine macroalgal species reveal coastal bacterial and animal communities associated with the alga. Finally, marine and limnic mussels provide an imprint of the surrounding bacterial and eukaryotic communities in coastal waters and rivers.

Using these data, we explore common patterns of biodiversity change across the tree of life in terrestrial, marine and limnic habitats in Germany over the past four decades. Recent work has highlighted different responses of biota to changing environmental conditions in the Anthropocene[13,16–23]. We explore the generality of these responses by testing three hypotheses for the temporal variation of biodiversity: (1) stressful conditions may have led to extinctions of species at individual sites, that is, local declines of α-diversity[16,20,23]; (2) alternatively, we test whether losses of species are countered by the immigration of novel taxa, leading to a pattern of biotic turnover (β-diversity)[17,19,22]. This turnover could occur (2a) gradually with the changing environment[17] or (2b) rapidly, when the community reaches a tipping point[19,22]. (3) Finally, we test whether the immigration of species across broad geographic scales, for example, of widespread invasive taxa, leads to a pattern of biotic homogenization, that is, a decrease of β-diversity across space[13,18]. To test these hypotheses, we developed a dynamic model for community assembly based on the equilibrium theory of island biogeography (ETIB)[24], which generates null expectations of diversity trends in the absence of disturbance. We further analyse the inferred patterns within the conceptual framework of Blowes et al.[21].

Results

Natural DNA samplers recover biodiversity across ecosystems

We metabarcoded 550 samples of archived natural sampler organisms from three marine, nine limnic and nine terrestrial sites in Germany (Fig. 1a and Supplementary Data 1). Using time series of leaves from trees (1985–2022), marine macroalgae (1985–2021) and marine (1985–2020) as well as limnic mussels (1994–2020), we reconstructed communities associated with these organisms at the time of sampling. Rarefaction and bootstrapping analyses indicated sufficient sequencing depth and sampling size for biodiversity estimations at both local and regional scales (Extended Data Fig. 1 and Extended Data Table 1). Our analysis recovered highly diverse prokaryote and eukaryote communities (Fig. 1b), a total of 66,184 zero-radius operational taxonomic units (OTUs) in 751 orders and 102 phyla. Tree leaves recovered 5,183 OTUs of bacteria in 94 orders, 6,250 fungal OTUs in 113 orders and 3,275 metazoan (mainly arthropod) OTUs in 24 orders. We found 5,474 bacterial OTUs in 101 orders and 787 metazoan OTUs in 78 orders in marine macroalgae. We found 21,266 OTUs of bacteria in 180 orders and 3,551 OTUs of microeukaryotes (mainly algae and protozoa) in 160 orders in marine blue mussels. In limnic zebra mussels, we found 14,292 bacterial OTUs in 184 orders, 5,587 microeukaryote (mainly algae and protozoa) OTUs in 173 orders and 523 metazoan OTUs in 71 orders (Fig. 1b and Supplementary Data 2).

The detected communities accurately represented their respective ecosystems and natural sampler organisms (Fig. 2 and Supplementary Data 3). For example, various typical coastal metazoans were found in bladderwrack samples and numerous OTUs of eukaryotic algae reflect the phytoplankton community surrounding mussels. Typical canopy-dwelling arthropods and leaf-associated fungi and bacteria were recovered from leaves (Supplementary Data 3). The OTUs detected in tree canopies showed a high specificity for their respective host tree, with each tree species harbouring a unique community of leaf-associated taxa (Extended Data Fig. 2). Typical monophagous arthropods or host-specific microorganisms were exclusively found on their respective host tree (Supplementary Data 3). The communities recovered from all sample types were also site-specific, with many taxa limited to single sites, probably indicating their specific

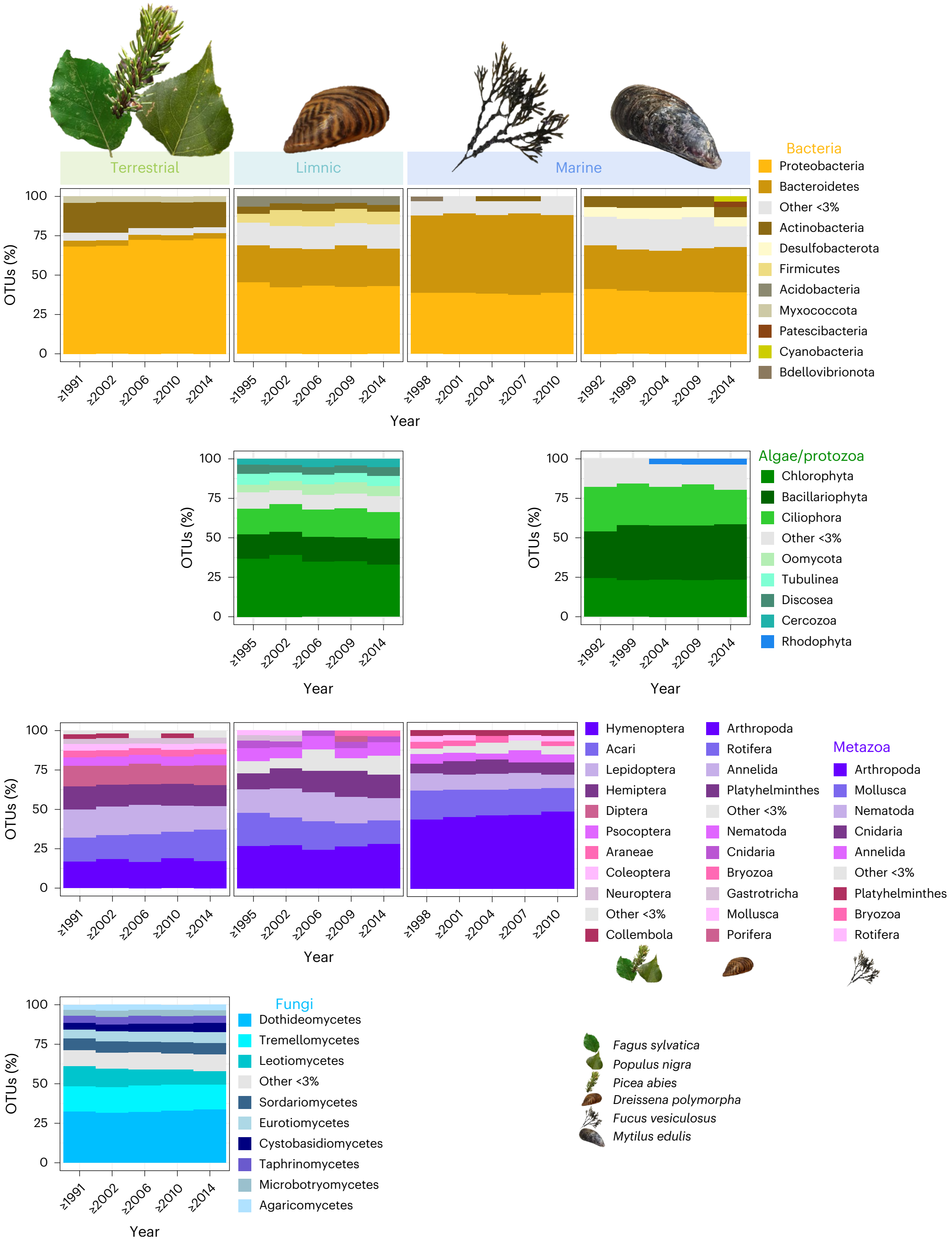

Fig. 2 | Taxonomic composition of bacteria, microeukaryotic plankton, metazoa and fungi detected in ESB samples from 1991 to 2021. Taxonomic composition is based on OTU occurrence and is shown for five time periods, each spanning several consecutive years per sampling type. Community composition is shown at phylum level for bacteria, microeukaryotic plankton and aquatic metazoans. Terrestrial metazoans are shown at order level and fungi at class level. Taxonomic groups are represented by different colour palettes. Icons refer to sampled species.

ecological requirements or biogeographic affinities. For example, highly disparate communities were associated with bladderwrack and blue mussels from the Baltic versus the Northern Sea; these two seas are distinguished by pronounced salinity differences[25]. Additionally, our two Northern Sea sampling sites, which are separated by about 200 km, harboured different sets of taxa. The same held true for the leaf-associated communities at different forest sites across Germany and the zebra mussel-associated communities in different river systems (Extended Data Fig. 2), which have different biogeographic affinities[26].

Natural DNA samplers reveal temporal biodiversity change

We analysed temporal patterns of α-diversity (local OTU richness), temporal β-diversity (changes in community composition over time), spatial β-diversity (biotic homogenization or differentiation

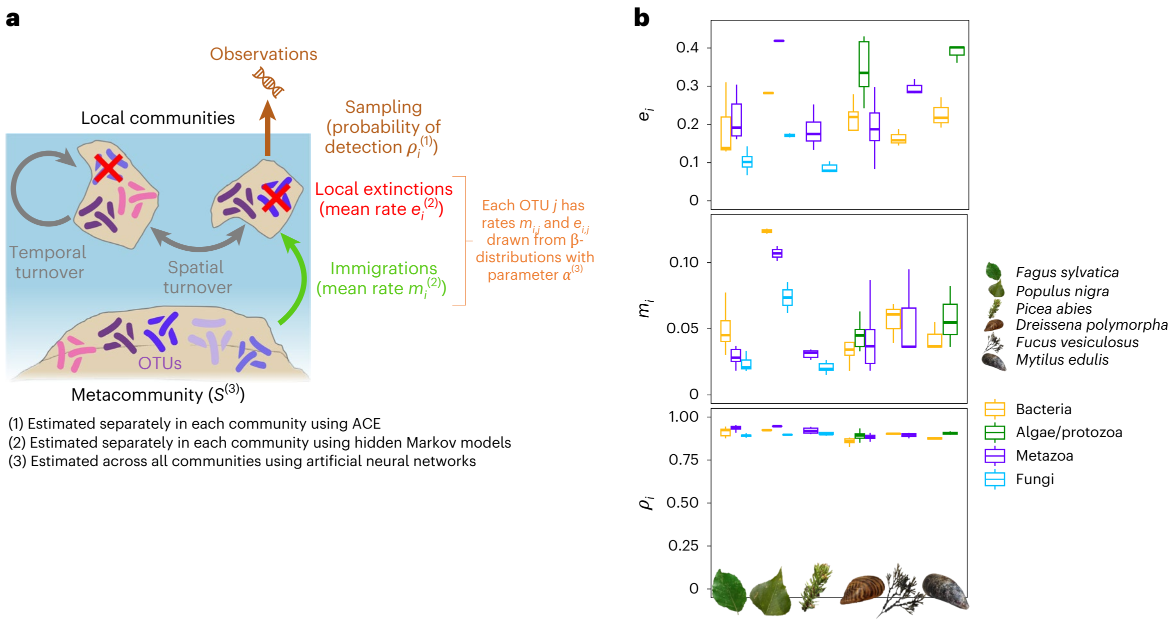

Fig. 3 | A model of community ecology to assess temporal and spatial change in communities associated with natural sampler organisms from the ESB. **a**, Schematic visualization of the non-neutral dynamic model for community assembly derived from the ETIB. **b**, Estimated parameters of extinction rate (e_i), immigration rate (m_i) and detection probability (ρ_i) in different taxonomic groups. Box plots show the median (centre line), the 25th and 75th percentiles (bounds of the box), and the minimum and maximum values (whiskers). No outliers were present in the data. For sample sizes underlying box plots see Data availability statement. Taxonomic groups are represented by different colours. Icons refer to sampled species.

of communities across sites) and γ-diversity (regional OTU richness). To evaluate the significance of the observed trends, we compared them with null expectations of community change in the absence of anthropogenic disturbances. These expectations were based on a dynamic model of community ecology that we developed, built upon the ETIB[17,24] (Fig. 3 and Methods). We validated this model using simulations (Extended Data Table 2 and Extended Data Fig. 3).

We identified no universal trend for α-diversity. Irrespective of ecosystems and taxonomic groups, richness remained stable, increased or declined (Fig. 4a,e, Extended Data Fig. 4a and Extended Data Table 3). A particularly pronounced drop in richness was found in marine prokaryotes. In contrast, richness strongly increased in limnic prokaryotes across the studied river systems. Interestingly, a reverse pattern was found for aquatic microeukaryotes, which showed α-diversity decreases in limnic habitats, but increases in marine sites (Fig. 4a,e). A more consistent pattern was found in terrestrial ecosystems, where most studied communities showed a slight increase of α-diversity across sites. This included a slight, albeit significant, increase in richness for some terrestrial arthropod communities (Fig. 4a,e and Extended Data Fig. 4a).

In contrast to α-diversity, a clear universal trend was found for changes in community composition over time (temporal β-diversity). This trend significantly deviated from the null expectations in all studied communities. We found considerable local extinctions of OTUs in all communities, which, however, were countered by the immigration of other taxa, leading to an out-of-equilibrium dynamic with a stronger-than-expected change in community composition (Fig. 4b,f, Extended Data Figs. 4b and 5a,c and Extended Data Table 3). Slightly distinct temporal dynamics of compositional change were observed across different taxonomic groups (Fig. 3b). In metazoans and microeukaryotes, very high rates of local extinction, combined with high rates of immigration, led to OTU-poor, rapidly changing communities. Bacterial communities were also characterized by high immigration rates, but had lower local extinction rates in comparison with metazoans and microeukaryotes. This resulted in more diverse, dynamic communities. In contrast, lower extinction and immigration rates observed in fungi (Fig. 3b) generated slower compositional change compared with other communities, but still faster than expected. The observed changes were gradual in all communities: no abrupt breaks of community composition were detected (Fig. 4b,f and Extended Data Figs. 4b and 5a,c). Also, the compositional change did not affect higher taxonomic ranks: the temporal composition of phyla, classes and orders associated with the natural samplers remained remarkably stable (Fig. 2). The changes in community composition can be seen at the level of thousands of individual OTUs within our data. Each sampler type and data set showed replacements of various OTUs, with immigrations and local extinctions approximately balanced (Extended Data Fig. 6). We also detected various novel colonizers, among them typical invasive species like the Pacific oyster (*Crassostrea gigas*) or plant pathogens infesting trees across Germany (Supplementary Data 4). At the same time, declines in the occurrence of several taxa are evident, for example, the common periwinkle (*Littorina littorea*) in marine ecosystems and the green silver-lines (*Pseudoips prasinana*) in forest ecosystems.

We then assessed the evidence of biotic homogenization by exploring patterns of spatial β-diversity. Similar to α-diversity, no universal trend across ecosystems and taxonomic groups was evident (Fig. 4c,g, Extended Data Figs. 4c and 5b,d and Extended Data Table 3). Most aquatic communities did not show a clear trend of spatial homogenization over time. Some became even more differentiated across space, for example, marine metazoans. The increasing spatial heterogeneity was especially evident in prokaryotes and microeukaryotes across limnic sites (Fig. 4c,g and Extended Data Fig. 5b,d). In contrast, a general pattern of homogenization across space was evident in terrestrial canopy ecosystems. Nearly all the taxonomic groups, from prokaryotes to fungi and arthropods, showed a (significant) spatial homogenization of communities over time in the terrestrial samples (Fig. 4c,g and Extended Data Figs. 4c and 5b,d). As in the temporal β-diversity, the observed change was gradual rather than abrupt.

We found no universal pattern of γ-diversity across ecosystems and different taxonomic groups (Fig. 4d,h, Extended Data Fig. 4d and Extended Data Table 3). In general, regional richness (γ-diversity)

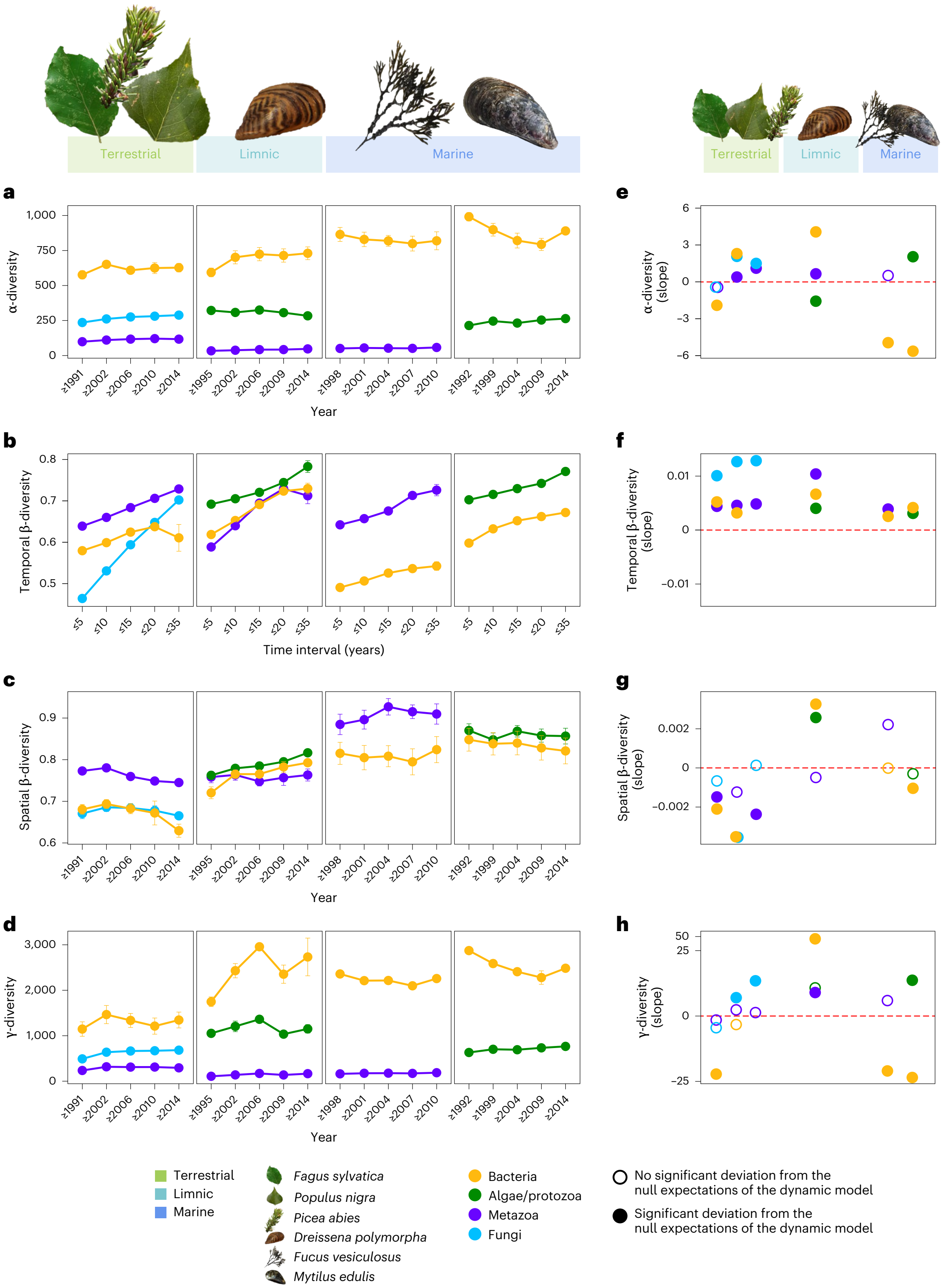

Fig. 4 | Multidecadal trends of α-diversity, temporal and spatial β-diversity, and γ-diversity in communities across the tree of life associated with natural DNA sampler organisms from the ESB. a, Trends of OTU richness (α-diversity) of the associated communities from 1991 to 2021. **b**, Temporal changes in community compositions (β-diversity measured using Jaccard distance) as a function of the time interval (in years) between samples from the same sampling site. **c**, Trends in spatial β-diversity (degree of dissimilarity in community composition between different sampling locations, measured using Jaccard distance) of the associated communities from 1991 to 2021. Results for temporal and spatial β-diversity are qualitatively similar when using only the turnover component of Jaccard distance for β-diversity (Extended Data Fig. 5). **d**, Bootstrap estimates of regional diversity (γ-diversity) for the associated communities from 1991 to 2021. All diversity indices were summarized as mean with standard error bars across sampling locations and/or time windows (and for terrestrial samples across tree species). For a more detailed visualization see Extended Data Fig. 4; for sample sizes underlying means with error bars see Data availability statement. **e**–**h**, Diversity trends from **a** (**e**), **b** (**f**), **c** (**g**) and **d** (**h**) reduced to their respective slopes. Filled circles indicate significant departures from the null expectations generated through the dynamic model for community assembly, suggesting an out-of-equilibrium dynamic. Different ecosystems and taxonomic groups are represented by different colours. Icons refer to sampled species.

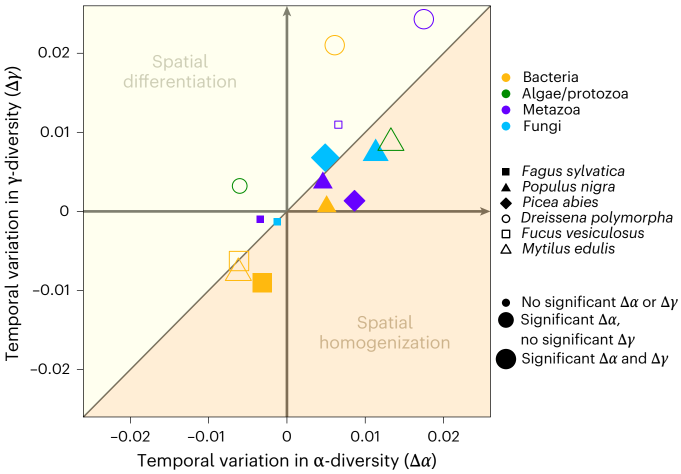

Fig. 5 | Temporal variation of γ-diversity (regional richness) as a function of the temporal variation of α-diversity (local richness). Following the conceptual framework of Blowes et al.[21], $\Delta\alpha < \Delta\gamma$ indicates spatial differentiation of community composition, whereas $\Delta\alpha > \Delta\gamma$ corresponds to spatial homogenization. The significance of the multidecadal variations of α- and γ-diversities of different communities associated with natural DNA sampler organisms from the ESB was assessed by measuring deviations from null expectations generated using the dynamic model for community assembly. Taxonomic groups are represented by different colours. Shapes refer to sampled species with their size indicating the significance level of $\Delta\alpha$ and $\Delta\gamma$.

trends roughly reflect local richness (α-diversity) trends, for example, with marine prokaryotes showing a strong decline, while a pronounced increase was found for limnic prokaryotes.

Finally, we explored the spatial variation of community composition within the conceptual framework of Blowes et al.[21]. This confirmed that most of the studied communities underwent a biotic homogenization (9/15 with $\Delta\alpha > \Delta\gamma$), while others (6/15 with $\Delta\alpha < \Delta\gamma$) experienced spatial differentiation (Fig. 5). Spatial differentiation was particularly significant in prokaryotes and metazoans in limnic sites and in fungi associated with Norway spruce, probably linked to the immigration of low-occupancy taxa across different sites ($0 < \Delta\alpha < \Delta\gamma$; Fig. 5). For instance, a bryozoan *Paludicella* OTU newly colonizing four out of nine sites in German river systems demonstrates this pattern of spatial differentiation. In contrast, spatial homogenization occurred in most terrestrial and marine communities associated with natural samplers. Yet, these communities exhibited variations in the type of homogenization. In most bacterial communities, the homogenization was owing to the local extinction of low-occupancy taxa at the different sites ($\Delta\gamma < \Delta\alpha < 0$; Fig. 5). This is exemplified by the disappearance of a *Wenyingzhuangia* (Bacteroidetes) OTU, which was unique to one of the North Sea bladderwrack sites, or the loss of an endophytic *Gluconacetobacter* (Pseudomonadota) OTU at the few beech sites previously inhabited by the genus. In other communities, homogenization can rather be linked to the widespread immigration of high-occupancy taxa ($0 < \Delta\gamma < \Delta\alpha$; Fig. 5), probably corresponding to invasive species or pathogens like *Taphrina*, a fungus newly infesting both our poplar sites (Supplementary Data 4).

Discussion

Standardized time series data are critical to understand biodiversity change in the Anthropocene[5,6,17]. These data, however, are lacking for most taxa and ecosystems[5,7–9]. Here we show that archived natural samplers provide biodiversity time series data of unprecedented standardization across ecosystems and the tree of life[10,13].

Biodiversity decline is often assumed to be driven by local losses of α-diversity[8,16,20,23,27,28]. However, our data do not support universal declines of α-diversity, refuting our first hypothesis. Most ecosystems did not lose diversity across individual sites; in fact, many communities even showed an increasing α-diversity. For instance, the analysis of archived leaf samples confirms the findings by Krehenwinkel et al.[13], suggesting that forest canopy arthropods are not affected by pronounced insect decline at local scales.

Instead of universal declines of α-diversity, we observe a widespread taxonomic replacement across all studied taxonomic groups. Our data indicate that thousands of species have disappeared from Germany's aquatic and terrestrial ecosystems in the past decades. The local extinctions of species, however, are countered by the immigration of novel, possibly better adapted taxa, supporting our second hypothesis[14,17]. This corresponds to an out-of-equilibrium dynamic in both aquatic and terrestrial ecosystems in Germany, potentially driven by anthropogenic disturbances[29]. The compositional change appears gradual across all taxonomic groups and ecosystems, following our hypothesis (2a). Also, taxonomic replacement mostly affects rather closely related taxa, with no distinct changes in higher-level taxonomic groups. This suggests that the studied communities have probably retained their ecological make-up and taxonomic replacements are functionally redundant[30–32]. Considering this background, we reject the hypothesis of rapid state shifts in communities that have reached tipping points (2b)[19,22]. The observation of a gradual compositional turnover as a predominant pattern of biodiversity change in the Anthropocene is well supported by other recent work[5,17,33,34]. The relatively similar rates of compositional change across all sampler organisms suggest a common driver, for example, climate change[2].

Our results highlight another facet of environmental change: gradual biotic homogenization across space[21,28,35], especially in terrestrial and marine ecosystems. In bacterial communities associated with beech leaves and marine samples, the observed homogenization is probably due to the local extinction of rare taxa[21]. In contrast, other communities showing biotic homogenization may have acquired novel widespread taxa. Such widespread generalist species frequently benefit from environmental change and replace more locally adapted, specialized species[21,36–38]. Consequently, the results support our third hypothesis for marine and especially terrestrial ecosystems. Interestingly, limnic ecosystems show contrasting patterns. The increasing geographic differentiation of aquatic communities in Germany over time might be caused by the ongoing spreading of invasive taxa and/or the immigration of novel taxa. The latter may be facilitated by artificial links between river systems, for example, the Rhine–Main–Danube Canal or shipping[39,40]. Results involving γ-diversity should, however, be interpreted with caution due to few sampling sites and their heterogeneous availability at different times (Supplementary Data 1) in some datasets. Also, some communities shifted from an upward to a downward diversity trend (or vice versa) over time. These limitations may have led to the slight inconsistencies in spatial β-diversity trends (Fig. 4g) and the framework of Blowes et al.[21] (Fig. 5) in limnic and beech-associated metazoans. Yet, the overall picture of either spatio-temporal homogenization or differentiation across different taxonomic groups and ecosystems is consistent across both approaches and supported by recent work on time series data from all across the globe[21,41].

The majority of the increasing or declining taxa that we observe are highly cryptic and rarely detected by monitoring programmes, which focus on prominent taxa like plants[42] or vertebrates[43]. Yet many of the taxa we observe represent critical elements in food webs, for example, phytoplankton in aquatic ecosystems or arthropods in tree canopies[44–46]. Future work should further explore the ecological role of the recovered taxa in their respective ecosystems and food webs. ESB samples can serve as an important early warning system for both the decline of local species and the emergence of pathogens or problematic invaders[13,14].

We here focused on ecosystems across Germany, but future work on natural sampler organisms from other ESBs may also enable the study of biodiversity change at broader geographical scales. ESBs have been established in various countries[47], holding the potential to explore global patterns of biodiversity change with natural samplers. Moreover, recent work has shown that DNA in natural samplers can show a remarkable temporal stability, even without long-term cold storage[48]. For example, community-level DNA has been successfully isolated from plant herbarium specimens[49], which opens up natural history collections as a promising source for future studies on biodiversity change. Natural sampler organisms thus hold great promise to provide the long-term global data that are so direly needed to understand and mitigate biodiversity decline.

Methods

Specimen bank data

This research complies with all relevant ethical regulations. All study protocols are approved by the German Environment Agency.

The German Environmental Specimen Bank (ESB) has been in operation since the early 1980s. The ESB collects samples of indicator species from various terrestrial and aquatic ecosystems. These species serve as accumulators of environmental chemicals and provide a detailed image of pollution and ecosystem health. To make pollution analysis comparable between years, ESB samples are collected according to highly standardized protocols. Samples are taken at the same time of the year, at identical sites and using identical protocols. Collection is done using sterile equipment to avoid carryover of even trace amounts of pollutants between samples. To ensure preservation of unstable chemical compounds, the samples are stored over liquid nitrogen after collection and for the long term, halting all chemical and biological degradation. To acquire an integrative view of pollution in an ecosystem, ESB samples are large, each one including hundreds to thousands of specimens or tissue compartments (leaves in case of trees)[10,50–55]. Each sample is cryomilled to a fine powder of a grain size of 200 µm, thoroughly homogenizing all traces of chemicals[56].

Recent work shows that ESBs are ideal for studies on biodiversity change. ESB indicator species can be considered natural community DNA samplers, which preserve an imprint of the surrounding biological community at the time of sampling. The highly standardized and contamination-free sampling and sample processing conditions, coupled with storage at ultra-low temperatures, make ESB samples perfectly suited for metabarcoding. The cryomilling of large ESB samples also guarantees an even distribution of community DNA traces among the sample and breaks open cell walls of various microorganisms, whose DNA is then uniformly released. Previous studies have already extensively tested and highlighted the suitability of different ESB samples for retrospective biodiversity monitoring[12–14]. Here, we use four different types of ESB samples from terrestrial, limnic and marine habitats as natural community DNA samplers to measure biodiversity change across four decades.

Tree leaves. The ESB collects leaves from three common tree species in Germany, namely European beech (*Fagus sylvatica*), Norway spruce (*Picea abies*) and Lombardy poplar (*Populus nigra*). The leaves are collected once annually or biannually and serve as samplers for aerial pollutants deposited on the leaves[51,52,54]. ESB leaf samples are collected from different forest ecosystem types, spanning a land use gradient from core zones of national parks to timber forests, forests neighbouring agricultural sites, and urban parks. Sample series from nine sites were included in this study, starting from 1985. Each sample contains hundreds of leaves from at least 15 individual trees, milled to a fine powder[51,52,54,56]. These samples contain DNA traces of all organisms that interacted with the tree canopy at the time of collection[13]. Here, we characterize communities of canopy-associated arthropods, fungi and bacteria. The results for arthropod diversity shown in our study represent a novel dataset compared with Krehenwinkel et al.[13], including additional and longer time series and an improved DNA extraction protocol to deal with polymerase chain reaction (PCR) inhibitors. Only the poplar datasets as well as the 16S ribosomal DNA (rDNA) datasets were taken from the original dataset by Krehenwinkel et al.[13].

Bladderwrack (*Fucus vesiculosus*). This macroalgae is widespread along the European coastline, where it makes up a substantial part of the biomass. Marine pollutants are enriched in the tissue of the algae, making it an ideal sentinel species for pollution monitoring[53]. Three sites have been sampled annually or biannually for bladderwrack thalli beginning in 1985. ESB samples from two North Sea sites are collected at intervals of two months, six times a year, and then merged into a pooled annual sample. The third site at the Baltic Sea is sampled twice a year. Bladderwrack is a critical species in coastal ecosystems, providing a habitat for countless taxa. All these taxa leave detectable DNA traces in the ESB sample[12]. Here we characterize communities of animals and bacteria that interacted with the bladderwrack.

Blue mussels (*Mytilus edulis*). This is the most common mussel in coastal ecosystems of northern and central Europe. Blue mussels constantly filter the water column for planktonic organisms and organic particles. In doing so, they enrich pollutants in their tissue, making them an excellent sentinel species for pollution monitoring[55]. The ESB has collected blue mussels at three coastal sites in Germany since 1985. The mussel's entire soft tissue including respiratory water is used for the ESB sample. Annual or biannual samples of hundreds of mussels are compiled from six sampling events at the North Sea and two at the Baltic Sea. With each mussel filtering roughly 1 litre of water per hour, these samples contain a comprehensive imprint of the annual planktonic biodiversity at the sampling site[12]. Here we characterize communities of eukaryotic plankton and mussel-associated bacteria.

Zebra mussels (*Dreissena polymorpha*). The limnic zebra mussel is an invasive species from the Black Sea region, which has colonized nearly all major rivers of Germany since the 1960s[57]. Like the blue mussel, zebra mussels are highly efficient filter feeders. Since the 1990s, zebra mussels are reared by the ESB on special plate stacks, which are then placed in four major German rivers for about one year, allowing the mussels to accumulate pollutants in their tissue. The mussels are then collected from the plate stacks, immediately deep-frozen and a sample of soft tissue including respiratory water is compiled from thousands of mussels[50]. The samples from nine sites used here provide an overview of limnic biodiversity from major rivers of Germany[12]. Here we characterize communities of animals, eukaryotic plankton and mussel-associated bacteria.

Laboratory workflow and sequence processing

Samples were processed as described in refs. 13,14. Work steps were performed on clean benches to avoid carryover and cross-contamination. We isolated DNA from 200 mg of homogenate from each sample. This amount was shown to be sufficient to recover the sample-associated diversity in ESB leaf samples[13] and is assumed to apply to all ESB sample types due to the identical grinding process[56]. DNA was extracted in one or two replicates depending on the sample type (Supplementary Data 5) using a CTAB protocol (OPS Diagnostics), which proved best suited to extract high-purity DNA from these sample types. The DNA extracts were then amplified for different DNA metabarcode markers to enrich various taxonomic groups from the samples (for a list of metabarcode markers and PCR conditions see Supplementary Data 5). PCR was performed using the Qiagen Multiplex PCR Kit in 10-µl volumes according to the manufacturer's protocols. Primers were chosen to amplify the associated community, but not the ESB indicator species itself, whose DNA dominates the extract. To characterize bacterial communities (all sample types), we amplified the V1 or V5–7 region of 16S rDNA[58–60]. For the bladderwrack and tree samples, mitochondrial and chloroplast DNA will be greatly overrepresented over bacterial DNA in the samples. We thus used primers that exclude chloroplast and mitochondrial amplification for these samples. This may also result in slightly lower species numbers of bacteria recovered, but should not affect the community composition within sample types. For terrestrial arthropods (tree leaf samples), we used a mitochondrial cytochrome oxidase I (COI) marker[48]. For fungi (tree leaf samples) we used the ITS1 region of the nuclear ribosomal cluster[61,62]. The variable V9 region of nuclear 18S rDNA was targeted to characterize communities of aquatic animals and eukaryotic plankton (bladderwrack and mussel samples[12]; for primer details, see Supplementary Data 5). PCR success was checked on 1.5% agarose gels, and the PCR products were then amplified in another round of PCR to add Illumina TruSeq adaptors and unique combinations of dual indexes[63] (Supplementary Data 5). All final libraries were pooled in approximately equimolar amounts, cleaned of leftover primers using 1X AMPure beads XP (Beckman Coulter) and then sequenced on an Illumina MiSeq using paired-end sequencing with 500-cycle V2 and 600-cycle V3 kits. To ensure reproducibility of our data and to recover rare species, we ran several PCR replicates for every sample, which were indexed and sequenced separately. The number of PCR replicates was adapted based on sample type and marker, and varied between three and six (Supplementary Data 5). Blank DNA extractions were included in every batch of extractions, and non-template control PCRs were run alongside all PCR reactions. All controls were sequenced along with the samples to provide a baseline for carryover or cross-contamination during processing.

Forward and reverse reads were merged using PEAR[64] with a minimum quality of 20 and a minimum overlap of 50 base pairs (bp). The merged reads were then quality-filtered by limiting the number of expected errors in a sequence to 1 (ref. 65) and transformed to FASTA format using USEARCH[66]. Primer sequences were trimmed off using Unix scripts. Long 18S rDNA amplicons (~350 bp) of limnic metazoan and microeukaryotic plankton generated from zebra mussels were trimmed to match the corresponding short amplicon of ~150 bp. As both metazoan and phytoplankton amplicons span exactly the same nuclear 18S rDNA region, all sequences were combined into one file. Likewise, reads generated from the three tree species were saved to one file for each marker. After trimming, the resulting file for each marker and sample type was dereplicated and clustered into zero-radius OTUs using the USEARCH pipeline. OTU tables were built for each sample type and marker, also using USEARCH. Taxonomy was annotated using blast2taxonomy script v1.4.2.[67] after BLAST searching[68] against the entire National Center for Biotechnology Information GenBank database for 18S rDNA and COI with a maximum number of ten target sequences. The SILVA database[69] was used for annotating 16S rDNA sequences, and the UNITE database[70] for fungal ITS1. The FungalTraits database[71] was used for the functional annotation of fungi. Taxonomic assignments based on BLAST hits with a base pair length of less than 80% of the amplicon length and/or less than 85% sequence identity were removed. We excluded all taxa except bacteria, algae/protozoa, metazoa or fungi from the respective datasets. Some fungal and bacterial DNA in the samples may also stem from small metazoans associated with the samples. These may bias the recovered diversity patterns from these microorganisms. To ensure independence between recovered biodiversity trends of different taxonomic groups, we functionally annotated fungi and bacteria in our terrestrial dataset and excluded those associated with arthropods. Results for the calculated diversity trends did not differ from those presented in Fig. 4. Furthermore, we used the FungalTraits[71] and PLaBAse[72,73] databases to check for the proportion of plant-associated fungi and bacteria in our terrestrial dataset. Of all OTUs with a genus-level annotation and a match in the respective reference database, we verified 34% (fungi) and 76% (bacteria) as plant-associated taxa.

Per OTU and sample replicate, we removed all reads below 3, as this is the read carryover between samples commonly observed in our workflows. The OTU tables were checked for contamination using negative controls by excluding OTUs present in the negative controls from the dataset (typical laboratory contaminants). We only detected negligible contamination in the samples. PCR replicates were merged and all datasets were checked for sufficient sequencing depth and sampling size (Extended Data Fig. 1; for resulting sampling and OTU count as well as number of phyla and orders see Supplementary Data 2 ‘Cleaned dataset’).

Statistical model and analyses of community diversity

For each sample type, we (1) only selected sites that were sampled for at least 5 years; (2) only kept sampling years represented by at least 50% of the sites; and (3) removed years that were isolated from the others (>2 years). We also removed samples with low read coverage (less than 50% of the median number of reads). Finally, because OTU read abundances from metabarcoding datasets are subject to many biases[74], we converted OTU abundances into binary presence/absence data and only analysed trends in terms of OTU occurrence. To limit cross-contamination, we considered an OTU as present in a sample if it represented at least 0.01% of the total reads. A total of 537 samples were included in the analysis (for resulting sampling and OTU count as well as number of phyla and orders per dataset; see Supplementary Data 2 ‘Filtered dataset (model)’ and Extended Data Table 1).

We measured community diversity trends in four different ways. First, in each community, in each year, we computed the α-diversity using the OTU richness as a measure of local diversity at a given time. Second, we computed β-diversities between pairs of communities (1) sampled at the same site in different years; or (2) sampled at different sites in the same year. Option (1) gives an idea of changes in community composition over time (temporal β-diversity), whereas (2) indicates changes in community composition across space (spatial β-diversity). We measured β-diversities using the full Jaccard distances (turnover and nestedness) and the turnover component of the Jaccard distances alone (R package betapart[75]). Last, for each dataset, each year, γ-diversity (regional diversity) was computed using bootstrapping with the specpool function (R package vegan[76]).

To identify temporal trends in these diversity indices, temporal models were fitted using mixed linear models accounting for the temporal autocorrelation between sampling years and the effect of the different sampling sites. We used the lme function (R package nlme[77]) with the corAR1 temporal correlation and the different sites as random effects. We fitted these temporal models with either the α-diversities, the temporal or spatial β-diversities, or the γ-diversities as response variables.

The significance of the observed trends was evaluated by comparing them with null expectations of community changes. Changes in community composition occur as a result of immigration and local extinction events, which are influenced by neutral and/or niche factors. This generates a dynamic equilibrium with ever-changing communities, even in the absence of any kind of disturbance. Following ref. 17, we built upon the ETIB[24] to set up a dynamic model for community assembly that generates null expectations of diversity trends in the absence of disturbance (Fig. 3a). The ETIB is a lineage-based model of species colonization of a local community (the island) from a metacommunity (the continent). In its simplest form[24], at each time step, it assumes that each OTU has a probability m_i to migrate from the metacommunity to the local community i, and once settled in the community, each OTU has a probability e_i to go extinct. The number of new immigration events (that is, of OTUs not already in the community i) per time step is given by $m_i(S - s_i)$, where S is the total number of OTUs in the metacommunity and s_i is the number of OTUs already present in community i; it declines as the number of OTUs in the community increases. The number of local extinction events per time step, given by $e_i s_i$, increases with the number of OTUs in the community. An equilibrium is reached when the number of immigration events per time step equals the number of extinction events, that is, $m_i(S - s_i) = e_i s_i$. The equilibrium number of OTUs in the community is given by $s_i = m_i S/(m_i + e_i)$. This simple form of

ETIB implies a linear decrease (respectively, increase) of the number of new immigration events (respectively, extinction events) per unit of time with the number of settled OTUs in the local community. It assumes that all OTUs have the same probabilities to migrate or go extinct (neutrality), and that these probabilities do not depend on the number of OTUs in the community, implying that there is a negligible influence of interspecific competition on immigration and extinction. It thus applies best to communities that are far from carrying capacity. This model has the advantage of being very straightforward to simulate using a simple discrete-time Markov chain[78].

Given the incomplete sampling of communities typically achieved with metabarcoding techniques, we assumed that each OTU present in community i is observed at each time step with a fixed probability ρ_i. This extra parameter can be handled using hidden Markov models. We assumed that the rates m_i, s_i and ρ_i vary from one community to the other due to various extrinsic and intrinsic factors (for example, distance to the metacommunity, community size and environmental factors).

Assuming neutrality, that is, that all OTUs have the same immigration and extinction rates, is a strong assumption often proved to be unrealistic[79]. We therefore relaxed the assumption of neutrality. In our non-neutral model, we assumed that immigration (respectively, extinction) rates for each OTU are sampled from a beta distribution with parameters α and $\alpha(1-m_i)/m_i$ (respectively, $\alpha(1-e_i)/e_i$). The rates are therefore sampled around m_i (respectively, e_i) with a variance that is inversely proportional to α: a large α corresponds to scenarios of neutrality whereas α closer to 0 indicates that immigration and extinction rates are very different across OTUs. While each OTU has specific immigration and extinction rates in each community, we assumed that the ranks of the OTUs in terms of immigration and extinction rates are conserved across communities (that is, an OTU with a low extinction rate in community i compared with other OTUs also has a low extinction rate in community j). We thus obtained a non-neutral model derived from the ETIB, which assumes that the presence of an OTU in a community results from the balance between immigration and local extinction, and that each OTU is characterized by specific rates of immigration and local extinction centred around the average rates. At equilibrium, some OTUs are more likely to be present in the community (for example, OTUs with higher immigration and lower extinction rates).

Instead of testing different parameter values chosen a priori[17], we implemented an inference strategy to adjust the model parameters to the empirical data using a sequential technique (Fig. 3a). First, in each community, the sampling fraction ρ_i was inferred using ACE (R package vegan[80]). Second, we estimated the average rates m_i and e_i by fitting the neutral model to each community using a hidden Markov model (R package seqHMM[81]). We used these estimates as community-specific estimates of the average rates of immigration and extinction of the non-neutral model. Third, given ρ_i, m_i and e_i, we used simulation-based inferences using artificial neural networks to estimate the parameters S and α. We generated a large number of simulated datasets by sampling S and $\log \alpha$ from uniform prior distributions and simulating the corresponding non-neutral model of community assembly, and for each of these simulations, we recorded α-, spatial and temporal β-, and γ-diversities through time across all the sampled sites. We specifically incorporated subsampling into the simulations (with probability ρ_i), such that not all OTUs present in the local communities are observed, mimicking the detection bias of metabarcoding: simulated α-, spatial and temporal β-, and γ-diversities are therefore directly comparable to empirical diversites. For S, we used a uniform prior distribution between the number of observed OTUs and three times the estimated γ-diversity; for $\log \alpha$, we used a uniform prior between 1 and 5. We started the simulations at year 1500 (providing ample time to ensure they reach equilibrium) with a random community composition at each site (each OTU has a probability s_i/S to be initially present in the community, where s_i is the theoretical number of species at equilibrium in the neutral model, given S, m_i and e_i: $s_i = m_iS/(m_i + e_i)$). Next, we simulated community composition over time in each site until 2023, sampled the communities according to ρ_i and recorded community composition through time for the years of sampling. We then computed for each simulation the α-, β- and γ-diversities: we used the same methods and sampling scheme as for empirical data (the number of sampling sites varied through time) to obtain comparable measures of α-, spatial and temporal β- as well as γ-diversities. We trained an artificial neural network to estimate S and $\log \alpha$ from time series of α-, β- and γ-diversities using the Python library Keras[82], with 100,000 simulations per dataset until reaching a sufficient predictive power. Once trained, the artificial neural network takes as input α-, β- and γ-diversities through time and outputs estimates of S and $\log \alpha$. We used a neural network with three intermediate layers, containing 132, 64 and 32 neurons respectively, and with exponential linear unit (ELU) activation functions. We prevented overfitting by using a dropout of 0.5 at each intermediate layer. Input and output data were scaled between 0 and 1 before fitting, and the simulations were split between the training set (90%) and the test set (10%). Once validated (Extended Data Fig. 3), we finally applied the trained neural network to our empirical data, and obtained corresponding S and $\log \alpha$ values.

Given the estimated S and $\log \alpha$ values, we simulated our non-neutral model 1,000 times with these parameters to generate time series of α-, β- and γ-diversities under an equilibrium model of community assembly. We then compared empirical and simulated temporal trends and considered an empirical trend to be significant if it fell outside the central 95% of the distribution of the simulated trends. P values were computed as the proportions of simulated trends that were higher or lower than the empirical trend. We interpreted significant deviations from the simulated equilibrium model of community assembly as indicative of out-of-equilibrium dynamics in the empirical data, potentially driven by anthropogenic disturbances.

We used simulations to test the validity of our approach that assesses the significance of the observed trends by comparing them to null expectations of community changes under the non-neutral dynamic model for community assembly. We simulated two scenarios: (1) a scenario without any disturbance; and (2) a scenario of biotic homogenization and regional diversity loss. To generate realistic simulations, we designed them using the number of sites, the total number of OTUs, the rates of immigrations and local extinctions, and the sampling probabilities estimated from the bacterial communities of beech (one of the largest datasets). In the first simulated scenario, we simply simulated community changes under the assumptions of our non-neutral dynamic model for community assembly—we therefore did not expect any deviations from the null expectations when applying our approach. Conversely, in the second simulated scenario, we simulated the regional extinctions of 10% of the OTUs and their replacement by widespread invasive OTUs across all communities—we therefore expected no variation of α-diversities, a decrease of spatial β-diversities (spatial homogenization) as well as a decrease of γ-diversities (regional diversity loss). We performed ten simulations for each scenario. When applying our approach, we correctly recovered the simulated scenario in almost all cases (Extended Data Table 2), confirming the validity of our approach.

To interpret the observed variations of community compositions, we used the conceptual framework of Blowes et al.[21]. For each sample type and taxonomic group, we plotted the temporal variation log γ-diversities as a function of the temporal variation of log α-diversities. By construction, $\Delta\alpha < \Delta\gamma$ indicates spatial differentiation of the community composition (increase of spatial β-diversities), whereas $\Delta\alpha > \Delta\gamma$ corresponds to spatial homogenization (decrease of spatial β-diversities).

Finally, we investigated the temporal trends of OTU occurrences, as some OTUs may be recent invaders and others may have gone extinct at the regional scale. For each sample type and taxonomic group, we only looked at OTUs present in at least 10% of the samples and across at least two sites. For each OTU, we first tested whether its occurrence in the communities tended to vary through time. To do so, we fitted a generalized linear mixed model with a binomial response (presence/absence of the OTU in a community) and considered the sampling site as random effects.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available via the Science Data Bank repository at https://doi.org/10.57760/sciencedb.13553 (ref. 83). Raw Illumina sequencing data are available in the European Nucleotide Archive repository under accession number PRJEB88877. Source data are provided with this paper.

Code availability

R code for data analysis and the dynamic model is available via the Science Data Bank at https://doi.org/10.57760/sciencedb.13553 (ref. 83).

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Acknowledgements

We thank K. Fischer for assistance with laboratory work. We want to thank A. Frohböse-Körner from the German Environment Agency (Umweltbundesamt) for her assistance in getting access to metadata for the samples. This work was funded by departmental funds of the department of Biogeography at Trier University and by the TrendDNA project of the German Environment Agency. We want to especially thank the ESB project group at Trier University and the German Environment Agency for providing the time series samples.

Author contributions

I.J., J.H., A.M., S.W., M.S., E.G., C.S., A.S., J.K. and N.S. performed the laboratory work and analysed the data. H.K., I.J., J.H., B.P.-L. and H.M. conceptualized the study, analysed the data and wrote the manuscript. B.P.-L. and H.M. developed the dynamic model for community ecology. S.K. performed the sequencing. M.P., R.K. and D.T. led the sampling. J.K. enabled access to the samples. All authors contributed to revising the final version of the manuscript.

Funding

Open access funding provided by Universität Trier.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41559-025-02812-6.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41559-025-02812-6.

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Peer review information *Nature Ecology & Evolution* thanks Jonathan Chase and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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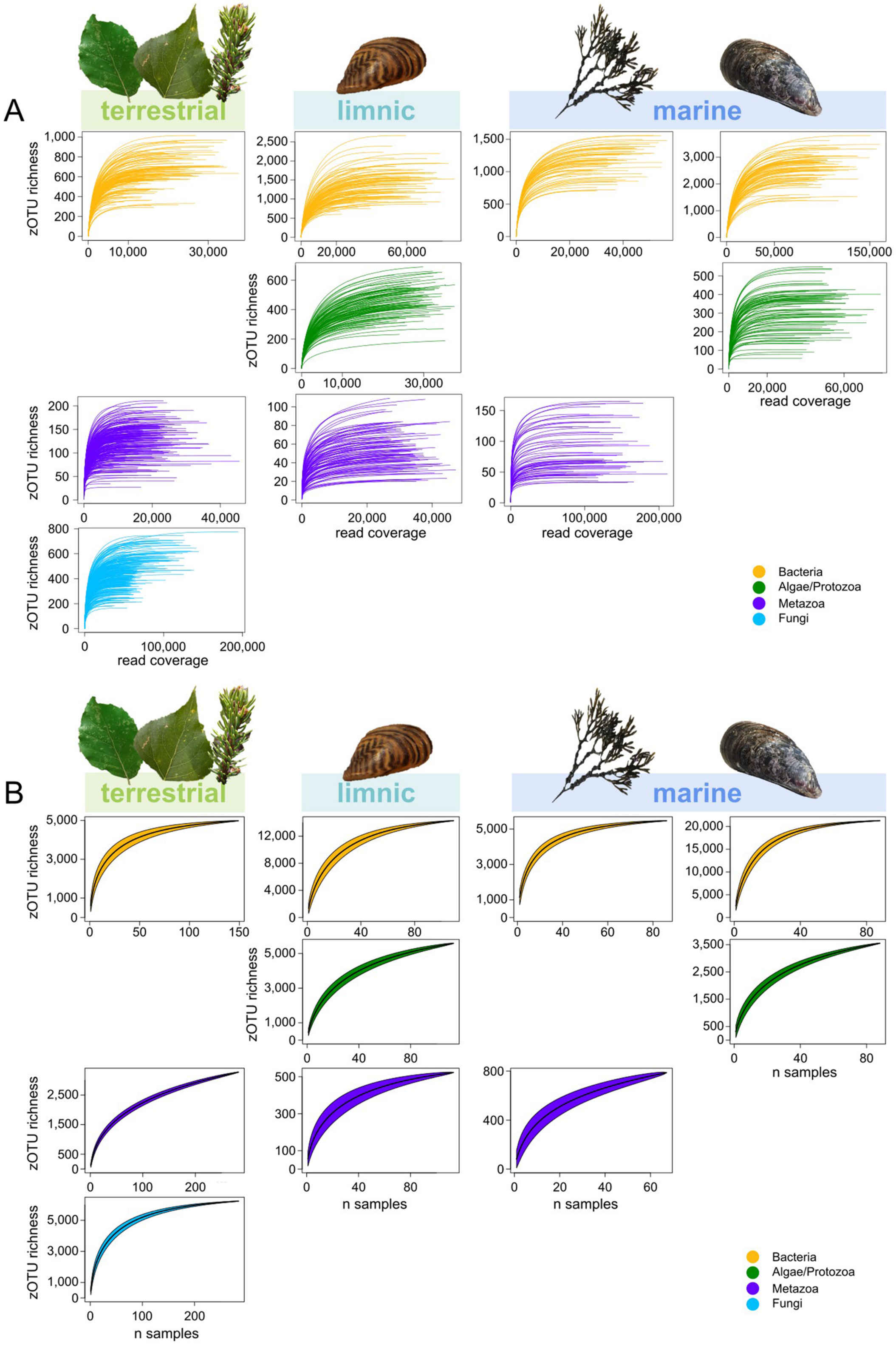
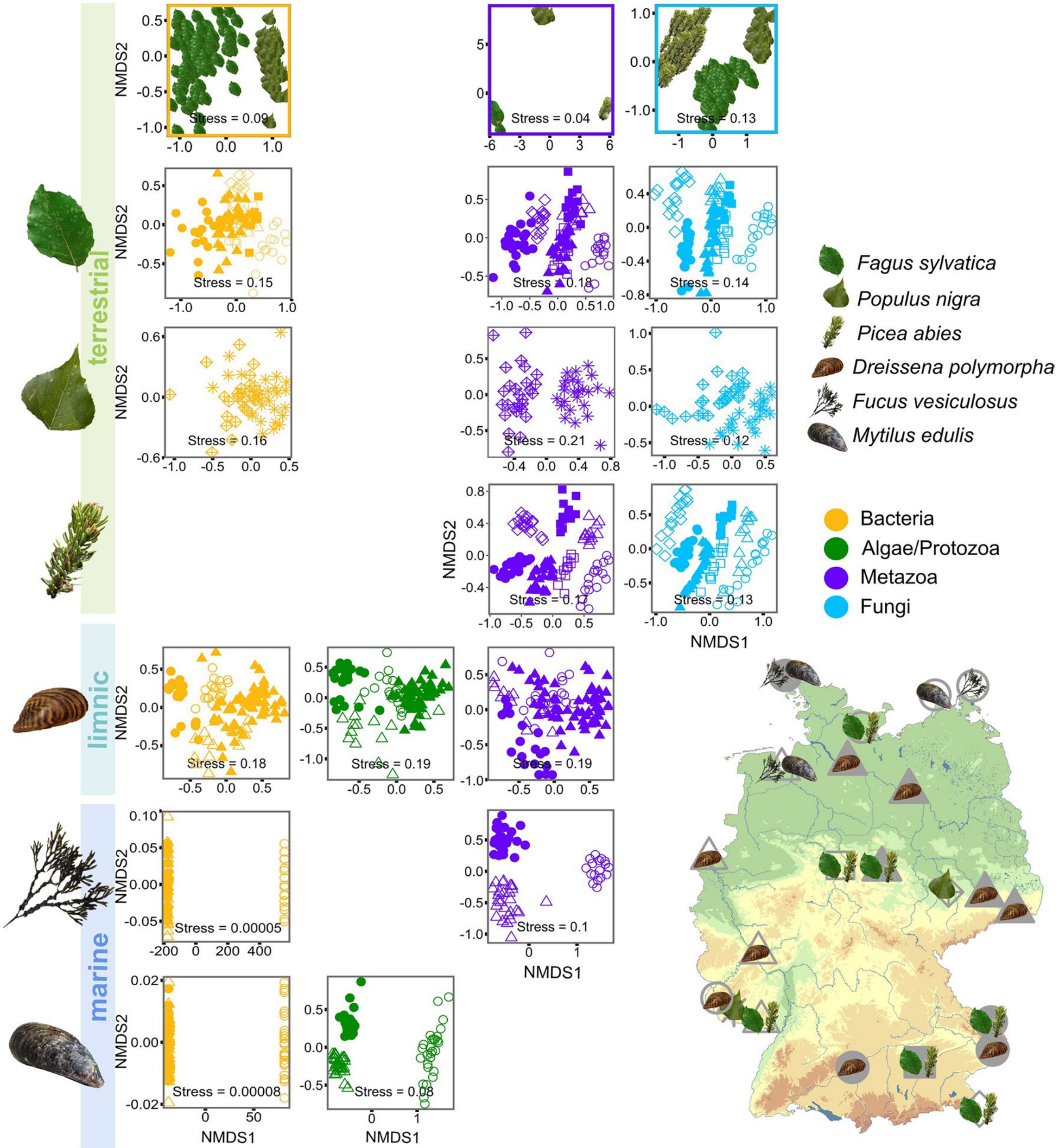
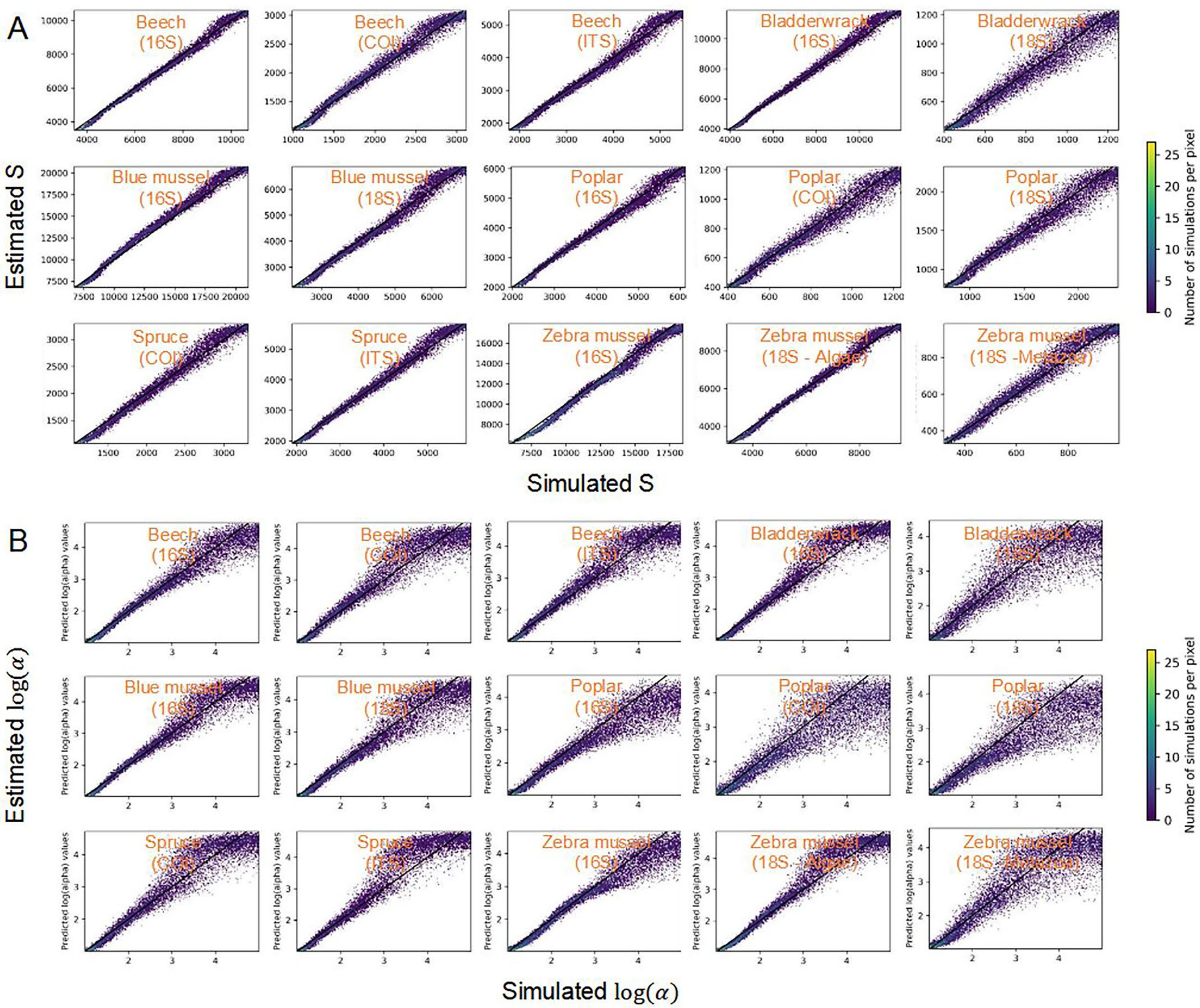


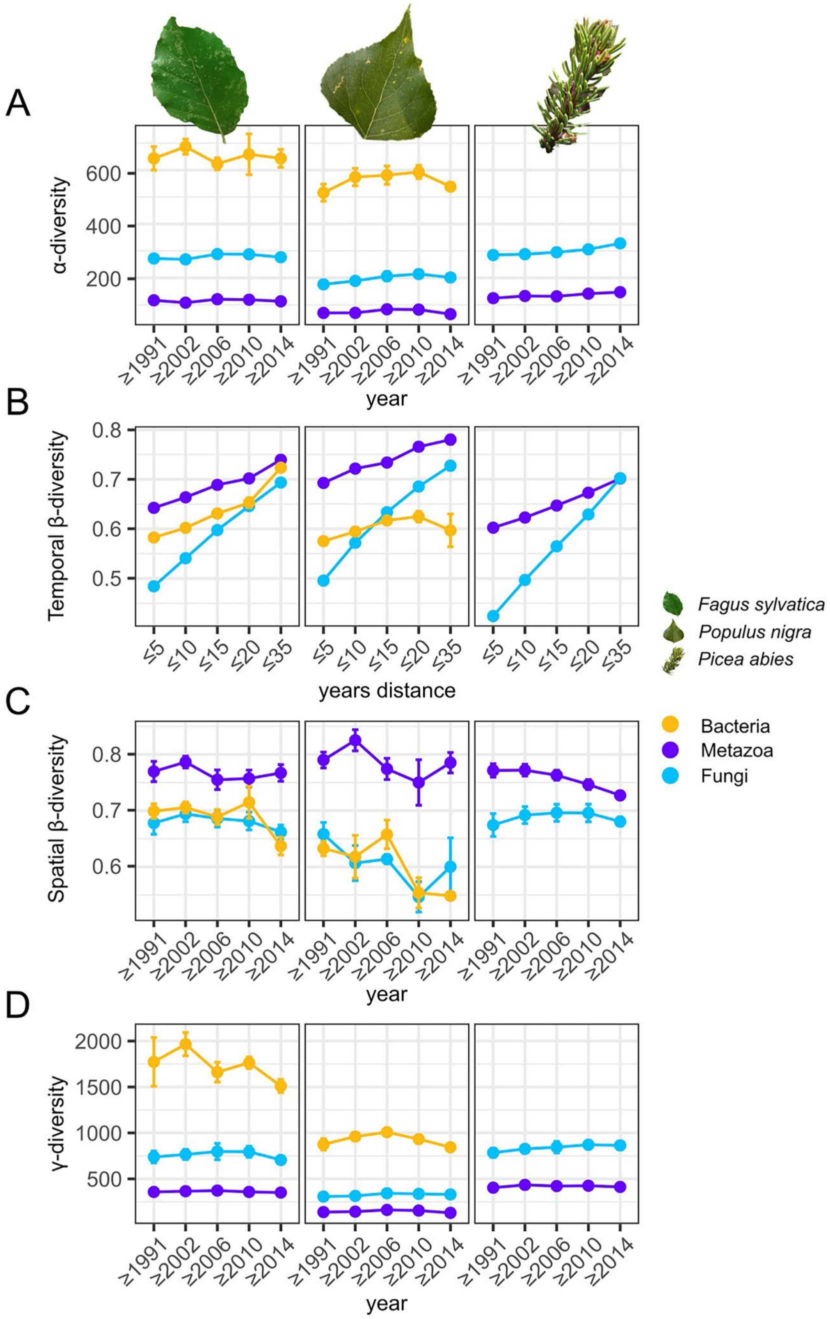
Extended Data Fig. 1 | Rarefaction and accumulation curves depict the saturation of OTU richness for each sampling type. a) Rarefaction curves showing OTU richness as a function of read coverage, with each curve representing an individual sample. **b**) Mean accumulation curves (central black line) and their standard deviation (colored area) from random permutations of the data illustrate the cumulative increase in OTU richness with each additional sample. Taxonomic groups are represented by different colors. Icons refer to sampled species.



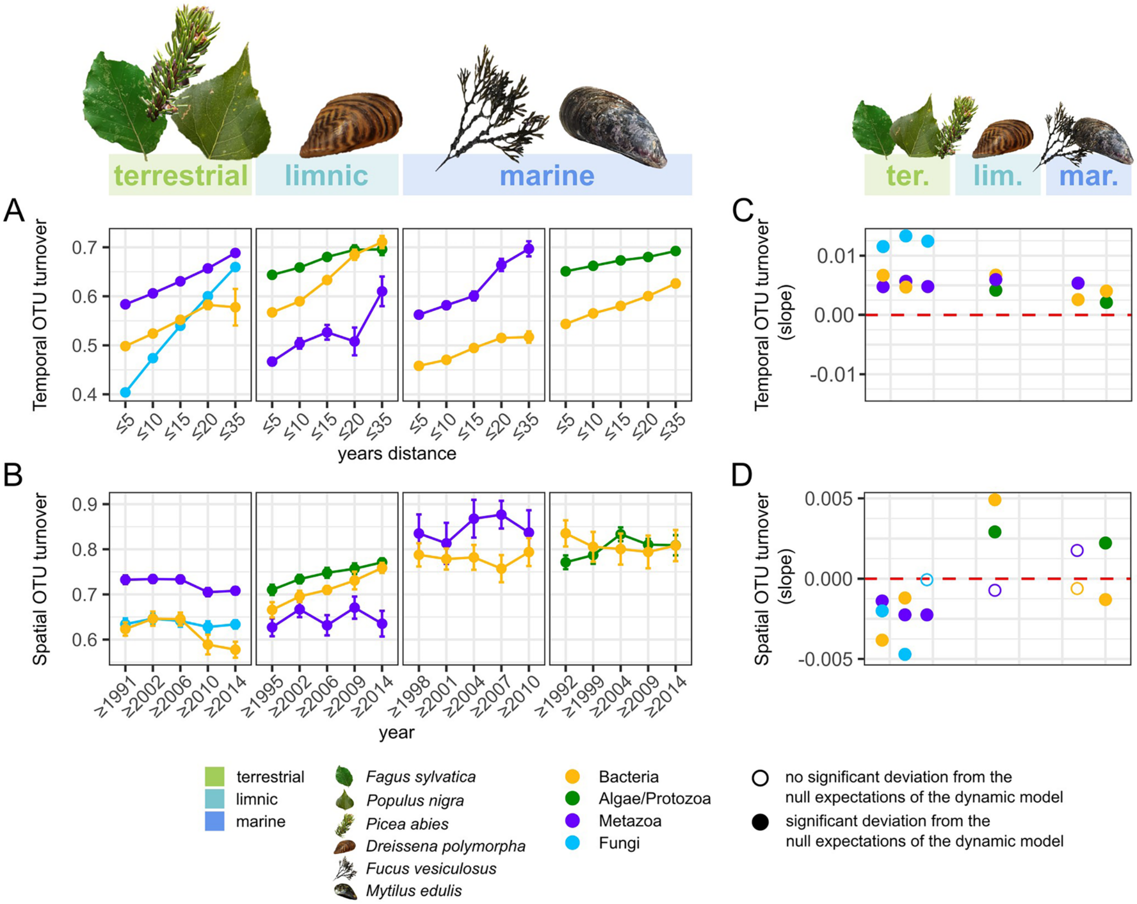
Extended Data Fig. 2 | Map of sampling sites and non-metric multidimensional scaling (NMDS) plots illustrating distinct bacterial, microeukaryotic plankton, metazoan and fungal communities at different sampling sites within terrestrial, limnic and marine ecosystems. The first row shows NMDS plots of Bacteria, Metazoa and Fungi associated with the leaves of three tree species - European beech, Norway spruce, and Lombardy poplar - where each tree species is represented by a distinct icon. Rows two through four illustrate the studied leaf-associated communities for each tree species separately. Rows five to seven show NMDS plots of Bacteria, Algae/Protozoa and Metazoa associated with limnic zebra mussels, as well as marine bladderwrack and blue mussel. Taxonomic groups are represented by colors. Icons refer to sampled species. Shapes denote the sampling location.



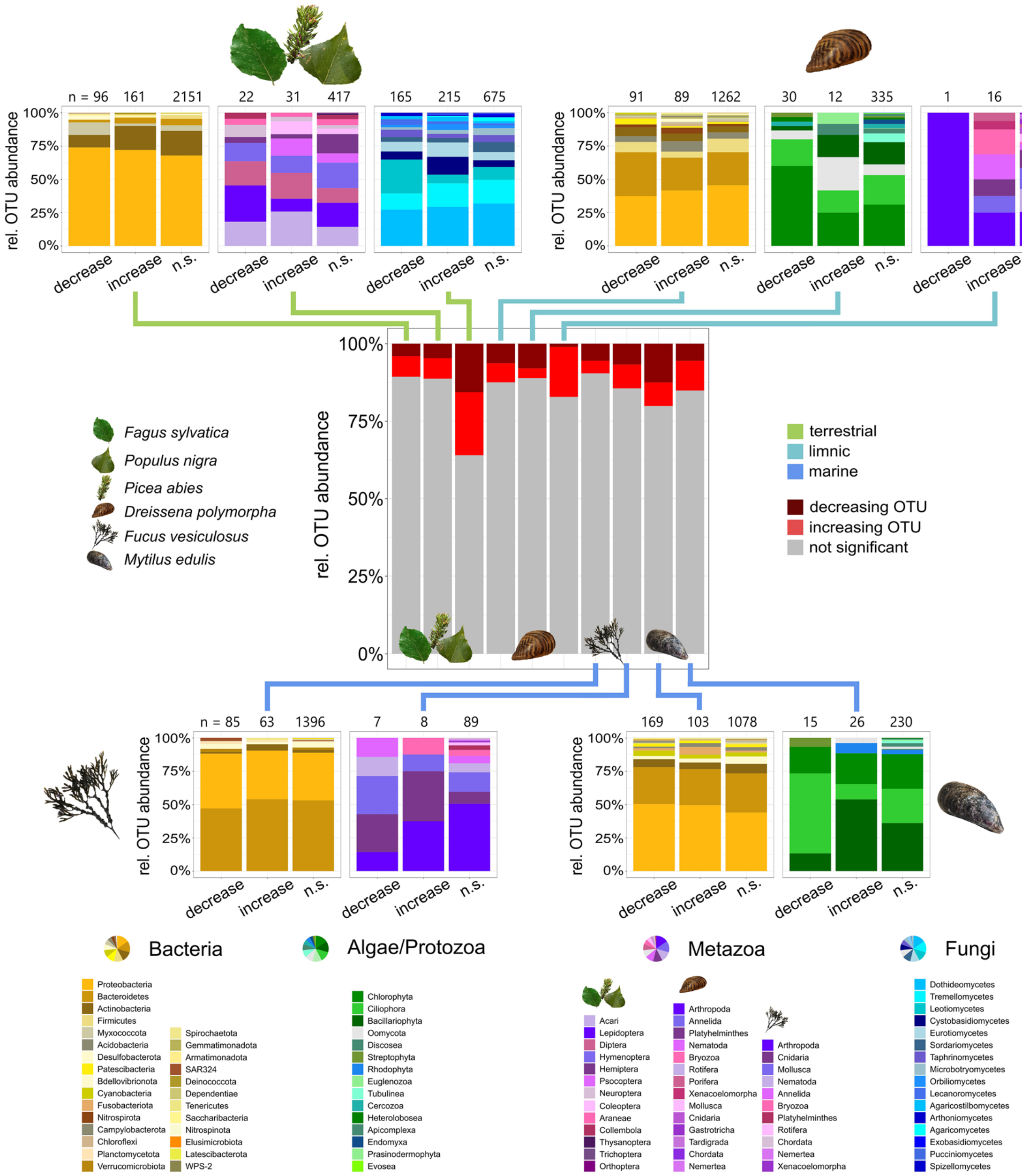
Extended Data Fig. 3 | Validation of parameter estimations using the artificial neural networks for the dynamic model of community assembly. a) Estimated values of the total number of OTUs in the metacommunity (S) as a function of the simulated one. **b**) Estimated values of parameters of the beta distribution (log(α)) as a function of the simulated ones. The black lines y = x represent perfect estimations.



Extended Data Fig. 4 | Multidecadal trends of α-diversity, temporal and spatial β-diversity and γ-diversity in communities across the tree of life associated with tree leaves, with each tree species presented separately. **a**) Trends of OTU richness (α-diversity) of the associated communities from 1991 to 2021. **b**) Temporal changes in community compositions (β-diversity measured using Jaccard distance) as a function of the time interval (in years) between samples from the same sampling site. **c**) Trends in spatial β-diversity (degree of dissimilarity in community composition between different sampling locations, measured using Jaccard distance) of the associated communities from 1991 to 2021. **d**) Bootstrap estimates of regional diversity (γ-diversity) for the associated communities from 1991 to 2021. All diversity indices are summarized as mean with standard error bars across sampling locations and/or time windows. For underlying sample sizes see data availability statement. Different taxonomic groups are represented by different colors. Icons refer to sampled species.



Extended Data Fig. 5 | Multidecadal trends of temporal and spatial OTU turnover in communities across the tree of life associated with natural DNA sampler organisms from the ESB. **a**) Temporal changes in community composition (β-diversity measured using turnover component of Jaccard distance) as a function of the time interval (in years) between samples from the same sampling site. **b**) Trends in spatial β-diversity (degree of dissimilarity in community composition between different sampling locations, measured using turnover component of Jaccard distance) of the associated communities from 1991 to 2021. All diversity indices were summarized as mean with standard error bars across sampling locations and/or time windows (and for terrestrial samples across tree species; for underlying sample sizes see data availability statement). **c**) + **d**) Diversity trends from **a**) + **b**) reduced to their respective slopes. Filled circles indicate significant departures from the null expectations generated through the dynamic model for community assembly, suggesting an out-of-equilibrium dynamic. Different ecosystems and taxonomic groups are represented by different colors. Icons refer to sampled species.



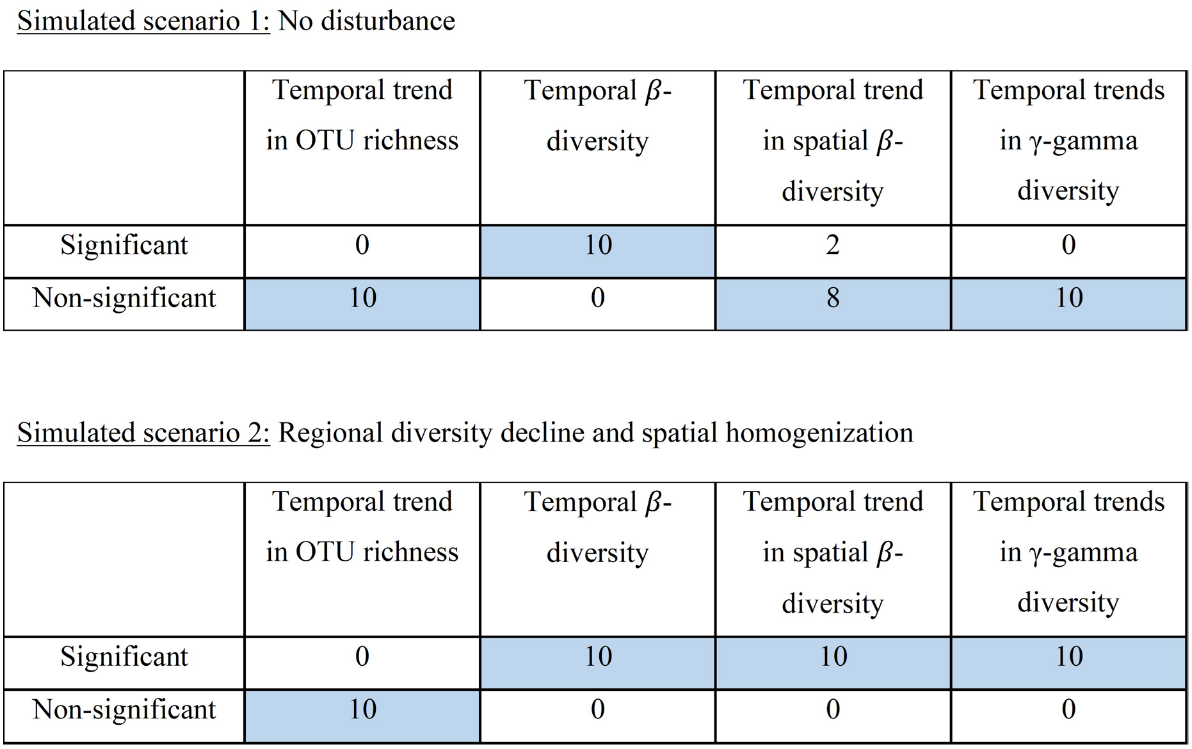
Extended Data Fig. 6 | Proportion of OTUs showing a significant increase, decrease, or no significant trend in occurrence over time and the respective taxonomic composition of each category (increase, decrease, n.s.) for each sampling type and taxonomic group (Bacteria, Algae/Protozoa, Metazoa, Fungi). The central chart represents the proportion of OTUs increasing, decreasing or showing no significant trend per taxonomic group and sample type. From each bar of the central chart a line (indicating the ecosystem type by its color) leads to one of the surrounding bar charts, which illustrates the taxonomic composition of each trend category (increase, decrease, n.s.). Community composition is shown on phylum level for bacteria, microeukaryotic plankton, and aquatic metazoans. Terrestrial metazoans are shown on order level and fungi on class level. Different ecosystems, trend categories and taxonomic groups are represented by different color palettes. Icons refer to sampled species.

Extended Data Table 1 | Estimates of regional diversity indicate that the sampling effort is sufficient to capture most of the diversity

Dataset	Number of observed OTUs	Estimated regional diversity
Beech (16S)	3,469	3,572
Beech (COI)	992	1,038
Beech (ITS)	1,773	1,829
Bladderwrack (16S)	3,865	3,976
Bladderwrack (18S)	399	417
Blue mussel (16S)	6,724	7,007
Blue mussel (18S)	2,207	2,311
Poplar (16S)	1,973	2,040
Poplar (COI)	395	412
Poplar (ITS)	761	788
Spruce (COI)	1,064	1,105
Spruce (ITS)	1,901	1,962
Zebra mussel (16S)	5,891	6,153
Zebra mussel (18S Algae)	3,043	3,183
Zebra mussel (18S Metazoa)	320	332

For each dataset, we reported the observed number of OTUs and the regional diversity estimated using bootstrapping (ACE estimates of γ-diversity).

Extended Data Table 2 | Validation of our approach using simulations



Simulated scenario 1: No disturbance

	Temporal trend in OTU richness	Temporal β-diversity	Temporal trend in spatial β-diversity	Temporal trends in γ-gamma diversity
Significant	0	10	2	0
Non-significant	10	0	8	10

Simulated scenario 2: Regional diversity decline and spatial homogenization

	Temporal trend in OTU richness	Temporal β-diversity	Temporal trend in spatial β-diversity	Temporal trends in γ-gamma diversity
Significant	0	10	10	10
Non-significant	10	0	0	0

For each simulated scenario, we reported the number of simulations with significant or non-significant temporal diversity trends evaluated by comparing them to null expectations under a non-neutral dynamic model for community assembly derived from the equilibrium theory of island biogeography (ETIB). Blue backgrounds indicate the results that are expected for each simulated scenario.

Extended Data Table 3 | Significance of the observed temporal diversity trends evaluated by comparing them to null expectations under a non-neutral dynamic model for community assembly derived from the equilibrium theory of island biogeography (ETIB)

Dataset	Temporal trend in α-diversity	Annual temporal β-diversity (mean β-diversity changes in 1-year intervals)	Temporal trend in spatial β-diversity	Temporal trend in γ-diversity
Beech (16S)	slope=-1.9, p=0.002	mean=0.59, p<0.001	slope=-0.0021, p<0.001	slope=-16.8, p<0.001
Beech (COI)	slope=-0.44, p=0.055	mean=0.63, p<0.001	slope=-0.0015, p=0.005	slope=-0.4, p=0.268
Beech (ITS)	slope=-0.42, p=0.137	mean=0.47, p<0.001	slope=-7e-04, p=0.055	slope=-1.22, p=0.154
Bladderwrack (16S)	slope=-4.95, p=0.001	mean=0.48, p<0.001	slope=0, p=0.489	slope=-14.37, p<0.001
Bladderwrack (18S)	slope=0.52, p=0.091	mean=0.63, p<0.001	slope=0.0023, p=0.077	slope=1.67, p=0.051
Blue mussel (16S)	slope=-5.64, p<0.001	mean=0.58, p<0.001	slope=-0.0011, p<0.001	slope=-19.91, p<0.001
Blue mussel (18S)	slope=2.04, p<0.001	mean=0.69, p<0.001	slope=-3e-04, p=0.168	slope=5.86, p<0.001
Poplar (16S)	slope=2.3, p<0.001	mean=0.57, p<0.001	slope=-0.0035, p<0.001	slope=-0.86, p=0.232
Poplar (COI)	slope=0.4, p=0.021	mean=0.68, p<0.001	slope=-0.0012, p=0.125	slope=0.62, p=0.056
Poplar (ITS)	slope=2.08, p<0.001	mean=0.46, p<0.001	slope=-0.0035, p<0.001	slope=2.17, p=0.003
Spruce (COI)	slope=1.12, p<0.001	mean=0.58, p<0.001	slope=-0.0024, p<0.001	slope=0.34, p=0.242
Spruce (ITS)	slope=1.51, p<0.001	mean=0.38, p<0.001	slope=1e-04, p=0.303	slope=5.7, p<0.001
Zebra mussel (16S)	slope=4.06, p<0.001	mean=0.6, p<0.001	slope=0.0034, p<0.001	slope=46.4, p<0.001
Zebra mussel (18S Algae)	slope=-1.57, p<0.001	mean=0.68, p<0.001	slope=0.0027, p<0.001	slope=3.75, p=0.644
Zebra mussel (18S Metazoa)	slope=0.65, p<0.001	mean=0.56, p<0.001	slope=-5e-04, p=0.354	slope=2.99, p=0.002

This table indicates the temporal trends in α-diversity (OTU richness through time; first column), the annual temporal β-diversity (mean β-diversity changes within one community in one year; second column), the temporal trends in spatial β-diversity (β-diversity across different sites through time; third column), and the temporal trends in γ-diversity (last column). Measures of β-diversity are computed using Jaccard distances. The significance of the empirical temporal trends was assessed by comparing them with the simulated trends. An empirical trend was considered significant if it fell outside the central 95% of the simulated distribution; p-values were computed as the proportions of simulated trends that were higher or lower than the empirical trend (see Methods). Green backgrounds indicate significant diversity increases toward the present, whereas purple backgrounds stand for significant diversity decreases. Gray backgrounds represent annual temporal β-diversity significantly larger than expected.

Chapter 4

eDNA metabarcoding of archived leaf samples reveals arthropod diversity decline in South Korean but not in German forest ecosystems

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Highlights:

- Comparison between trends in arthropod communities of two distant yet similar long-term monitoring archives
- Retrospective biodiversity trends of terrestrial arthropod communities over the last decade in South Korean ecosystems
- Declines in general α-diversity
- Discussion of the importance of long and complete time series as well as reference databases

Received: 30 November 2024 | Accepted: 23 June 2025

DOI: 10.1111/icad.12860

Insect Conservation and Diversity

Royal Entomological Society

ORIGINAL ARTICLE

eDNA metabarcoding of archived leaf samples reveals arthropod diversity decline in South Korean but not in German forest ecosystems

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Funding information

Ministry of Environment (MOE) of the Republic of Korea, Grant/Award Number: NIER-2020-01-01-058

Editor: Raphael K. Didham and Associate Editor: Diana E. Bowler

Abstract

1. Widespread arthropod decline has been reported in numerous studies. However, due to a lack of standardised time series data and a strong geographic bias of available time series, the global extent of this phenomenon is not well understood.
2. While pronounced insect decline in intensively used landscapes is well documented, recent studies have found relatively stable arthropod diversity in forests.
3. Here, we explore 11 years of arthropod diversity change in forests of different levels of anthropogenic disturbance in Germany and South Korea. By eDNA metabarcoding leaf material from the two countries' environmental specimen banks (ESBs)—highly standardised pollution monitoring archives—we reconstruct communities of hundreds of arthropod species.
4. Samples from both ESBs recover a diverse and functionally similar assemblage of plant-associated arthropods. The data from Germany show a temporally stable α-diversity in both anthropogenically disturbed and near-natural landscapes. However, across our sampling locations in South Korean communities, forest arthropod α-diversity declined by, on average, 61% (95% confidence interval: 42.12%–74.99%) between the start and end of the decade.
5. Our results show that the extent of arthropod decline can vary considerably by geographic region and ecosystem and highlight the immense value of biological archives to support long-term biodiversity research.

KEYWORDS

eDNA, environmental specimen bank, forest arthropod communities, metabarcoding, α-diversity decline

INTRODUCTION

Massive losses of arthropod biomass and diversity have been reported worldwide in recent years (Habel et al., 2019; Hallmann et al., 2017;

Julian Hans and Manuel Stothut contributed equally to this study.

Lewinsohn et al., 2022; van Klink et al., 2020). Numerous potential reasons are discussed for this insect decline, ranging from climate change to light pollution, land use intensification and the more frequent use of pesticides (Jaureguiberry et al., 2022; Owens et al., 2020; Uhler et al., 2021; Van der Sluijs, 2020). However, our understanding of this phenomenon is still limited by a lack of standardised, geographically widespread and long-term data on arthropod community diversity (Thomas et al., 2019; Welti et al., 2021). Available time series are usually short, limited to a few sites, lack standardisation, or focus on only a few target taxa. The lack of time series data has led some authors to suggest that observed patterns of arthropod decline might only reflect localised trends or simply be part of long-term population fluctuations (Cameron & Sadd, 2020; Colla & Packer, 2008; Gonzalez et al., 2016; MacGregor et al., 2019; Newton et al., 2023; Wagner et al., 2021).

A promising source of the time series so direly needed to understand patterns of insect decline is environmental DNA (eDNA) from biological archives of plants. When an insect interacts with a plant, it leaves a trace of its DNA, for example, in the form of saliva or faeces (Krehenwinkel, Weber, Künzel, & Kennedy, 2022). This trace can be enriched from the plant tissue and the species identified by metabarcoding, allowing accurate reconstruction of the interacting arthropod community (Weber et al., 2024). The arthropod eDNA on plant material can be remarkably stable, persisting for multiple decades under proper storage conditions, for example, in herbarium collections (Stothut et al., 2024). Even more promising are environmental specimen banks (ESBs), long-term archives of environmental samples, which were initially founded for pollution monitoring (Greaves et al., 2016; Rüdel et al., 2013; Schladot et al., 1992). They have been operating around the globe for more than a decade and have collected samples under highly standardised procedures. These samples are often stored at low or ultra-low temperatures, offering optimal conditions to preserve eDNA (Küster et al., 2015; Rüdel et al., 2015; Zizka et al., 2022). Several ESBs collect large numbers of leaves from forest ecosystems as an indicator of atmospheric deposition of pollutants (Luo, 2018). Moreover, these leaves also contain traces of the interacting community of arthropods at the time of collection (Krehenwinkel, Weber, Broekmann, et al., 2022).

There are a total of 28 ESBs worldwide (Zhao et al., 2015), offering the opportunity to track biodiversity changes. Two ESBs that closely resemble each other in terms of sample types and sampling are the German ESB organised by the German Environment Agency (Umweltbundesamt) and the National Environmental Specimen Bank of South Korea (Korean ESB) at the National Institute of Environmental Research. Both have been in operation for more than 10 years and collect canopy leaves from sites across land cover gradients, from conurbations and agricultural areas to timber forests and national parks. The sampling design, sample processing and sample storage of the Korean ESB is harmonised with that of the German ESB (Klein et al., 2018; National Institute of Environmental Research, 2016a, 2016b; Tarricone et al., 2018a, 2018b). Hence, eDNA from leaf samples of both ESBs provides a unique opportunity to explore long-term forest arthropod diversity trends across ecosystem types (hereafter land use types) in two distant countries. To date, this study is unique in terms of collaboration between ESBs to analyse community trends.

Recent work suggests that insect decline is particularly prevalent in open landscapes exposed to intensive land use, while its effect is less pronounced in more natural ecosystems (Newbold et al., 2015; Outhwaite et al., 2022; Seibold et al., 2019). Especially in dense forests, the extent of insect decline seems to be less pronounced, with some studies even suggesting no temporal losses of forest arthropod α-diversity (Junk et al., 2024; Krehenwinkel, Weber, Broekmann, et al., 2022; Stothut et al., 2024). Considering this background, we here test the hypotheses that (1) no significant decline of arthropod α-diversity will be detected in near-natural forest landscapes of Germany and Korea and (2) a significant decline will be detected in anthropogenically disturbed forest landscapes.

To test these hypotheses, we reconstruct arthropod biodiversity in forest canopy ecosystems of South Korea and Germany by eDNA metabarcoding of archived leaf samples collected in two land use types. Here, we defined core zones of national parks and timber forests as near-natural land use type and forests near agricultural and urban areas as anthropogenic land use type. In total, 28 sampling locations across the two countries were analysed, with 10 near-natural and 7 anthropogenic land use types in Germany and 5 near-natural and 6 anthropogenic land use types in South Korea. In doing so, we provide a highly standardised time series from 2012 to 2022 of arthropod community diversity.

METHODS

Environmental specimen banks and sample collection

The National Environmental Specimen Bank of South Korea (Korean ESB) was established in 2012, while the German Environmental Specimen Bank (German ESB) has been operating since 1985, with both ESBs conducting leaf material collection from tree canopies. ESBs are archives for environmental samples, which are collected using highly standardised protocols (Fliedner et al., 2022). The aim of the ESBs is to assess background contamination with pollutants at sampling locations representing the country's main land use types (i.e., forests in national parks, timber forests, forests near agricultural areas and in urban parks) with the respective samples. To find representative areas for the land use types, potential sampling locations were mapped and analysed by scientific staff. These locations were chosen prior to the initial routine sampling procedure. Trees in the sampled ecosystems are exposed to anthropogenic chemicals which deposit in the tissue and/or on the surface of the leaves, providing insight into the health and status of the ecosystem by these natural samplers. The samples are always collected at the same time of year and in the same locations using highly clean materials to prevent cross-contamination with even very low concentrations of environmental chemicals. Each year, different trees of the same population are sampled to minimise organism-specific biases in pollutant accumulation. This provides a realistic representation of the forest's pollution load rather than

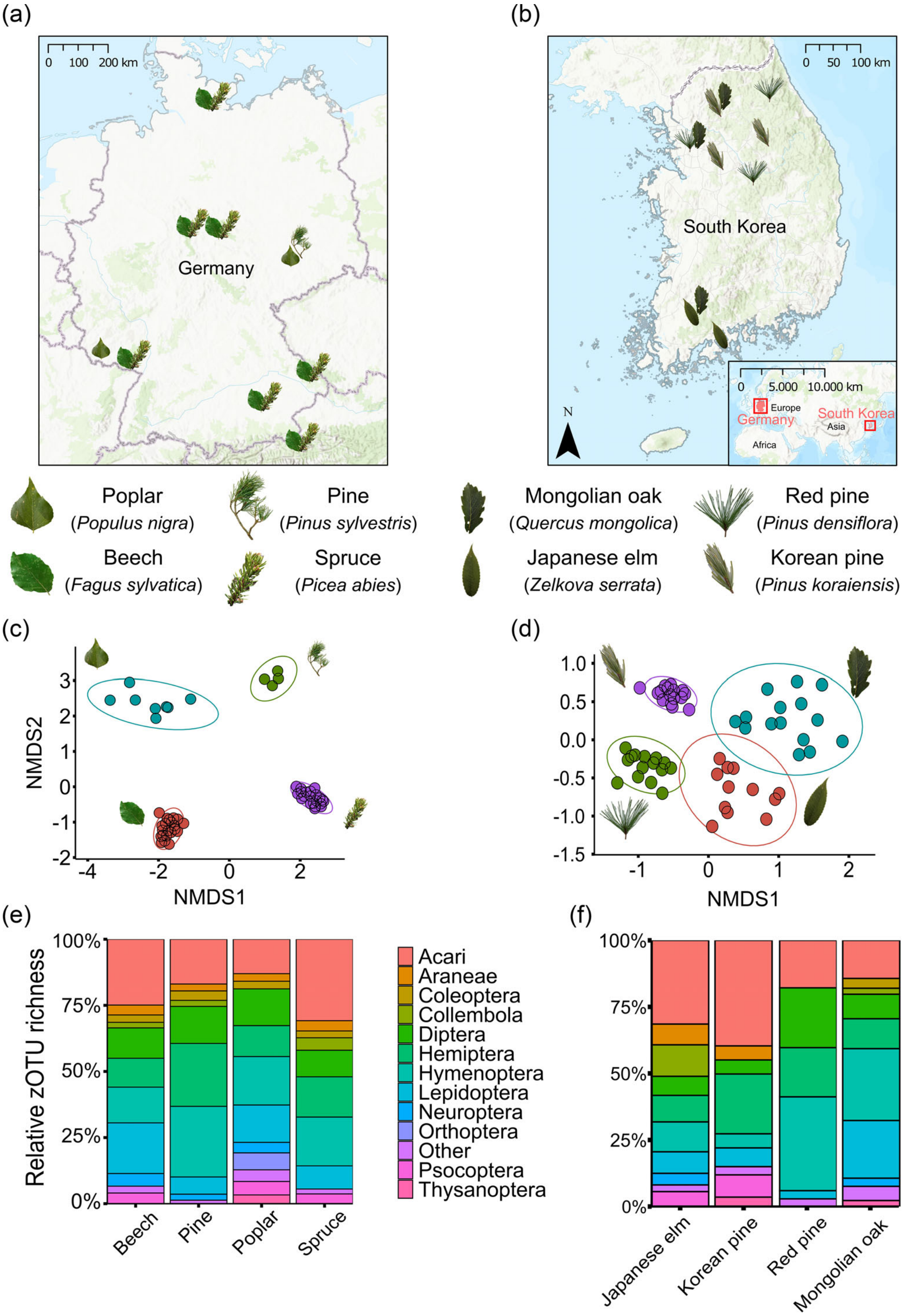

FIGURE 1 The selection of sampling sites and the recovery of diversity and interactions within canopy-associated arthropod communities from leaf material. (a) Sampling sites of the four tree species (European beech *Fagus sylvatica*, black poplar *Populus nigra* 'Italica', Norway spruce *Picea abies*, and Scots pine *Pinus sylvestris*) sampled by the ESB in Germany. (b) Sampling sites of the four tree species (Mongolian oak *Quercus mongolica*, Japanese zelkova *Zelkova serrata*, Japanese red pine *Pinus densiflora*, and Korean pine *Pinus koraiensis*) sampled by the ESB in South Korea. (c,d) NMDS plots showing tree-specific arthropod community composition for (c) the German Environmental Specimen Bank (German ESB) and (d) the National Environmental Specimen Bank of South Korea (Korean ESB). (e,f) Barplots showing recovered taxon composition of zOTUs across the tree species in samples from (e) the German ESB, (f) the Korean ESB. Percent composition was calculated using count data, that is, the frequency with which a given zOTU was detected in a given sample. Taxa amounting to less than 2% of the total zOTU number are merged as 'Other'.

site-specific conditions. The samples are stored at ultra-low temperatures over liquid nitrogen directly after collection in the field, which effectively halts both chemical and biological processes. In order to obtain a representative picture of the studied site, sample amounts are large, with around 1.1 kg of fresh leaves collected for each sampling location per year. Here, fresh material is collected from 10 to 15 trees, depending on the sampling location and pooled to one sample, representing the whole study area. Every sample, each made of hundreds of leaves, is cryo-milled to a fine powder with a grain size of 200 μm, ensuring the thorough homogenisation of all chemical traces present on and in the leaves (Rüdel et al., 2008, 2015).

Recent studies demonstrate that tree samples of ESBs are not only suitable for monitoring environmental chemicals, but also for biodiversity monitoring (Junk et al., 2024; Krehenwinkel, Weber, Broekmann, et al., 2022). Trees are natural DNA samplers that preserve an imprint of the eDNA of the organism interacting with them at the time of sampling and hence reflect the current state of arthropod biodiversity (Krehenwinkel, Weber, Broekmann, et al., 2022). The utilisation of contamination-free sampling techniques in combination with the storage of samples at ultra-low temperatures enables metabarcoding to be performed on these samples. Cryo-milling of the samples not only ensures the even distribution of chemical pollutants but also of nucleic acids. The perfect preservation and the homogeneous distribution of DNA in the samples provide a unique opportunity to conduct biomonitoring studies. The efficacy of this approach has already been demonstrated in previous studies (Junk et al., 2023; Weber et al., 2023).

Here, we use 59 leaf samples from 11 sampling locations of the Korean ESB collected at six available sampling time points between 2012 and 2023: 2012, 2014, 2015, 2016, 2020, and 2022/2023 (Table S1, Figure 1b). Four South Korean tree species were sampled: Mongolian oak (*Quercus mongolica*), Japanese zelkova (*Zelkova serrata*), Korean pine (*Pinus koraiensis*) and Japanese red pine (*Pinus densiflora*). These tree species were selected due to their biological and ecological importance for South Korean forest ecosystems. While all sampling locations are in forests, land cover types within a 10 km radius differ (Table S1). The sampling was designed according to the guidelines of the National Institute of Environmental Research (National Institute of Environmental Research, 2016a, 2016b). As *Q. mongolica* and *Z. serrata* were not sampled in 2022, and *P. koraiensis* and *P. densiflora* were absent in 2023, we analysed the four tree species of these years jointly.

Further, we chose 54 leaf samples from 17 sampling locations of the German ESB collected at the same time points as the Korean ESB (Table S1, Figure 1a). Four German tree species were sampled: European beech (*Fagus sylvatica*), Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*) and black poplar (*Populus nigra* 'Italica') (Klein et al., 2018; Tarricone et al., 2018a, 2018b). Leaves are collected annually or every 2 years, like the Korean ESB samples, with samples dating back to 1985. A complete time-series analysis of zero-radius operational taxonomic units (hereafter zOTUs) richness trends in German canopy-associated arthropod communities is provided (Figure S1; see Krehenwinkel, Weber, Broekmann, et al., 2022 for further information). Like the Korean ESB samples, the German ESB leaf samples represent forests of different land use types (Table S1). Detailed information for all samples analysed in this study, including sampling location, year, respective species, latitude and longitude of sampling location, month of sampling, and mean annual temperature, if accessible, are listed in Table S2.

Molecular processing

Two hundred milligram (±10%) of leaf powder was weighed out without interrupting the cold chain by processing the samples on liquid nitrogen. DNA extraction was performed following the CTAB protocol according to the manufacturer (OPS Diagnostics, New Jersey, USA). For the PCR, we used the primer pair NoPlantF_270 (RGCHTTYCCHCGWATAAAYAAYATAAG) and mlCOIintR_W (GRGGRTAWACWGTTCAWCCWGTNCC), amplifying a 116-bp fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) (Krehenwinkel, Weber, Künzel, & Kennedy, 2022). PCR duplicates were generated for each sample. One microliter of template DNA was used in a 10-μl reaction, following the Qiagen Multiplex PCR kit protocol, with 35 cycles and an annealing temperature of 46°C (Qiagen, Hilden, Germany). PCR success was checked on 1.5% agarose gels. We then performed an index PCR as described above with six cycles at an annealing temperature of 56°C to attach sequencing adaptors to each sample. All samples received unique combinations of forward and reverse TruSeq indexing primers (Illumina, San Diego, CA, USA). The final libraries were checked for amplification success on 1.5% agarose gels and pooled into approximately equal amounts based on the intensity of the gel bands. Final pools were cleaned using 1X AMPure XP magnetic beads (Beckman Coulter, California, USA) and were sequenced on an Illumina MiSeq using several 500-cycle V2 kits, with an estimated sequencing depth of 20,000 reads per PCR replicate, which has been shown to saturate the recovered diversity in this sample type (Krehenwinkel, Weber, Broekmann, et al., 2022). In addition to all experiments, 32 negative controls of all DNA extractions and PCRs were prepared and sequenced alongside the samples to evaluate levels of carryover and contamination. Details of the laboratory workflow are provided (Table S3).

Sequence processing

Sequence analysis followed Krehenwinkel, Weber, Künzel, and Kennedy (2022). Reads were demultiplexed by dual index combination using CASAVA (Illumina) with no mismatches allowed, and merged using PEAR (Zhang et al., 2014) with a minimum quality of 20 and a minimum overlap of 50. Batch quality filtering and conversion to FASTA format were performed using the FASTX Toolkit (Gordon & Hannon, 2011), with a threshold of a minimum of 90% of bases > Q30. Sed was used to trim primer sequences, allowing degenerate sites to vary and retaining only sequences that began with the forward primer and ended with the reverse primer. Next, reads were dereplicated using USEARCH (Edgar, 2010) and were then clustered into zOTUs using the unoise3 command (Edgar, 2016). Chimeric sequences were removed de novo during zOTU clustering. We identified zOTUs to the lowest possible taxonomic level using BLAST search (Altschul et al., 1990) against the National Centre for Biotechnology Information (NCBI) database (downloaded January 2023) retaining the top 10 hits to check for conflicting assignments. Only zOTUs that were assigned to an arthropod sequence with a minimum length match of 54 bp were kept for further analysis. For the taxonomic annotation of zOTUs, species were identified at a minimum of 99% similarity to a reference sequence, genera at 98%, families at 95% and orders at 85% based on the methods described in Stothut et al. (2024). USEARCH was used to build an OTU table. Further, ecological annotations for all recovered taxa were assigned using the databases 'Plant parasites of Europe', 'Global Biodiversity Information Facility' (GBIF) and 'iNaturalist' (Ellis, 2020; GBIF, 2020; iNaturalist, 2023).

Statistical analysis

The statistical analysis was performed in RStudio, 2022.7.1.554 (RStudio Team, 2022) and R 4.2.2 (R Core Team, 2022). For data management and plotting, the package tidyverse v2.0.0 was used (Wickham et al., 2019). Based on recovered read numbers in controls, we set all read occurrences below four to zero to prevent possible index carryover between samples. This was done as the highest read occurrence in our negative control was three. All replicates of the same sample were merged. As *Q. mongolica* and *Z. serrata* were not sampled in 2022, and *P. koraiensis* and *P. densiflora* were absent in 2023, we analysed these years jointly.

To check whether sequencing depth was sufficient, rarefaction curves were created using the package vegan v2.6-4 (Oksanen, 2020), showing a saturation for all samples. As metabarcoding data can suffer from quantitative biases, we only used presence/absence-informed measures of diversity in downstream analyses. Following this, non-metric multidimensional scaling plots (NMDS; $k = 2$, trymax $= 999$, binary Jaccard distance) were created in vegan (Oksanen et al., 2007) to visualise community differentiation (Figure 1c,d). Effects of tree species, sampling location, land use type and year on the ordination were tested with permutational analysis of variance (PERMANOVA) using the package vegan. We defined land use types as near-natural or anthropogenic forest landscapes, with the first representing mostly undisturbed forests (i.e., timber forest and core zones of national parks) and the latter representing forest types with significant anthropogenic disturbance (forest close to agricultural areas or in urban parks). Land use types, that is, near-natural and anthropogenic forest landscapes, differ in their size, with near-natural landscapes exhibiting larger connected forests than anthropogenic landscapes. The difference between tree species was further tested post-hoc using the pairwise Adonis package (Martinez Arbizu, 2020).

Subsequently, trends of zOTU richness (α-diversity) over time were analysed. The α-diversity was calculated as the total zOTU richness of each site within each year. To test which model is best-suited, we first checked for overdispersion (Table S4). Since all values indicated overdispersion, we tested negative binomial generalised linear mixed models (GLMMs) including sampling location as a random effect and land use, year and tree species as fixed effects for both countries separately, instead of a Poisson model. The estimated variance of the random effect was small for both countries (Germany: 0.005; South Korea: 5.1×10^{-94}). The low variance indicates that the model may be over-parameterised, which increases the risk of instability. Further, it suggests that there is little site-specific variation in Korea, which is why we decided to use negative binomial generalised linear models (GLMs) instead for further analyses. We first tested for an interaction term of year and land use type, as well as the effect of tree species as a fixed factor. However, the interaction term was not significant (Germany: $p = \text{f}$; South Korea: $p = 0.227489$), indicating no differences in trends between near-natural and anthropogenic forest landscapes. As a result, the interaction term was excluded from the final models, which included year, land use type and tree species as fixed effects in the analysis of α-diversity trends (Table 1), using the default settings of R stats (v4.2.2).

Further, to check for consistency between zOTU and arthropod species richness trends, α-diversity trends over the time were additionally conducted. For this approach, we filtered zOTUs with a minimum match of at least 99% identity to a reference sequence and a minimum 54 bp length, and sequences belonging to the same species were merged. GLMs with the fixed factors year, land use type and tree species were tested here.

The model was fitted for each dataset, that is, Korean and German ESB data separately, using the glm.nb function (R package MASS) (Venables & Ripley, 2007) with a log link function, allowing the model coefficients to be interpreted as multiplicative effects on the mean richness.

Lastly, we computed β-diversities between pairs of communities sampled at the same sampling location in different years, that is, changes in community composition over time (temporal β-diversity). We measured β-diversity using the full Jaccard distance (R package vegan). Here, we used linear models (LMs), as the Q–Q plots indicated a Gaussian distribution of the residuals and the Breusch–Pagan test revealed no significant heteroscedasticity (Germany *p*-value $= 0.05952$, South Korea *p*-value $= 0.1314$).

RESULTS

Tree-specific arthropod communities

We used a total of 113 samples from archives of the German and South Korean ESB from 28 different sampling locations. By eDNA metabarcoding these samples, we were able to reconstruct the community associated at the time of sampling. The saturation of all rarefaction curves indicated a sufficient sequencing depth for each sample allowing for further data analysis. We recovered 659 zOTUs from the Korean ESB, and 1113 zOTUs from the German ESB. Four arthropod

TABLE 1 Negative binomial generalised linear model (GLM) to analyse the effects of year and tree species on zOTU richness of different land use types in the German and Korean Environmental Specimen Bank (ESB) time-series.

Predictors	Land use type	Estimate	Std. error	*p*-value	Observation	Region
Year	Near-natural	−0.0825	0.0271	**>0.01**	27	South Korea
TreeSpecies Korean_Pine	Near-natural	−0.7120	0.2928	**>0.05**	27	South Korea
TreeSpecies Mongolian_Oak	Near-natural	−0.2823	0.2833	0.3191	27	South Korea
TreeSpecies Red_Pine	Near-natural	0.0492	0.2334	0.8332	27	South Korea
Year	Anthropogenic	−0.1294	0.0263	**>0.001**	32	South Korea
TreeSpecies Korean_Pine	Anthropogenic	−0.6386	0.2589	**>0.05**	32	South Korea
TreeSpecies Mongolian_Oak	Anthropogenic	0.2500	0.2462	0.3099	32	South Korea
TreeSpecies Red_Pine	Anthropogenic	0.0149	0.2946	0.9595	32	South Korea
Year	Near-natural	−0.0024	0.0111	0.8318	34	Germany
TreeSpecies Spruce	Near-natural	0.0507	0.0782	0.5162	34	Germany
Year	Anthropogenic	0.0235	0.0282	0.4044	20	Germany
TreeSpecies Pine	Anthropogenic	0.3761	0.1601	**>0.05**	20	Germany
TreeSpecies Poplar	Anthropogenic	−0.1529	0.1449	0.2913	20	Germany
TreeSpecies Spruce	Anthropogenic	0.1572	0.1619	0.3317	20	Germany

Note: Significant *p*-values are printed in bold.

classes, 16 orders, 100 families, 128 genera and 97 species were identified in Korean ESB samples, with Hymenoptera being the richest order (198 zOTUs in 23 families). The German ESB samples included four classes, 20 orders, 162 families, 323 genera and 346 species of arthropods, also with Hymenoptera being the richest order (240 zOTUs in 26 families). The tree species showed relatively similar order-level composition in Germany and Korea (Figure 1e,f). For both countries, presence-absence data were summed for each order to calculate an occurrence-based frequency, with Acari emerging as the most frequent group, followed by Hymenoptera and Hemiptera. Moreover, similar taxa appeared in comparable abundances in both time series. The detected arthropod species were ecologically diverse and included predators, parasites, parasitoids and phytophages (Figure S5). Each tree species showed a species-specific arthropod community in both Korea and Germany (Figure 1c,d, PERMANOVA *p*-value < 0.01 All). Further, we found significant effects of sampling location (PERMANOVA Korean ESB *p*-value < 0.01, German ESB *p*-value <0.01) and land use type (PERMANOVA Korean ESB *p*-value < 0.01, German ESB *p*-value < 0.05) on the ordination. The effect of the sampling year was solely significant for the Korean ESB (PERMANOVA Korean ESB *p*-value < 0.05, German ESB *p*-value = 0.314). Many characteristic arthropod species for certain tree species were detected, such as *Pineus boerneri*, a pest which infests pines and was a very abundant species on Korean *Pinus densiflora* (Cardoso et al., 2021; Havill et al., 2023), and *Tinocallis viridis*, an aphid found on elm, which is highly associated with plants belonging to the family Ulmaceae (Lee et al., 2017). The recovered arthropod communities also closely reflected their respective origins in Europe and Asia. For example, we found typical South Korean species, like *Peridea lativitta*, *Myrmarachne japonica* and *Stomaphis japonica*, in the Korean ESB samples. At the same time, German leaf samples showed the presence of typical European taxa, like the Norway spruce shoot gall midge *Piceacecis abietiperda* and the beech gall midge *Mikiola fagi*. A list of zOTUs found with 100% match to a reference sequence over 100% of the amplicon length are further provided (Table S5). Additionally, a list with zOTUs (100% match to a reference sequence over 100% of the amplicon length) and arthropod species exclusively found in anthropogenic or near-natural forest landscapes is accessible (Table S6). This was done for the German and South Korean dataset separately.

Temporal biodiversity trends

The zOTU richness of arthropod communities derived from samples from the German and Korean ESB was analysed from 2012 to 2022

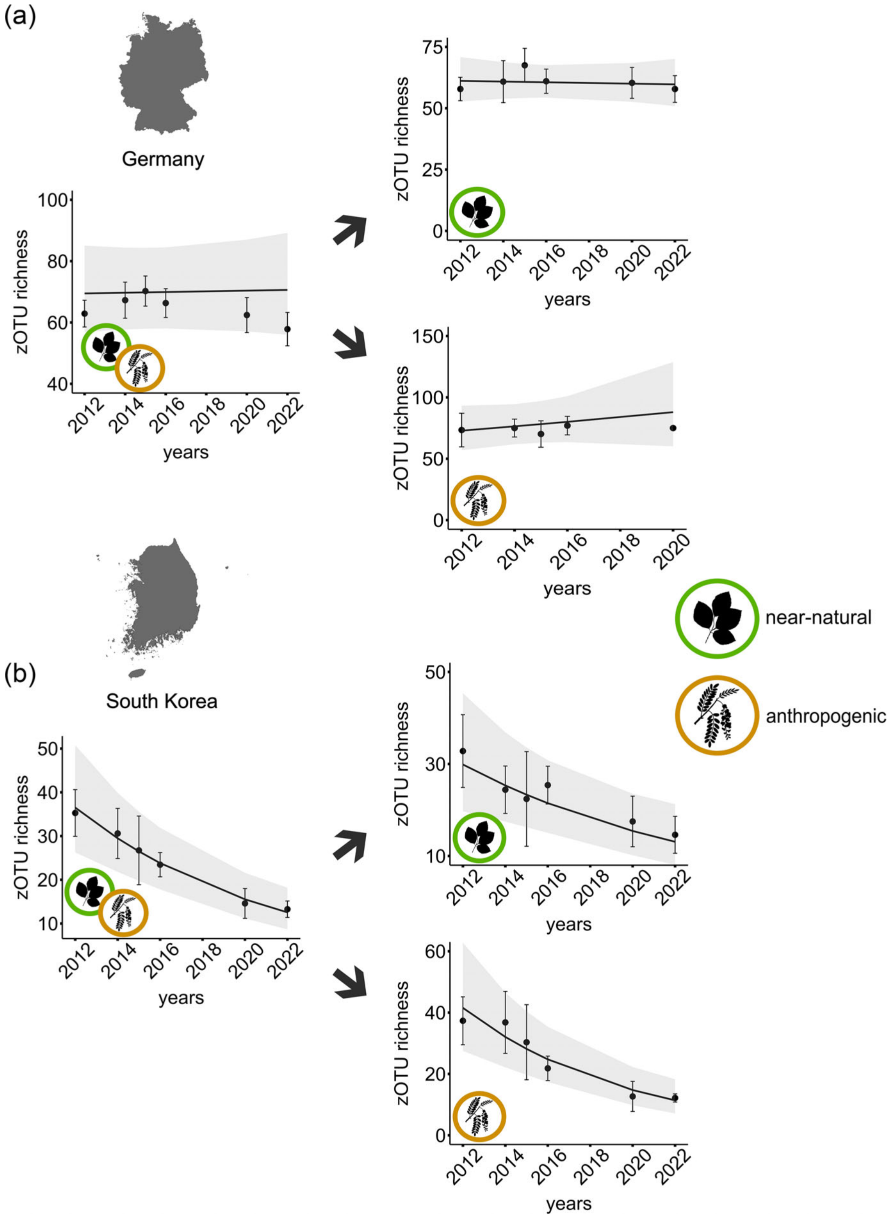

FIGURE 2 Trends of zOTU richness in forest arthropod communities over 11 years in Germany and South Korea. (a,b) Trends of zOTU richness in (a) German ESB samples and (b) Korean ESB samples. Trends are shown for near-natural (*top*) and anthropogenic classified forest landscapes (*bottom*) for both ESBs. The solid dots represent predicted means of summarised zOTU richness values across sampling locations and time windows, and vertical lines indicate 95% confidence intervals. The data were analysed using a generalised linear model (GLM). Icons refer to analysed forest landscape.

(Figure 2). Aside from some temporal fluctuations, α-diversity in Germany showed no significant change over the years, with values remaining stable from 2012 to 2022 (Figure 2a, left) ($\text{slope}_{\text{year}} = 0.002$, p-value $= 0.877$, $R^2 = 0.04$). When analysing land use types separately, the same trends emerge (Figure 2a, right; near-natural: $\text{slope}_{\text{year}} = -0.002$, p-value $= 0.83$, $R^2 < 0.01$; anthropogenic: $\text{slope}_{\text{year}} = 0.02$, p-value $= 0.404$, $R^2 = 0.06$). In contrast, the Korean ESB data showed a pronounced and statistically significant decline in zOTU richness over the same period (Figure 2b, left). The overall richness of canopy-associated arthropods of South Korea decreased by 61% (95% confidence interval: 42.12%–74.99%) over the past 11 years in the study sites, with an average of 35 zOTUs at the beginning and 13 zOTUs at the end of the survey ($\text{slope}_{\text{year}} = -0.107$, p-value <0.01, $R^2 = 0.08$). The same declining trends occur in near-natural (52% decline, 95% confidence interval: 3.54%–80.69%; $\text{slope}_{\text{year}} = -0.083$, p-value <0.01, $R^2 = 0.06$) and in anthropogenic forest landscapes (66% decline, 95% confidence interval: 42.61%–78.56%; $\text{slope}_{\text{year}} = -0.13$, p-value <0.01, $R^2 = 0.1$) in South Korea (Figure 2b, right). The same trends occur when analysing at the species level, with German communities remaining largely stable, while Korean communities experience significant declines (Figure S2; Germany: $\text{slope}_{\text{year}} = -0.003$, p-value $= 0.759$, $R^2 = 0.06$; South Korea:

$\text{slope}_{\text{year}} = -0.109$, p-value <0.01, $R^2 = 0.08$). The trend in Korean communities is mainly driven by anthropogenic sites (near-natural: $\text{slope}_{\text{year}} = -0.05$, p-value $= 0.176$, $R^2 = 0.03$; anthropogenic: $\text{slope}_{\text{year}} = -0.155$, p-value <0.001, $R^2 = 0.13$). Meanwhile, German communities exhibit similar non-significant trends in both land use types (near-natural: $\text{slope}_{\text{year}} = -0.0008$, p-value $= 0.94$, $R^2 = 0.04$; anthropogenic: $\text{slope}_{\text{year}} = -0.017$, p-value $= 0.497$, $R^2 = 0.08$).

The temporal trends in zOTU richness for the four most common arthropod orders (Acari, Hymenoptera, Hemiptera, and Lepidoptera) were additionally analysed (Figure S3). In the German ESB samples, zOTU richness remained stable across all orders throughout the study period (Acari: $\text{slope}_{\text{year}} = -0.007$, p-value $= 0.53$, $R^2 = 0.12$; Hymenoptera: $\text{slope}_{\text{year}} = 0.025$, p-value $= 0.25$, $R^2 = 0.08$; Hemiptera: $\text{slope}_{\text{year}} = 0.0001$, p-value $= 0.993$, $R^2 = 0.12$; Lepidoptera: $\text{slope}_{\text{year}} = 0.002$, p-value $= 0.853$, $R^2 = 0.12$). While minor fluctuations were observed, no significant changes over time were detected. In contrast, all four arthropod orders in the Korean ESB samples exhibited significant declines in zOTU richness over the same period (Acari: $\text{slope}_{\text{year}} = -0.09$, p-value <0.01, $R^2 = 0.09$; Hymenoptera: $\text{slope}_{\text{year}} = -0.166$, p-value <0.01, $R^2 = 0.15$; Hemiptera: $\text{slope}_{\text{year}} = -0.08$, p-value <0.01, $R^2 = 0.06$; Lepidoptera $\text{slope}_{\text{year}} = -0.188$, p-value <0.01, $R^2 = 0.19$). When analysing the land use types separately, the same trends appear. While all four orders show, in most cases, significant declines in near-natural and anthropogenic landscapes in South Korea (exception Hemiptera near-natural: $\text{slope}_{\text{year}} = -0.05$, p-value $= 0.17$, $R^2 = 0.09$), German communities exhibit little yet statistically insignificant changes.

Despite the pronounced changes of α-diversity in the analysed time series, the relative order-level composition of the arthropod communities remained stable in both Korea and Germany (Figure S4a,b). The same is true for the functional traits of the recovered taxa, which remained largely unchanged over time in their relative abundance (Figure S4c,d).

Finally, we investigated whether changes in community composition occur over time. Recently published studies suggested a biotic turnover in different ecosystems (Dornelas et al., 2014; Krehenwinkel, Weber, Broekmann, et al., 2022). Here, we find no significant changes in Korean or German arthropod communities (Figure S5; South Korea: $\text{slope}_{\text{year}} = 0.006$, p-value $= 0.068$, $R^2 = 0.03$; Germany: $\text{slope}_{\text{year}} = 0.003$, p-value $= 0.407$, $R^2 < 0.01$). When analysing land use types separately, only near-natural landscapes in Germany exhibit significant trends ($\text{slope}_{\text{year}} = 0.006$, p-value <0.05, $R^2 = 0.08$). This result for the shortened German time series contrasts with previous findings, where a significant turnover in canopy-associated arthropod canopies was revealed, indicating an effect of the length of the time series and the number of comparisons on our results (Junk et al., 2024; Krehenwinkel, Weber, Broekmann, et al., 2022).

DISCUSSION

By using eDNA from archived leaves from pollution monitoring archives, we reconstruct 11 years of tree canopy-associated arthropod community diversity of selected forests in Germany and South Korea. Samples from ESBs are exceptionally well suited for long-term biodiversity trend analyses since samples are collected in a highly standardised manner and stored at ultra-low temperatures. This has the advantage that biological and chemical processes are completely stopped and a snapshot of the community at the time of sampling is recorded. Furthermore, the application of the same sampling protocols enables long-term analysis with minimal sampling bias, while optimally storing and processing large amounts of sampling material in defined sampling distances. The successful application of ESB samples for biodiversity trend analysis has been demonstrated in several studies (Junk et al., 2023, 2024; Krehenwinkel, Weber, Broekmann, et al., 2022).

The diverse communities of recovered arthropods reflect their respective geographic origin and tree species very well. Using this dataset, we test the hypotheses that Korean and German arthropod communities in (1) near-natural forest landscapes are largely unaffected by insect decline (Crossley et al., 2020; Krehenwinkel, Weber, Broekmann, et al., 2022), while (2) those in anthropogenically disturbed woodlands have declined in recent years (Zhou et al., 2023). In line with previous results (Junk et al., 2024; Krehenwinkel, Weber, Broekmann, et al., 2022), selected German forests indeed show no temporal declines in near-natural landscapes over the 11-year study period, supporting our first hypothesis. Interestingly, an arthropod decline was also not observed in anthropogenically disturbed forests in Germany, leading to the rejection of our second hypothesis. In Korean forests, however, we find a significant α-diversity decline, affecting both near-natural and anthropogenic landscapes but appearing stronger in the latter land use type. The observed differences could be partially explained by the number of locations included in this study. While the Korean time series comprised approximately equal numbers of near-natural and anthropogenic sites (5 vs. 6), near-natural landscapes were slightly overrepresented in the German dataset (10 vs. 7). As more data typically lead to clearer trends, we would expect to detect a trend in the German time series, where more sites were included. Although fewer sites were analysed in the Korean dataset, we already observe a significant decline for both land-use types, albeit with relatively wide confidence intervals. While the number of locations appears to influence the results to some extent, we assume that the observed differences are likely due to factors other than the number of sampling sites alone.

The biological richness of different taxonomic arthropod groups in Asia has been subject to numerous surveys in the past (Lim et al., 2014; Park, 2010, 2011; Roh et al., 2015; Song & Park, 2017). Yet, these studies are limited in terms of their scope by focussing on single taxonomic groups, low number of locations or having a short survey length. Recently, Kwon et al. (2023) showed the impact of temperature and precipitation on species distributions and abundances using 5 years of data. This study gives a first impression about possible changes and declines of arthropod communities in the future while covering multiple years. Another recent study on agricultural systems in East Asia has suggested considerable arthropod decline in the last two decades (Zhou et al., 2023). Our data support this statement and show that selected forests in South Korea also undergo a strong arthropod diversity decline. It is striking that Korean forests already showed a significantly lower arthropod richness than the German sites, even at the beginning of the time series. This is despite the fact that the sampling schemes of both specimen banks are similar (Klein et al., 2018; National Institute of Environmental Research, 2016a, 2016b; Tarricone et al., 2018a, 2018b). However, we cannot rule out that slight differences in the sampling and sample processing approaches between the two countries' ESBs have contributed to the lower detected richness in South Korea. The declines in Korean forests in this study may have started much earlier than our first datapoint in 2012, possibly explaining the lower values at the start of the survey. This assumption is reinforced by a recent study that demonstrated a decline in the diversity of macromoth communities from 2005 to 2017 in South Korea (Choi et al., 2019). In fact, insect decline has been ongoing for several decades in Germany (Habel et al., 2019; Hallmann et al., 2017). Similar patterns could therefore occur in different habitats, which need to be confirmed in future studies.

In line with the declining trends in zOTU richness, we find similar trends at the species level. The lower species numbers in South Korea are possibly due to incomplete reference databases for South Korean communities. The German Barcode of Life is one of the best developed databases, probably leading to more identifications of arthropod species than in South Korea. Trends at the species level should therefore be interpreted with caution. Irrespective of the cause of the differences in species richness between the two countries, identical sampling procedures are applied in both specimen banks, and zOTU and species richness trends both indicate a decline. We thus consider the observed trends in Korea to be reliable. Different decline rates between South Korean and German forest arthropods could be driven by different regional influences of the sites. One example in this context is the ongoing urbanisation of South Korea (Showket et al., 2021), with urban areas in Korea having expanded considerably over the last decade (OECD, 2019). Indeed, canopy-associated arthropods showed a particularly sharp decrease from 2012 to 2022 in urban areas around the city of Seoul, as indicated by our data (data not shown). The ongoing decline of arthropods in urban areas of Korea is supported by a study by Kwon et al. (2016) who revealed a decline of soil arthropods from 1998 to 2007 in the same area. Recently, light pollution was identified as a major, often overlooked driver of insect declines, which is directly linked to urbanisation (Owens et al., 2020). Numerous studies have provided information on the negative effect of artificial light on different taxonomic groups (Grubisic et al., 2018; Hölker et al., 2010; Linares Arroyo et al., 2024; Longcore et al., Longcore & Rich, 2004). A recent study by Hakbong et al. (2021) further analysed the impact of artificial light at night on insect community composition. Here, they compared communities in the Gwangneung Forest Biosphere Reserve, an important area for biodiversity protection in South Korea, with three areas that are increasingly affected by artificial light at night. It appears reasonable that light pollution might have an effect on arthropod communities in our study areas.

Reasons for arthropod declines may also operate at a larger scale than regional land cover, such as climate change (Marta et al., 2021). South Korea's land temperature has risen more than the global average in recent decades (Kim et al., 2023), which has profound impacts on local arthropod communities in Korea (Kwon et al., 2016). Furthermore, enhanced atmospheric nitrogen deposition is known to be a driver of plant biodiversity losses, which potentially induce cascading negative effects on associated communities (Staude et al., 2020; van der Plas et al., 2024). While both countries show similar levels of atmospheric nitrogen deposition (Schwede et al., 2018), this alone appears not to be a main driver. Another potential factor might be the introduction of invasive species (Choi et al., 2021; Choi & Park, 2012; Lister & Garcia, 2018), often linked with climate change, which could contribute to these declines. The main drivers have yet to be identified, but it is reasonable to assume that the loss we see is caused by many different factors that are reinforcing each other rather than one specific factor.

Besides biotic and abiotic factors, recent work suggests that reported diversity or abundance declines in arthropods may be caused by short time series (Bang & Faeth, 2011; Didham et al., 2020; Hausmann et al., 2022; MacGregor et al., 2019). Arthropod populations are known to undergo pronounced temporal fluctuations. The observation of only short snapshots may lead to false assumptions. The influence of the length of time-series data becomes apparent in our dataset when analysing the changes in community composition (i.e., temporal β-diversity). Here, we find no significant changes in South Korean or German arthropod communities. This result for the German time series is mainly driven by the number of comparisons within each sampling location. When analysing the full time series over 30 years, a highly significant turnover is evident (see Junk et al., 2024; Krehenwinkel, Weber, Broekmann, et al., 2022). The temporal turnover of community composition is well supported by other work (Blowes et al., 2019; Dornelas et al., 2014; Magurran et al., 2010). Therefore, it cannot be ruled out that the length and completeness of the time series have an influence on the trends described here. Future analysis of Korean ESB samples collected in the coming years will help to provide the answer whether these trends are persisting or reversing or could be mainly driven by the length of the time series.

Our work highlights insect decline as a complex phenomenon of global importance, affecting forests with varying degrees of anthropogenic influence alike. Our findings of an arthropod biodiversity decline in selected Korean forests underscore the value of long-term sample storage in specimen banks for biodiversity surveys worldwide. The long-term data from selected ESB sampling locations in Germany and South Korea impressively demonstrate the need for representative monitoring programmes at national and international levels. Regulatory monitoring of terrestrial ecosystems has so far been less comprehensive and systematic than the monitoring of air, freshwater and marine ecosystems. As a result, there is not only a lack of long-term monitoring data, but also a lack of baselines for assessing changes in biodiversity. The objective should be to generate robust data sets for reliable statements about changes in biological diversity of terrestrial ecosystems.

AUTHOR CONTRIBUTIONS

Julian Hans: Conceptualization; methodology; data curation; investigation; formal analysis; visualization; writing – original draft; writing – review and editing; project administration. **Manuel Stothut:** Conceptualization; methodology; data curation; investigation; formal analysis; visualization; project administration; writing – original draft; writing – review and editing. **Caroline Schubert:** Investigation; methodology. **Elisabeth Gold:** Methodology; investigation. **David Chung:** Conceptualization; methodology; investigation; writing – review and editing. **JangHo Lee:** Conceptualization; methodology; investigation; writing – review and editing. **Jan Koschorreck:** Conceptualization; writing – review and editing. **Susan Kennedy:** Methodology; writing – review and editing. **JungKeun Oh:** Conceptualization; methodology; investigation; writing – review and editing. **Thomas Udelhoven:** Methodology; writing – review and editing. **Henrik Krehenwinkel:** Conceptualization; funding acquisition; investigation; methodology; supervision; project administration; resources; writing – review and editing.

ACKNOWLEDGEMENTS

We thank Petra Willems, Karin Fischer and Sabine Naber for help during laboratory sample processing. We thank Sven Künzel (Max Planck Institute for Evolutionary Biology, Plön) for generating the sequences. We thank Yannis Schöneberg (Department of Biogeography, University of Trier) for support in data analysis. We thank Jinwon Seo for collecting and providing the Korean samples. The black species icons of Figures 2 and S3 were obtained from https://www.phylopic.org/ and are dedicated to the public domain. This work was funded by departmental funds of the Department of Biogeography at Trier University and by the TrendDNA project of the German Federal Environment Agency (Umweltbundesamt). We want to especially thank the ESB project group at Trier University and the Umweltbundesamt and all project partners of the German Environmental Specimen Bank for providing the time series samples. This work was supported by the National Institute of Environment Research (NIER) funded by the Ministry of Environment (MOE) of the Republic of Korea (grant no. NIER-2020-01-01-058). Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Science Data Bank at https://doi.org/10.57760/sciencedb.15787.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Trends of zOTU richness of forest arthropod communities over three decades in German ESB samples. The solid dots represent predicted means of summarised zOTU richness values across sampling locations and time windows, and vertical lines indicate 95% confidence intervals. The data were analysed using a generalised linear model (GLM).

Figure S2. Trends of species richness of forest arthropod communities over 11 years in (a) German ESB samples and (b) Korean ESB samples. The solid dots represent predicted means of summarised species richness values across sampling locations and time windows, and vertical lines indicate 95% confidence intervals. The data were analysed using a generalised linear model (GLM).

Figure S3. Trends of zOTU richness of the four most abundant orders in forest arthropod communities over 11 years in German ESB and Korean ESB samples. The solid dots represent predicted means of summarised zOTU richness values across sampling locations and time windows, and vertical lines indicate 95% confidence intervals. The data were analysed using a generalised linear model (GLM). Icons refer to analysed arthropod order.

Figure S4. Taxonomic and functional trait composition of canopy-associated arthropod communities in German and Korean ESB samples. (a,b) Barplots showing recovered taxon composition of zOTUs across time in samples from (a) the German ESB and (b) the Korean ESB. Taxa amounting to less than 2% of the total zOTU number are merged as 'Other'. (c,d) Barplots showing percent composition of taxa representing different functional traits across time, for (c) German ESB samples and (d) Korean ESB samples.

Figure S5. Temporal changes in community compositions (β-diversity measured using Jaccard distance) as a function of the time interval (in years) between samples from the same sampling site. The solid dots represent predicted means of summarised Jaccard diversity indices across sampling locations and time windows, and vertical lines indicate 95% confidence intervals.

Table S1. Sampling locations and associated tree species of the South Korean and German Environmental Specimen Banks (ESB).

Table S2. List of all samples including sampling location, year, respective species, latitude and longitude of sampling location, month of sampling and mean annual temperature if accessible.

Table S3. Details of the laboratory workflow.

Table S4. Values for testing of overdispersion.

Table S5. List of all zOTUs that match the reference sequence to 100% over 100% of the amplicon length.

Table S6. List of all zOTUs and arthropod species that are exclusively found in near-natural and anthropogenic landscapes.

How to cite this article: Hans, J., Stothut, M., Schubert, C., Gold, E., Chung, D., Lee, J. et al. (2025) eDNA metabarcoding of archived leaf samples reveals arthropod diversity decline in South Korean but not in German forest ecosystems. *Insect Conservation and Diversity*, 1–13. Available from: https://doi.org/10.1111/icad.12860

Chapter 5

Shifts in functional groups of tree-associated fungi reveal decreasing tree health in German forest ecosystems

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Highlights:

- Comparative analysis of changes in fungal communities associated with a non-native (*Picea abies*) and native tree species (*Fagus sylvatica*) in German ESB samples
- Shifts of functional fungal group as potential indicators of changes in tree health
- Stable α-diversity trends in the native species, in contrast to significant increases in the non-native
- Both tree species exhibit compositional turnover over the four-decade study period
- Unfavourable functional groups significantly increased in spruce but not in beech, suggesting stress and declining health in the non-native species

Shifts in functional groups of tree-associated fungi reveal decreasing tree health in German forest ecosystems

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Abstract

Forests are among the most important ecosystems on earth, where trees fulfil key ecological functions. Climate change, with rising temperatures, reduced water availability and more frequent extreme weather events, poses considerable challenges for these systems. This affects not only the tree species themselves but also associated groups of organisms such as fungi. This hyper diverse group plays essential roles for trees and entire ecosystems. They facilitate nutrient uptake in trees and contribute to plant health by providing defence mechanisms against pathogens. Changes within diversity and functional fungal groups could be early indicators of declining tree health. In this study, we analyse standardised environmental samples from the German Environmental Specimen Bank, a long-term monitoring programme. The routinely sampled tree species beech (*Fagus sylvatica*) and spruce (*Picea abies*) allow a direct comparison between a native species and a species occurring outside its natural range. The study covers a period of nearly four decades in German forest habitats from 1985 to 2022. The results show significant changes in the fungal communities of both tree species over the study period. Functional groups experience differing trends for the two tree species: While hardly any changes were found in beech, significant increases of ecologically relevant groups such as plant pathogens and litter saprotrophs occurred in spruce communities. These changes within fungal communities may reflect imbalances caused by declining tree health. For the first time, this study allows temporally resolved analyses of associated fungal communities with linking these changes to reduced tree health, which can have cascading effects on entire forest ecosystems.

Introduction

Forests cover nearly 30% of the terrestrial land area (United Nations 2017) and fundamentally shape ecosystems through their structural and functional traits (Ehbrecht et al. 2021; Maynard et al. 2022; Ray et al. 2023). Within the environment, trees are involved in pivotal processes, such as the water and carbon cycles (Reichstein et al. 2013; Hoek van Dijke, Anne J. et al. 2022). They further contribute to ecosystem services, such as the flow of energy in forest ecosystems (Richter and Billings 2015), the regulation of the local climate (Schwaab et al. 2021) and air quality (Sonwani et al. 2022). In addition, their structural diversity allows trees to create suitable conditions for many species, facilitating biodiversity and interactions with various organism groups like other plants, fungi and metazoa (Baldwin et al. 2006; Toju et al. 2014). The development of forest stands is significantly influenced by biotic stresses such as pests and diseases. Abiotic influences, including climate change, drought and storms, further shape the dynamics and resilience of forests (Seidl et al. 2017; Anderegg et al. 2020).

In the temperate forests of Central Europe, the two tree species European beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*) are among the most dominant ones. In Germany alone, beech accounts for 16% of forest cover and spruce for 25% (Bundesministerium für Ernährung und Landwirtschaft 2023). Although both tree species are widespread in German forests, they have different responses to environmental influences due to their biological characteristics. The beech is naturally occurring in Germany and represents the potential natural vegetation of Central European forests (San-Miguel-Ayanz et al. 2021). Here, they form the climax community in most regions (Fuchs et al. 2024). Due to the longer post-glacial presence compared to spruce, beech trees were able to develop a broad ecological valence and are therefore more tolerant to different environmental conditions (San-Miguel-Ayanz et al. 2021). Furthermore, beech trees are relatively long-lived, reaching ages of up to 300 years and provide a habitat for many different groups of organisms. The spruce, on the other hand, is naturally limited to colder regions, such as Scandinavia, Eastern Europe and higher elevations in Central Europe (San-Miguel-Ayanz et al. 2021). While spruce naturally thrives in these high montane areas and boreal forests, forestry plantations extended its range to lower latitudes and altitudes. Especially in Germany, spruce is often cultivated in monocultures, where the lack of tree diversity increases its vulnerability to biotic and abiotic stresses (Felton et al. 2010). As spruce has only recently been planted in these new areas, its adaptation strategies to comparatively new environmental conditions are not yet as pronounced as those of beech. Due to their different ecological characteristics as well as their introduction to Central European regions, it can be assumed that beech trees have better adaptation strategies to local climatic conditions than spruce trees.

Particularly in times of climate change, with rising temperatures and more frequent extreme weather events, biocenoses are increasingly facing major challenges (Spiecker 2000). This applies to tree species, which must adapt to these changing conditions, along with their associated communities. One striking example of an associated group of organisms is the fungal kingdom, a hyper diverse group occupying a wide range of ecological niches. Its high adaptability to diverse climatic conditions has enabled fungi to occur worldwide and in every ecosystem (Větrovský et al. 2019). Here, they fulfil pivotal roles as symbionts, like as epi- and endophytes, pathogens and decomposers, constantly shaping their environment (Casadevall et al. 2008). Alterations in fungal composition may be a response to environmental stressors, like pathogen outbreaks after severe droughts. Changes in the number of species and their functional groups could allow for conclusions about tree health and thus entire forests. With this information, valuable insights into the resilience of forests under changing climatic conditions are provided (Gómez-Aparicio et al. 2022).

In the face of ongoing long-term environmental changes, the evaluation of impacts on ecosystems requires long, standardized and representative biodiversity assessments, which are currently largely lacking (Magurran et al. 2010; Bálint et al. 2018). Reliable conclusions and predictions about microbiome-derived forest health trends are therefore scarce. Many existing studies are limited in scope, often focusing on specific regions or fungal groups, which restricts comprehensive evaluations (Fang et al. 2023). This data gap impairs the ability to detect early warning signs of forest health decline and hinders the development of effective conservation and management strategies. Environmental Specimen Bank (ESB) samples offer a promising solution to this issue. These long-term pollution monitoring archives store samples from indicator organisms collected across diverse ecosystems (Fliedner et al. 2022). Together with the indicator species, associated organisms and their DNA traces are perfectly preserved (Zizka et al. 2022). The standardized collection and storage at ultra-low temperatures of these samples allow for retrospective analyses, facilitating comprehensive ecological assessments. DNA metabarcoding enables the analysis of changes in different groups of organisms associated with tree species (Krehenwinkel et al. 2022; Junk et al. 2024), and can thereby provide insights into forest health.

Here, we use spruce and beech samples from the German ESB to perform DNA metabarcoding to characterise changes in canopy-associated fungal communities, covering a period of nearly four decades. Since the impact of environmental changes is assumed to be much stronger in non-native spruce, we directly compare the trends of fungal communities of spruce to the native beech. To investigate this, we test three hypotheses:

The fungal communities on spruce needles respond stronger to environmental changes than those of beech leaves, causing:

1) fungal species extinctions at individual sites, resulting in a stronger local decline of α-diversity in spruce than in beech.

2) an enhanced exchange of species, driven by local extinctions and the immigration of new species, resulting in a biotic turnover, i.e. shifts in temporal β-diversity.

3) increased shifts in functional fungal trait groups, especially in saprotrophs and plant pathogens.

Methods

2.1. Environmental Specimen Banks and sample collection

The German ESB was established in 1985 and has been systematically collecting leaf material from tree canopies. Environmental specimen banks act as archives for environmental samples collected using standardized protocols (Fliedner et al. 2022). By analysing environmental chemicals within and on the surface of leaves from diverse ecosystems, ESBs provide valuable insights into ecosystem health, largely through the passive collection of airborne pollutants. Each year, samples are collected at the same sites and time using sterile materials to prevent even minimal chemical-contamination. After collection, samples are rapidly stored at ultra-low temperatures over liquid nitrogen gas phase, effectively stopping all chemical and biological activity. To ensure the samples accurately represent the sampled sites, each tree sample contains at least 60 g of leaf material per tree to collect 1.1 kg of fresh material which is then mixed to one homogenate. These samples are ground to a fine powder (200 µm grain size) using a cryo-mill, enabling a homogeneous distribution of all chemical components and nucleic acids (Rüdel et al. 2008; Rüdel et al. 2015).

For this study, we analysed 232 leaf samples from 14 different ESB sampling sites in Germany, covering a 38-year period from 1985 to 2022. Samples were taken from two German tree species: European beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*), with both species consistently sampled at the same sites and in close spatial relation (Klein et al. 2018; Tarricone et al. 2018). Leaves were collected either annually or biennially across a range of ecosystem types, covering forests in core zones of national parks, timber forests and forests close to agricultural areas and urban parks.

2.2. Molecular processing

We extracted DNA from 200 mg of homogenized material per sample, using a CTAB protocol (OPS Diagnostics, New Jersey, USA), which proved best suited to extract high-purity DNA from these sample types (Krehenwinkel et al. 2022; Junk et al. 2024). DNA was extracted in duplicate runs per sample. To analyse fungal DNA, we targeted the ITS1 region of the nuclear ribosomal DNA cluster, employing the primer pair ITS1f (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC), which amplifies a 264-bp fragment (White et al. 1990; GARDES and BRUNS 1993). Each sample was processed in duplicate PCRs, resulting in quadruplicates per sample. We used 1 µl of template DNA in a 10-µl reaction volume, following the Qiagen Multiplex PCR kit protocol with 35 cycles and an annealing temperature of 55 °C (Qiagen, Hilden, Germany). PCR amplification success was confirmed on 1.5% agarose gels. We then conducted an index PCR, using six cycles with a 56 °C annealing temperature to attach unique combinations of forward and reverse TruSeq indexing primers (Illumina, San Diego, CA, USA). The final libraries were verified for amplification success on 1.5% agarose gels and pooled into approximately equimolar quantities based on the gel band intensities. The pooled libraries were purified using 1X AMPure XP magnetic beads (Beckman Coulter, California, USA) and sequenced on an Illumina MiSeq with 600-cycle V3 kits. Throughout all procedures, negative controls were included for DNA extractions and PCRs, which were sequenced alongside the samples to assess potential carryover and contamination.

2.3. Sequence processing

Sequences were processed as described in Junk et al. (2024). In brief, forward and reverse reads were merged using PEAR (Zhang et al. 2014) with a minimum quality of 20 and a minimum overlap of 50 bp. The merged reads were then quality-filtered by limiting the number of expected errors in a sequence to 1 (Edgar and Flyvbjerg 2015) and transformed to fasta format using USEARCH (Edgar 2010). Primer sequences were trimmed off using UNIX scripts. After trimming, the resulting file was dereplicated and clustered into zero radius OTUs (hereafter zOTUs) using the USEARCH pipeline. zOTU tables were built for each sample type, also using USEARCH. Taxonomy was annotated using the blast2taxonomy script v1.4.2. (Schöneberg 2023) for BLAST searching (Altschul et al. 1990) against the entire UNITE database (Abarenkov et al. 2024) for fungal ITS sequences. The taxonomic information with the highest level of sequence similarity among the first ten hits for the lowest taxonomic level possible was accepted. All sequences not belonging to the kingdom of fungi were removed from the dataset. The FungalTraits database (Põlme et al. 2020) was used for the functional annotation of fungi.

2.4. Statistical analysis

All statistical analyses were conducted in R 4.4.3 (R Core Team 2024) and RStudio 2023.12.1.402 (RStudio Team 2023) using the packages vegan 2.6-6.1 (Oksanen et al. 2001) and tidyverse 1.3.0 (Wickham et al. 2019). First, Pearson correlations were calculated to verify the consistency between all four replicates of each sample. All replicates within each sample with a correlation equal or higher than 80% were merged into one single sample by summing the reads. Samples in which not all replicates had a correlation of at least 80% were excluded from further analyses. Further, zOTUs were processed as described in Junk et al. (2024). An additional data cleaning was performed, i.e. for each sample type, we exclusively (i) selected sites that had been sampled for at least five years, (ii) retained only sampling years that were represented in at least 50% of the sites, and (iii) excluded years that were isolated from the others (>2 years apart). Additionally, samples with low read coverage (less than 50% of the median number of reads) were removed. Since zOTU read abundances in metabarcoding datasets are subject to various biases, zOTU abundances were converted into binary presence/absence data, and only trends in zOTU occurrence were analysed. To minimize cross-contamination, a zOTU was considered present in a sample if it accounted for at least 0.01% of the total reads (further detailed information in Junk et al. (2024)).

Community diversity trends were measured in three ways. Detailed information is listed in Junk et al. (2024). First, α-diversity using the zOTU richness as a measure of local diversity at a given time in each community in each year was calculated. Then, spatial and temporal β-diversities trends were calculated between pairs of communities. For spatial β-diversity trends, pairs of communities sampled in the same year at different sites were used, while for temporal trends pairs at the same sites in different years were considered. The spatial β-diversity hence revealed information about changes in community composition across space, while the temporal β-diversity revealed information about changes in community composition over time. For β-diversity trends the full Jaccard distances were used. We used mixed linear models accounting for the temporal autocorrelation between sampling years and the effect of the different sampling sites for identifying temporal trends in these diversity indices. The lme function (R-package nlme (Pinheiro and Bates 1999)) with the corAR1 temporal correlation and the different sites as random effects for α-diversity and the pairwise comparison between sites as random effects for β-diversity were applied.

Results

In total, 2754 zOTUs were recovered, with 5 phyla, 27 classes, 107 orders, 298 families, 752 genera and 1029 species being identified. Pleosporales was the richest order in beech (193 zOTUs in 22 families) and Helotiales the richest order in spruce (143 zOTUs in 14 families). The recovered taxa were species specific and showed location-specific fungal communities (Figure 1 A&B; PERMANOVA < 0.05 all). Numerous detected taxa were restricted to individual sites. Diverse groups of plant pathogens, epiphytes and mycoparasites like *Botrytis cinerea*, *Trichoderma* sp. and *Thyridium* sp. were detected. Many fungal orders were found in spruce and beech samples (Figure 1 C&D). Notably, while the relative richness of fungal orders associated with beech remained stable, spruce-associated communities displayed temporal increases and decreases. Specifically, Pleosporales demonstrated an increasing trend in richness over time, whereas Helotiales exhibited a decline throughout the study period.

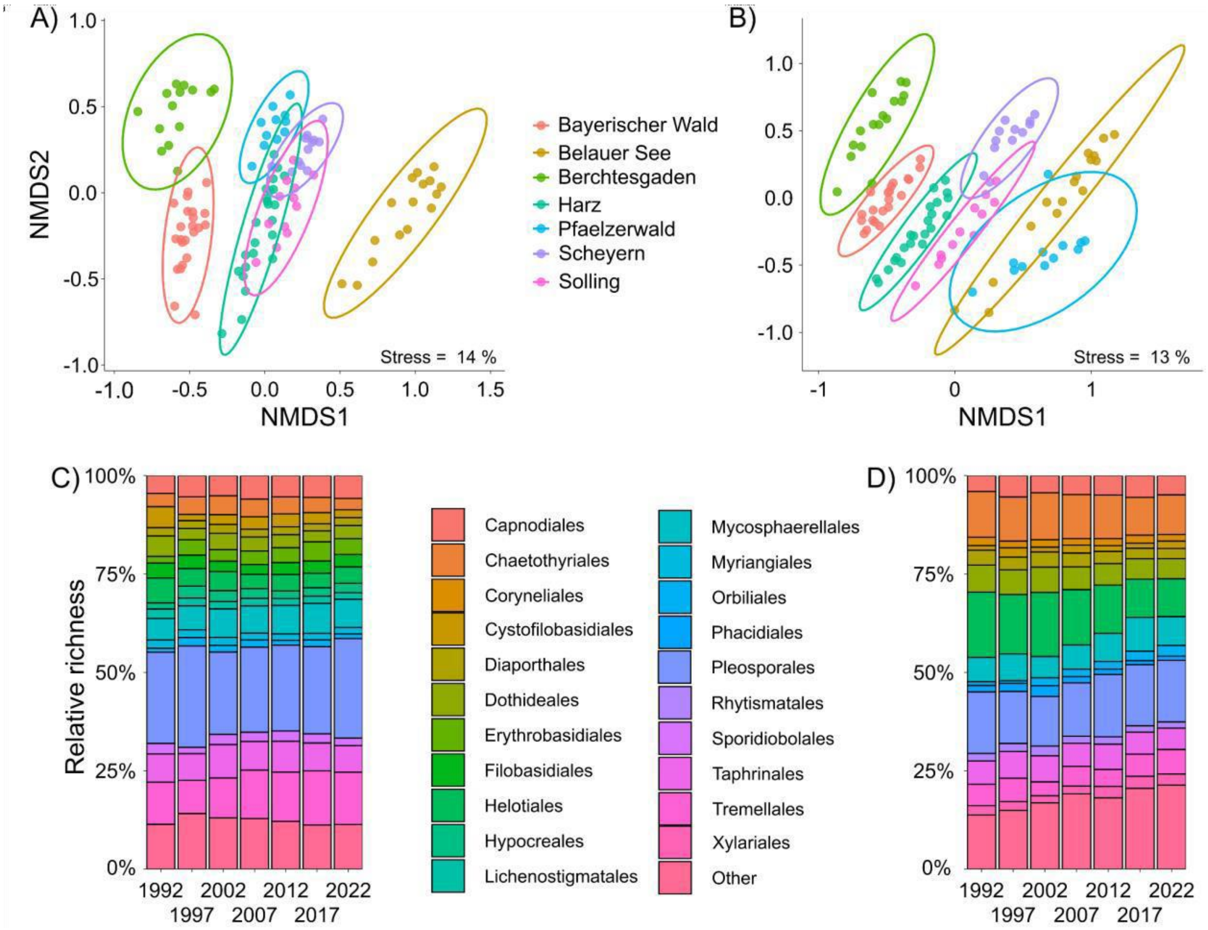

Figure 1: Non-metric multidimensional scaling (NMDS) plots illustrating fungal communities and the taxonomic composition of fungal orders. Communities associated with beech (A, C) and with spruce (B, D).

Temporal diversity trends across tree species

The α-diversity (local zOTU richness) of beech and spruce exhibited distinct trends over three decades, indicating differences in the long-term dynamics of fungal communities associated with the tree species (Figure 2A). While fluctuations in richness values were observed for both beech and spruce throughout the study period, beech-associated fungal richness remained relatively stable ($R^2 = 0.001$, p-value > 0.05), suggesting no significant directional changes over time. In contrast, spruce-associated fungal richness exhibited a significant increasing trend over time ($R^2 = 0.089$, p-value < 0.01). However, it is noticeable that the zOTU richness shows fluctuations for both tree species at all locations. The richness increases or decreases at times and then changes its trend (Supp. Fig. 1, Supp. Fig. 2).

Regarding trends in temporal β-diversity (shifts in community composition over time) both tree species experience significant changes. Here, a temporal shift in community composition is observed for both tree species (Beech $R^2 = 0.79$, p-value < 0.001; Spruce $R^2 = 0.68$, p-value < 0.001, Figure 2B). This trend is more pronounced in spruce than in beech. While communities associated with spruce sampled only with a small yearly distance tend to be more similar than those in beech, this pattern reverses over longer time spans, with greater temporal dissimilarity observed in spruce. Extended time intervals between sampling events in spruce, in particular, lead to higher dissimilarity values. Furthermore, this shift in community composition occurs gradually instead of abruptly, suggesting a continuous transition rather than a sudden tipping point. This becomes apparent, when all locations are analysed separately (Supp. Fig. 3, Supp. Fig. 4). This gradual change in community composition is evident across all locations for both beech and spruce and aligns with recent work in other ecosystems (Dornelas et al. 2014; Blowes et al. 2019). The spatial changes within beech and spruce communities only show little change, however, not statistically significant (Supp. Fig. 5).

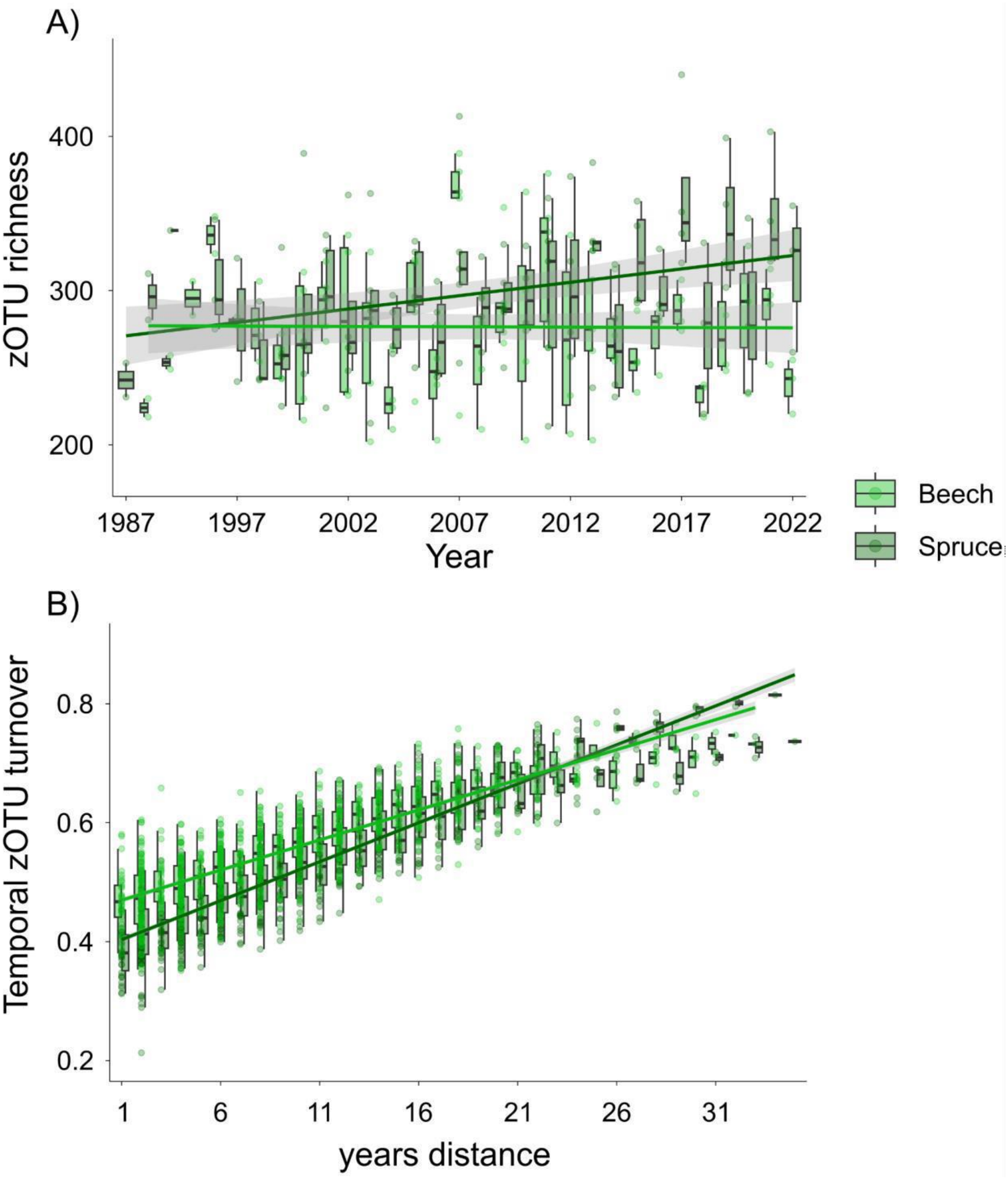

Figure 2: Temporal changes of fungal communities associated with beech and spruce. A) Trends of zOTU richness (α-diversity) of the associated communities from 1987 to 2022. B) Temporal changes in community composition (β-diversity) as a function of the time interval (in years) between samples from the same sampling site. All diversity indices were summarized as mean with standard error bars across sampling locations or time windows.

Similar patterns of compositional change are evident in the functional groups of fungi. While no significant changes are observed in any tree species-relevant group for beech, notable shifts occur in spruce-associated fungal groups (Figure 3). Over time, significant increases in abundance are detected in mycoparasitic, epiphytic, and plant-pathogenic fungal groups, whereas saprotrophic groups remain largely unchanged. The increase in specific functional groups suggests a shift in the overall fungal community structure in spruce.

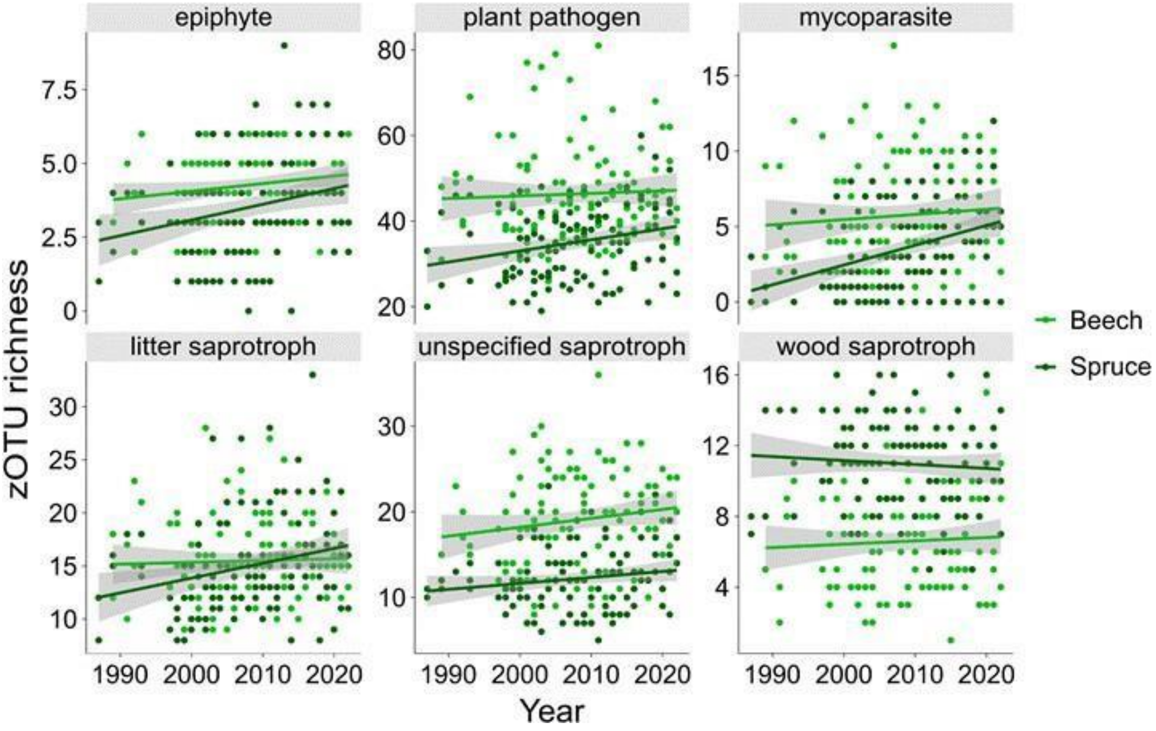

Figure 3: Temporal changes of fungal functional trait groups associated with beech and spruce.

Discussion

The assessment of biodiversity change in the Anthropocene requires standardized time series data, which are currently mostly lacking (Magurran et al. 2010; Bálint et al. 2018). In this study, we demonstrate the great potential of German ESB samples to detect changes in canopy-associated fungal communities and their functional groups and by this draw conclusions about tree health.

The initial hypothesis of this study proposed that fungal communities exhibit a significant decline in species richness at local scales. Recent work suggests that the observed loss of biodiversity is primarily driven by losses in local species diversity (Dirzo et al. 2014; Wagner et al. 2021a; Wagner et al. 2021b; Mikryukov et al. 2023). However, our results do not support this assumption, leading to the rejection of our first hypothesis. While beech-associated fungal communities remained relatively stable over time, spruce communities even showed a significant increase in species richness at four out of the seven sites analysed.

Instead of a decline in richness, we find a significant biotic turnover in both communities. The loss of many taxa appears to be compensated by the immigration of new, likely closely related species. This becomes apparent, as higher taxonomic groups of fungi remain largely unchanged, with no entire groups disappearing or newly emerging in our dataset. While the taxonomic groups remain largely stable, an exchange of closely related taxa which are potentially fulfilling similar ecological functions, is assumed. Moreover, this turnover appears to occur gradually rather than rapidly, supporting our second hypothesis. Similar trends have recently been documented in other systems (Magurran et al. 2010; Dornelas et al. 2014; Blowes et al. 2019). Given that these changes occur across both tree species and all study sites, we assume that a common underlying driver may be responsible for the observed patterns. Climate change has frequently been identified as a potential driver of such shifts in community composition.

Similar to the shifts in community composition, we find significant changes within the functional groups of fungi. For this approach, the ecological important groups of epiphytes, phytopathogens, mycoparasites and saprotrophs were examined. While fungal communities of beeches showed little to no change in these groups, spruce communities exhibited substantial increases. The observed increase in litter saprotrophic fungi suggests an accumulation of dead plant material over time. This trend is likely driven by the increasing frequency and severity of extreme weather events associated with climate change. Repeated episodes of drought stress have been reported in spruce stands in recent years (Ditmarová et al. 2010). While droughts have naturally occurred in Germany in the past, ongoing climate change is projected to cause a long-term decline in groundwater levels across many regions of the country (Wunsch et al. 2022). This is problematic, as spruce trees are known to be particularly sensitive to water availability (Wallin et al. 2002). Rising temperatures in combination with reduced water availability are therefore expected to deteriorate growing conditions for spruce stands in Germany. Negative effects on spruce stands caused by droughts in Germany are already visible, with spruce having the highest dieback rate compared to tree species such as oak, beech or pine (Bundesministerium für Ernährung und Landwirtschaft 2023). In addition, increasing bark beetle infestations further induce stress for spruce stands (Plath et al. 2024). This stress inevitably results in potential infection with other plant pathogens. Healthy trees normally produce defence compounds such as phenols. Under drought stress, weakened trees tend to reduce or even cease the production of these defence compounds, thereby creating favourable conditions for pathogen infestation. The significant increase in pathogenic fungi in spruce indicates a decline in tree health that can affect entire forest ecosystems. Additionally, with changing environmental conditions, the introduction of invasive species becomes more frequent (Finch et al. 2021). With available ecological niches, these non-native species can easily establish and thus alter entire ecosystems. The community change potentially triggered by these species is apparent in our results. However, the effects on trees and tree health are difficult to predict and require comprehensive long-term monitoring approaches.

Environmental stresses are likely to contribute to shifts in community composition, as reflected in the functional groups, potentially indicating early signs of ecological disturbance. The analysis of functional fungal groups thus holds considerable potential as an early indicator of declining tree health. By identifying shifts in these groups at an early stage, forest management strategies could be implemented proactively to prevent widespread tree mortality, thereby helping to preserve entire forest ecosystems and their associated functions.

Data availability

Raw sequencing data, OTU tables, R code for data analysis and the dynamic model are available in the Science Data Bank (https://doi.org/10.57760/sciencedb.23683). Private link for editors and reviewers: https://www.scidb.cn/en/s/f6FrQf.

Acknowledgements

We thank Karin Fischer and Sabine Naber for assistance with laboratory work. This work was funded by departmental funds of the department of Biogeography at Trier University and by the TrendDNA project of the German Environment Agency (Umweltbundesamt). We want to especially thank the ESB project group at Trier University and the German Environment Agency for providing the time series samples.

Author contributions

JH, MS, EG and CS performed the laboratory work and analysed the data. HK, JH and MS conceptualized the study, analysed the data, and wrote the manuscript. BP and HM developed the dynamic model for community ecology. SK performed the sequencing. JK and AK enabled access to the samples. All authors contributed to revising the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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General discussion

Long-term monitoring data are currently lacking for many species and ecosystems, making it difficult to draw reliable conclusions about biodiversity change in the Anthropocene. In this thesis, I demonstrated the great potential of environmental samples from the German Environmental Specimen Bank (ESB) to conduct time series analyses of biodiversity change of sample associated communities using a metabarcoding approach. The ESB's highly standardised sample collection and processing, combined with the storage at ultra-low temperatures, enable retrospective analyses across space and time.

In Chapter one, I provided a detailed overview of the metabarcoding approach, outlining its methodological framework, strengths, and limitations. In the subsequent chapters, the application of this method to generate high-resolution biodiversity data was presented. For the first time, it was possible to assess biodiversity change across the tree of life in German ecosystems over the past four decades. A direct comparison of two harmonised ESBs further enabled a global perspective on specific patterns of community change.

Although these samples provided insights into long-term trends in German sample-associated communities for the first time, some limitations occurred. The length and continuity of the time series proved critical in detecting biodiversity trends. This is particularly evident when comparing the Korean ESB data, which spans eleven years, to the German time series covering nearly four decades. Arthropod communities exhibit long-term fluctuations (Macgregor *et al.* 2019), which is visible in German communities (Junk *et al.* 2024). To achieve a comparison between the two datasets, the German time series was restricted to the same time window as the Korean data. Within this shortened timeframe, no significant changes were observed in arthropod communities from German agricultural sites. However, this finding contrasts with earlier studies (Krehenwinkel *et al.* 2022) and is primarily caused by the limited time span analysed. This example underscores the critical importance of long-term and continuous data for accurately assessing biodiversity trends. Continued biodiversity monitoring in Korea by the Korean ESB is therefore essential, to validate the current results and to determine whether the observed changes reflect long-term trends or are part of natural community fluctuations.

Another major limitation is the availability and completeness of reference databases, which are essential for reliable biodiversity assessments. While the taxonomic information in databases for German arthropod species is comparatively good, databases for Korean species are less well developed. This difference becomes evident when comparing trends between the two datasets on species levels. While arthropod communities in Germany exhibited moderate species richness values, those in Korea seemed to be less diverse. This is probably due to incomplete reference databases rather than true biological patterns. A more comprehensive database would facilitate stronger and more reliable interpretations of biodiversity change in the last decade. The issue of database completeness extends beyond arthropods and affects other taxonomic groups as well, such as fungi. Although fungi play critical roles in ecosystem functioning, taxonomic and functional information about this group remains scarce (Phukhamsakda *et al.* 2022). This limitation is evident in Chapter five, where I analysed changes in functional groups of fungi associated with a native and a non-native tree species in Germany. The aim was to draw conclusions about tree health based on compositional changes of the functional fungal groups. While the decline of tree health was reflected in the increase of less favourable functional groups in the non-native species, the lack of detailed taxonomic and functional trait data limits the strength of such conclusions. More comprehensive reference databases will be essential to fully realise the potential of fungal community analyses in ecological monitoring.

Despite certain limitations, samples from the German ESB offer numerous opportunities for future research. With climate change and global warming affecting communities worldwide, analysing the responses of organisms to environmental stresses has become increasingly critical. Whole genome sequencing combined with epigenetic and transcriptomic analysis provides a powerful approach to investigate stress responses, for example to elevated temperatures (Gleason & Burton 2015; Xu *et al.* 2023). By sequencing the complete genome of a species, it is possible to identify and investigate specific regions responsible for mediating responses to external stressors. For example, the eelpout (*Zoarces viviparus*) is routinely sampled by the ESB at two locations in the North Sea and one in the Baltic Sea. In recent work, a high-quality genome assembly for this species was published (Fuhrmann *et al.* 2024). With this reference genome, detailed investigations into how the species responds to thermal stress, potentially induced by climate change, becomes applicable. One approach is the analysis of DNA methylation patterns. During this chemical process, methyl groups are added to the DNA, resulting in changes in the appearance and structure of the DNA (Pal *et al.* 2015). This can directly affect gene regulation and transcription, with heat stress acting as a trigger for the up- or down regulation of specific genes (Liu *et al.* 2023). As the eelpout and other marine organisms are expected to experience increasing stresses due to elevated temperatures (Gruber *et al.* 2021), ESB samples provide the great opportunity to link climatic information with molecular stress responses in marine organisms.

Like in aquatic ecosystems, terrestrial organisms face various challenges in times of ongoing climate change. With rising temperatures, these communities are already experiencing significant changes (Khaliq *et al.* 2024). Trees, in particular, are expected to experience increased mortality rates due to drought and temperature stress, which can trigger cascading effects throughout entire ecosystems (Anderegg *et al.* 2013). To detect early signs of stress, transcriptomic analysis has proven to be a valuable tool. The beech (*Fagus sylvatica*), which is routinely sampled by the ESB at seven sampling locations across Germany, is particularly well-suited for this research. As this tree species forms the natural climax community in many German regions (Fuchs *et al.* 2024), impacts on numerous other systems are expected. Comparing gene expression patterns across sites enables the identification of regional differences as well as early stress responses.

The analysis of interaction networks within biotic communities is another valuable area of research. While recent work has provided first insights into plant-arthropod interactions (Stothut *et al.* 2024a; Stothut *et al.* 2024b), knowledge about microbial networks remains comparatively limited. New studies have explored alterations in network complexities under varying environmental conditions, with differing results (Hernandez *et al.* 2021; Zhai *et al.* 2024). For instance, microbial networks in the rhizosphere either increased (Zhai *et al.* 2024) or decreased their complexity (Hernandez *et al.* 2021) in response to external stresses. These contrasting findings highlight the need for further research to draw robust conclusions about how microbial network structures change over the time, triggered by environmental factors.

The results of the projects in this thesis already showed the great potential of ESB samples for relevant issues of our time. Nevertheless, some questions could only be partially answered in these studies. The centrepiece of this thesis is the spatial and temporal change of biotic communities across the tree of life, addressed in Chapter three. Although initial assumptions about drivers have been identified and discussed, the question of which factors are the main drivers of changes in biological communities remains open. In the meta-analysis of Jaureguiberry *et al.* (2022), various drivers for global diversity loss and their respective contributions were discussed. While land-use change was identified as the main driver for terrestrial and limnic ecosystems, direct exploitation and climate change were the main drivers in marine environments. For Germany, high-resolution climate data are provided by the German Meteorological Service (Deutscher Wetterdienst - DWD). Together with data on pollutant concentrations in the environment, which are routinely analysed and published by the ESB, the identification of drivers responsible for the observed changes in German communities should be explored. It can be assumed that it is a combination of several factors, such as climate change, increased eutrophication and land- and sea-use change, which are already known to be major drivers of biodiversity change (Davison *et al.* 2021; Jaureguiberry *et al.* 2022; Wang *et al.* 2021).

Although the ESB samples already cover large areas in Germany, a generalisation of the observed patterns should be approached with caution. Ideally, the aim should be to include a broader range of species and additional sampling locations in order to strengthen the findings. One possibility would be to include other existing monitoring programmes run by German authorities. For example, the ‘Monitoring der biologischen Vielfalt in Agrarlandschaften (MonViA), has been monitoring habitat, species and genetic diversity in agricultural sites under the coordination of the Julius Kühn Institute since 2019 (MonViA Verbundprojekt 2024). This is just one of many official monitoring initiatives. The integration and potential harmonisation of the numerous programmes could provide an opportunity to investigate changes in biotic communities throughout Germany in the Anthropocene. Cooperation between authorities would be a treasure for addressing the major environmental and ecological challenges of our time.

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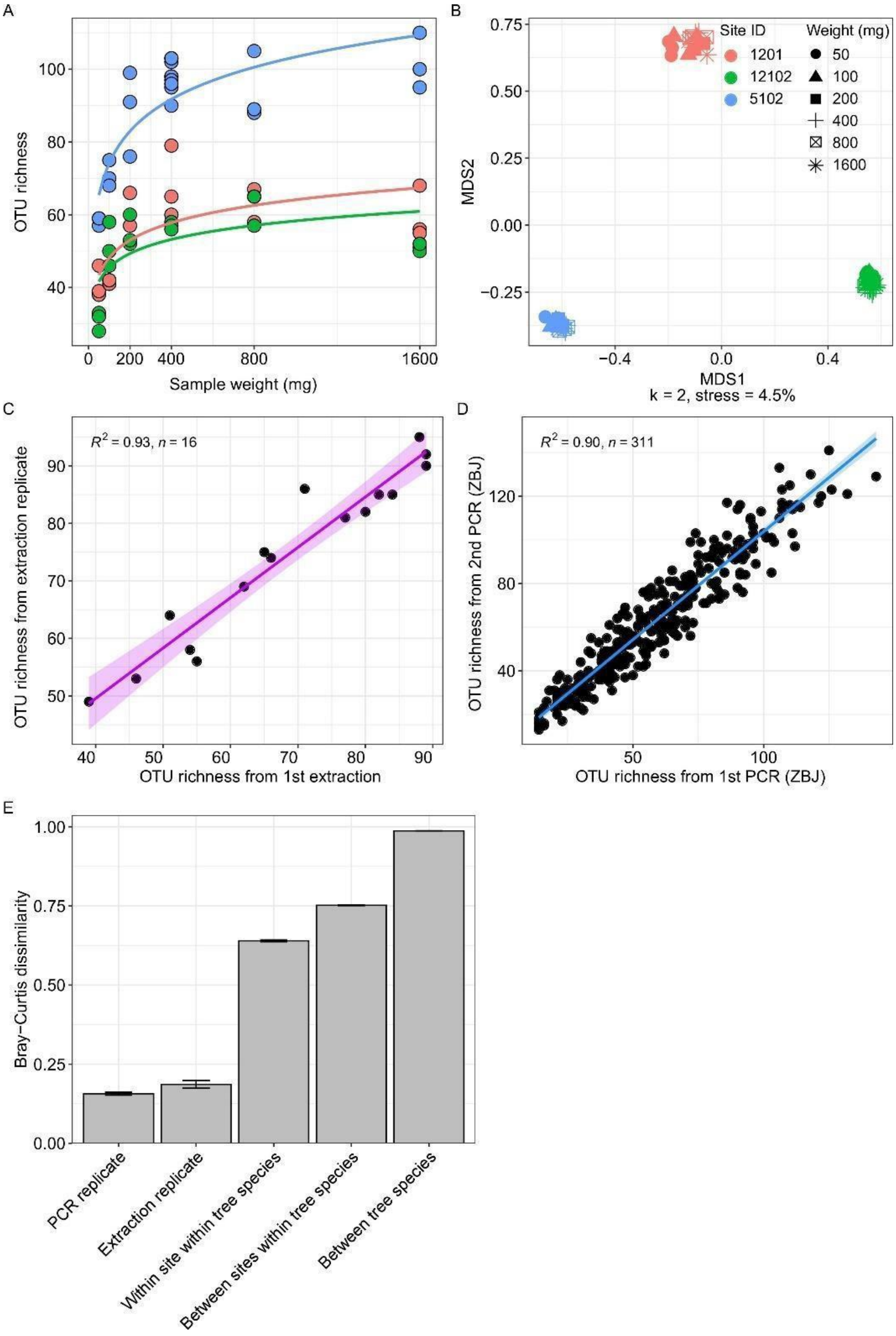
Supplements

Supplementary material

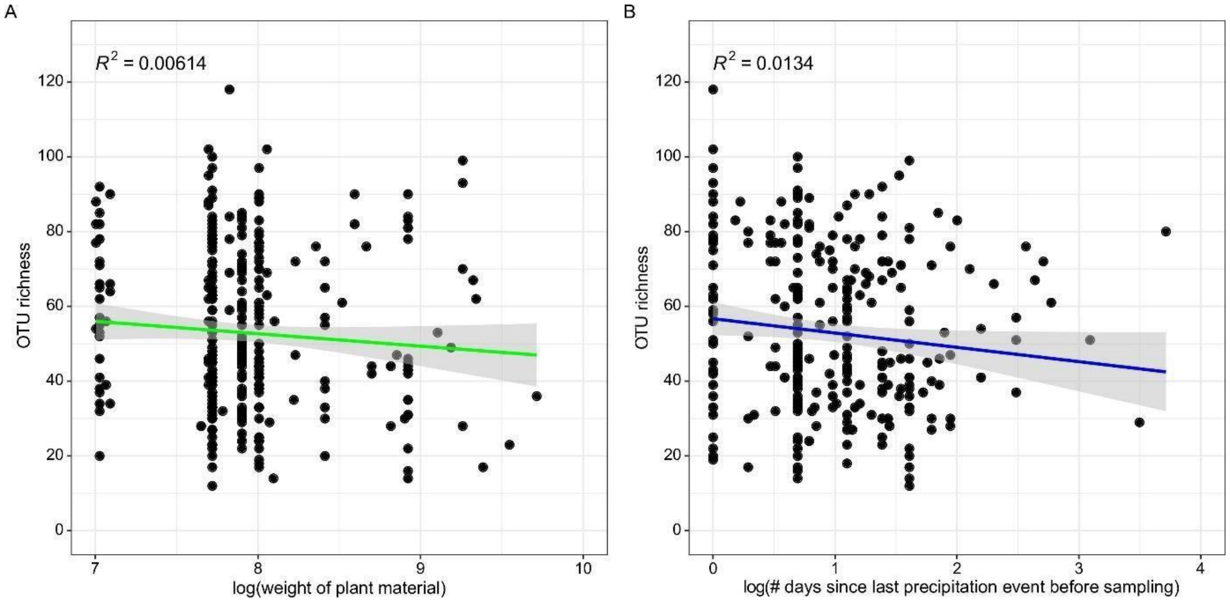
Chapter 2

Chapter two has been published in the journal *eLife*. All data, on which the study is based, has been uploaded to the Dryad Digital Repository (https://doi.org/10.5061/dryad.x0k6djhmp).

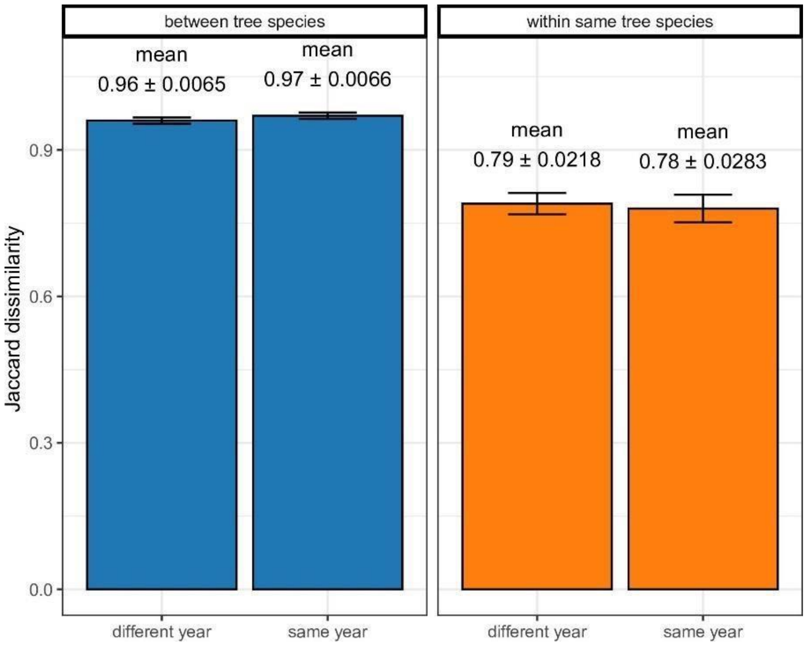
Appendix



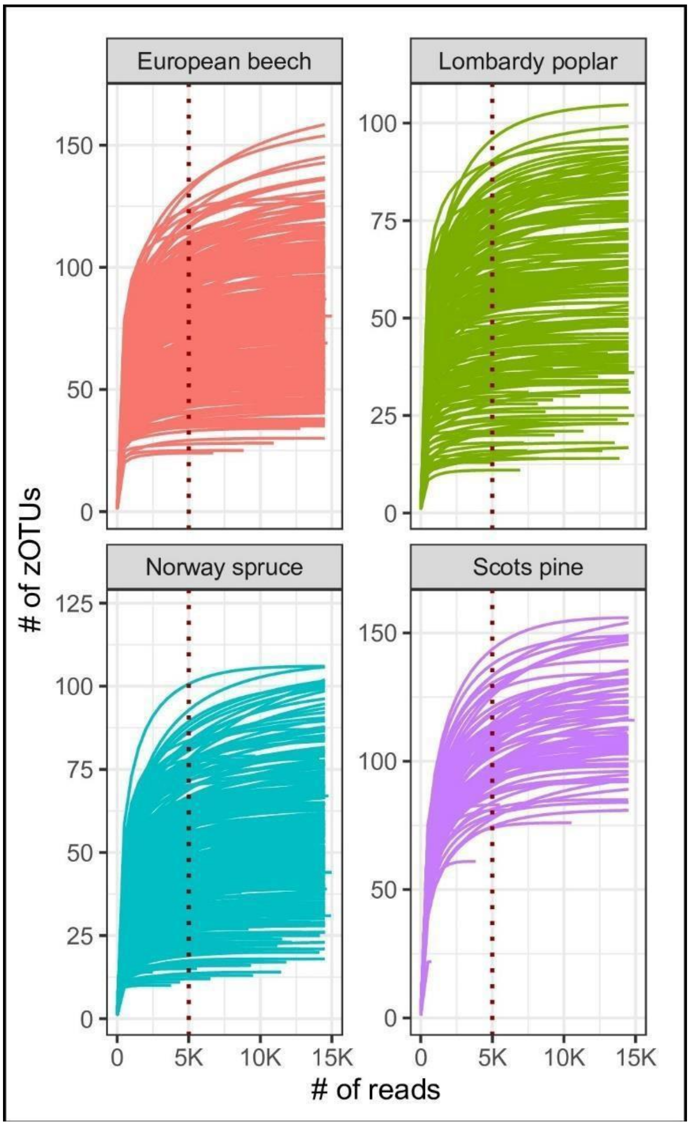
Supp. Figure 1.1: Effect of replication and sample input on recovered arthropod diversity. (A) Saturation curves of recovered arthropod OTU richness for three sampling sites as a function of the amount of leaf homogenate used for DNA extraction. Extractions were made in triplicate for each input weight. (B) NMDS plot for the same samples. Homogenate samples cluster by sampling site, while the amount of leaf homogenate used for DNA extraction has no discernable effect on β-diversity. (C) Correlation of OTU richness obtained from extraction replicates. (D) Correlation of OTU richness obtained from PCR replicates. (E) Average β-diversity dissimilarity (and 95 % confidence interval) between PCR replicates, extraction replicates, different years within a site, different sites within a tree species and between tree species.



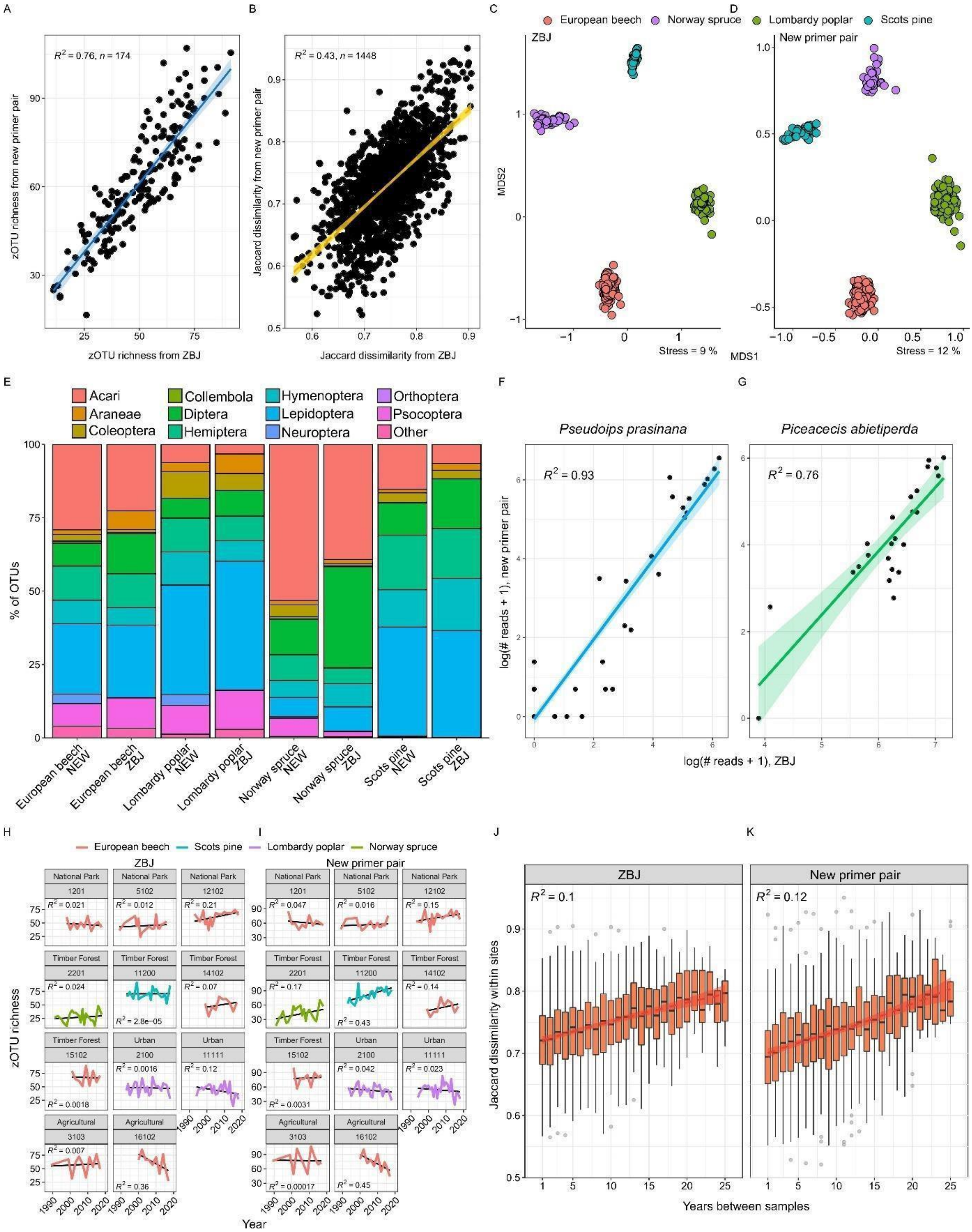
Supp. Figure 1.2: Effect of weight of plant material and precipitation events before sampling on the recovered arthropod diversity. (A) Correlation between the amount of leaf material (wet weight of leaves, in grams) in a homogenate sample and recovered arthropod community richness. No significant effect of increasing leaf amount on diversity was recovered, suggesting that the large number of leaves collected – several thousand per ESB sample – is sufficient to saturate the recovered diversity. (B) Correlation between richness and the number of days between last precipitation event and sampling event. The number of days between rainfall and sampling did not affect the recovered diversity.



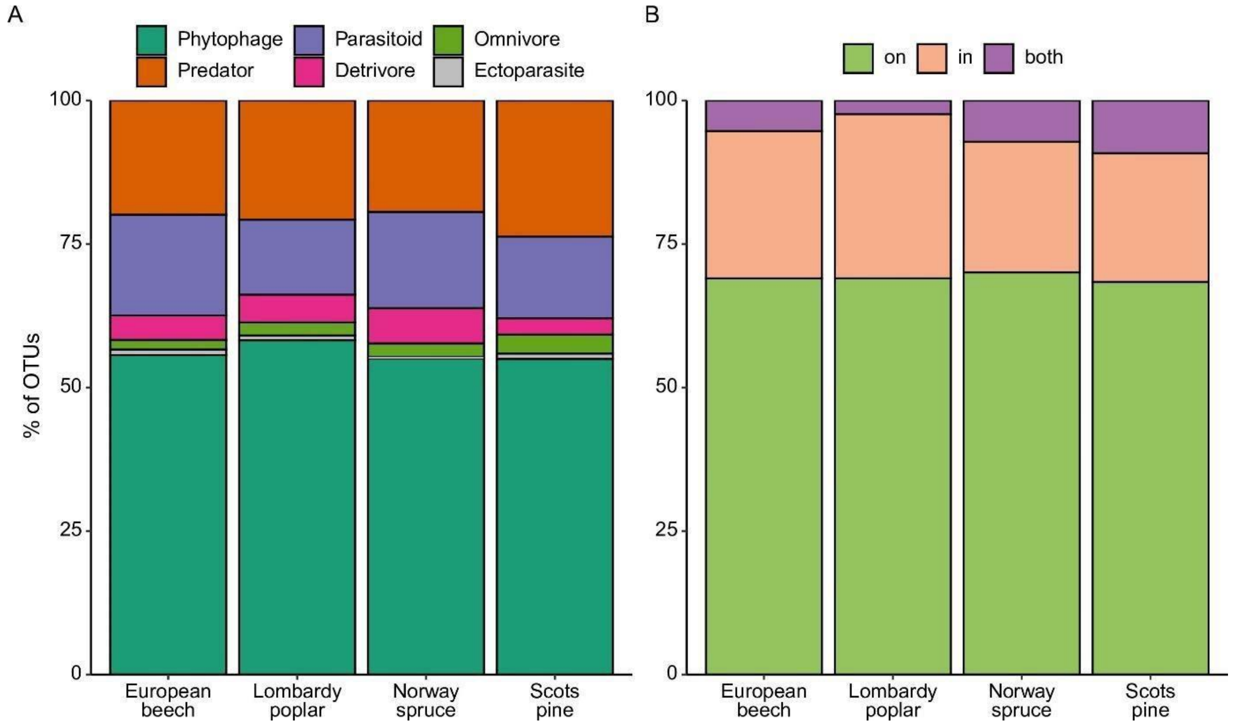
Supp. Figure 1.3: Contamination check in the cryomill. The plots show average β-diversity (and 95 % confidence interval) between ESB tree samples processed right after each other in the cryomill (same year) and for tree samples from the same site, which were not processed consecutively in the mill. The blue plot shows β-diversity between tree species while the orange one shows β-diversity within tree species. Assuming cross-contamination by the mill, samples processed consecutively should show a significant reduction of β-diversity.



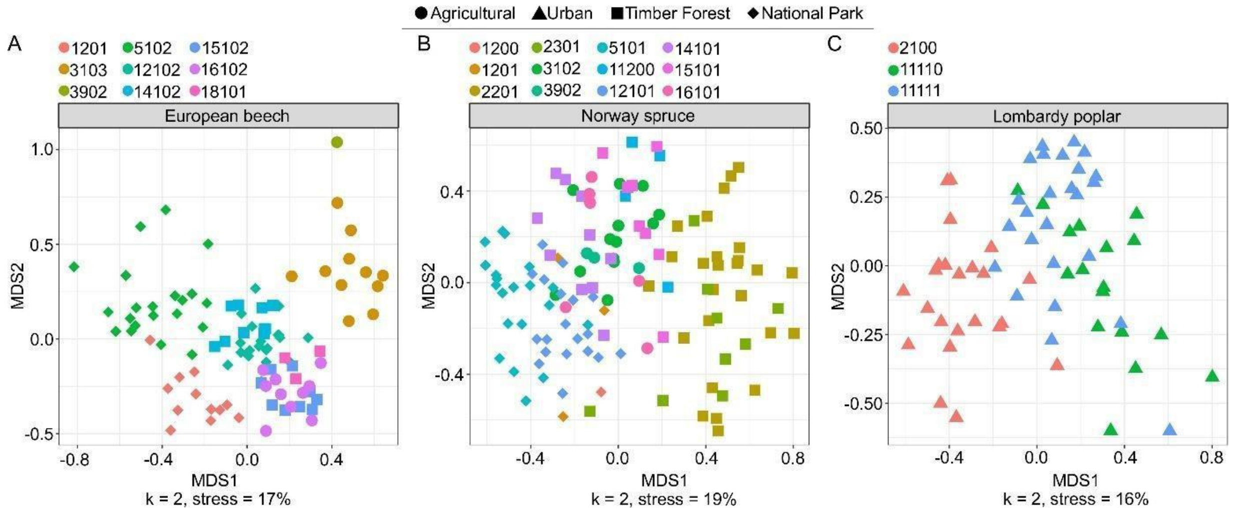
Supp. Figure 1.4: Rarefaction curves for all analysed samples. The dotted lines show the sampled read abundance for each of the two PCR replicates of each sample.



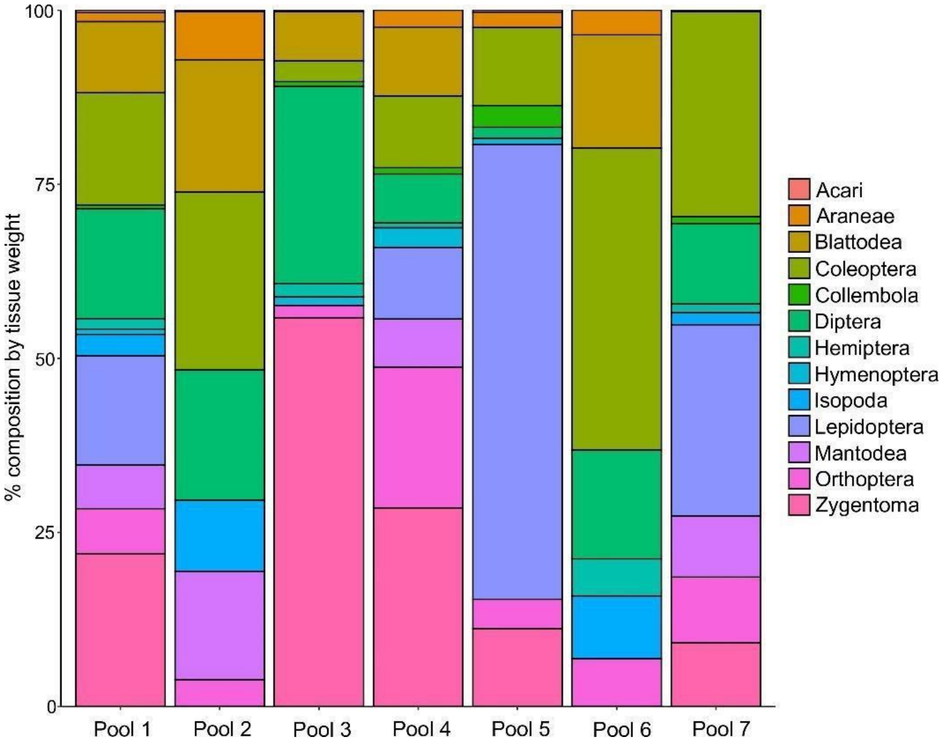
Supp. Figure 1.5: Comparison of recovered taxonomic composition and diversity patterns for the two COI markers (ZBJ-ArtF1c/ZBJ-ArtR2c vs. NoPlantF_270/mICOIintR_W) used in this study. (A) Correlation of zOTU richness and (B) β-diversity between samples. (C, D) NMDS ordination of samples. (E) Order-level taxonomic composition by tree species. (F, G) Comparison of single species read abundances of two exemplary taxa. (H, I) zOTU richness over time for the eleventime series analyzed for both markers. (J, K) Temporal community turnover: Jaccard dissimilarity within sites compared against temporal distance between sampling events.



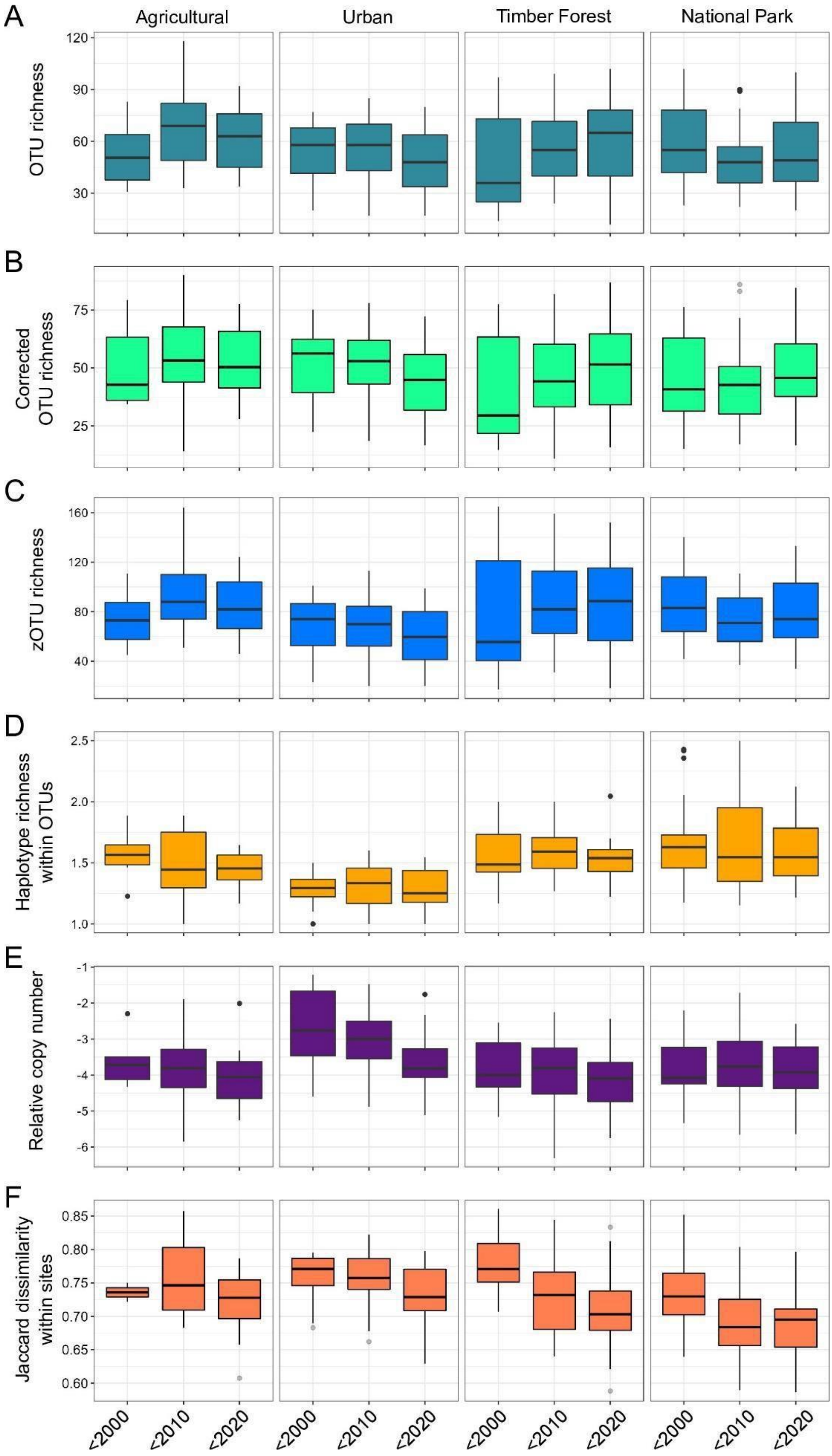
Supp. Figure 1.6: Ecological diversity of arthropod species recovered from the four tree species. (A) Relative abundance of arthropod OTUs in different feeding guilds. (B) Relative abundance of arthropod OTUs whose DNA likely originated from outside or inside the sampled leaves.



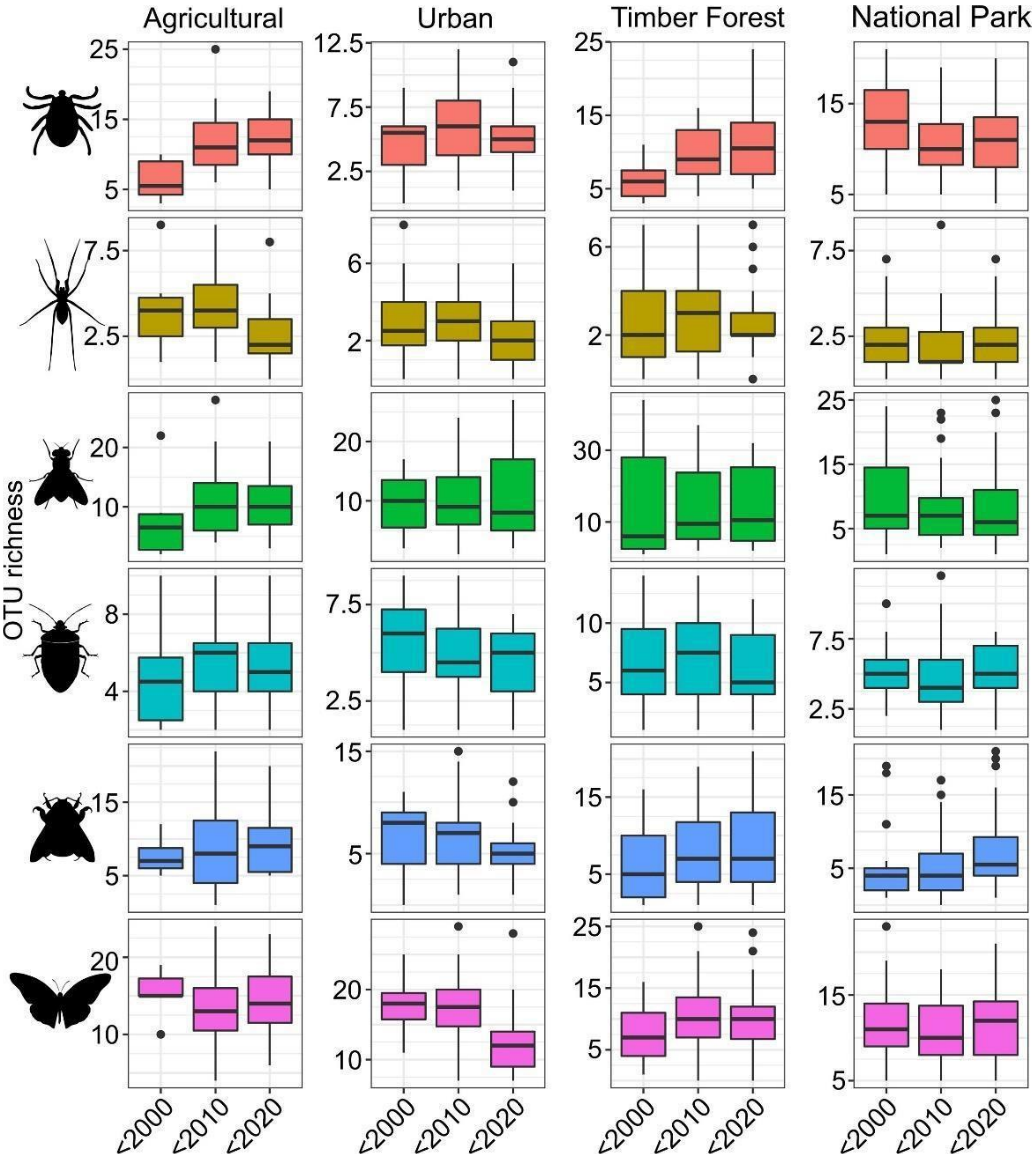
Supp. Figure 1.7: NMDS showing arthropod community differentiation by site, separated by tree species. (A) European Beech, (B) Norway Spruce, and (C) Lombardy Poplar. Color represents site and shape represents land use type.



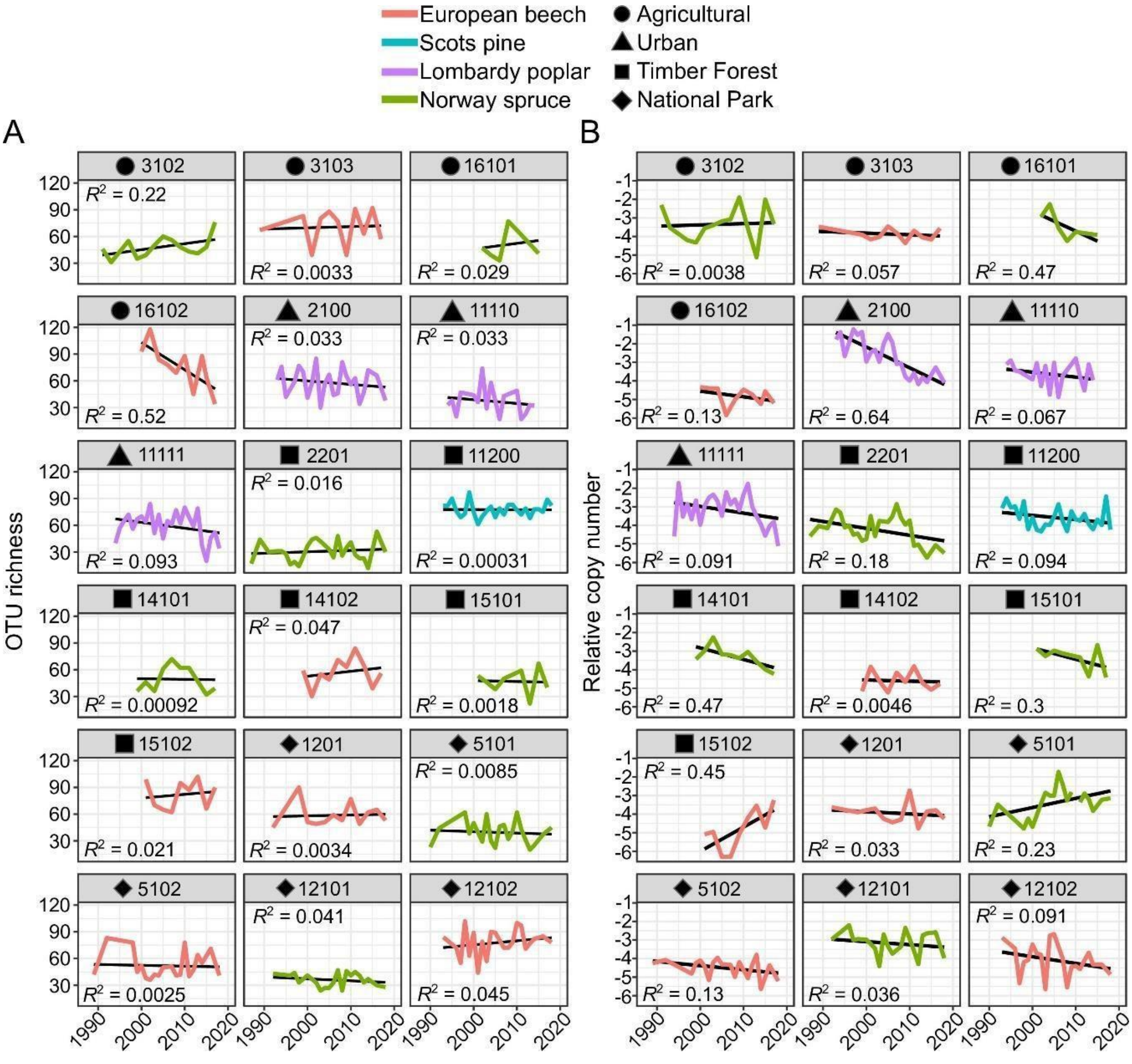
Supp. Figure 1.8: Taxonomic composition of the mock communities used to test the qPCR assay. Order-level composition of the seven mock communities (Pools 1–7) used to test our quantitative PCR (qPCR) assay. Each community contained different DNA proportions of species from 13 different orders and three classes across the arthropod tree of life.



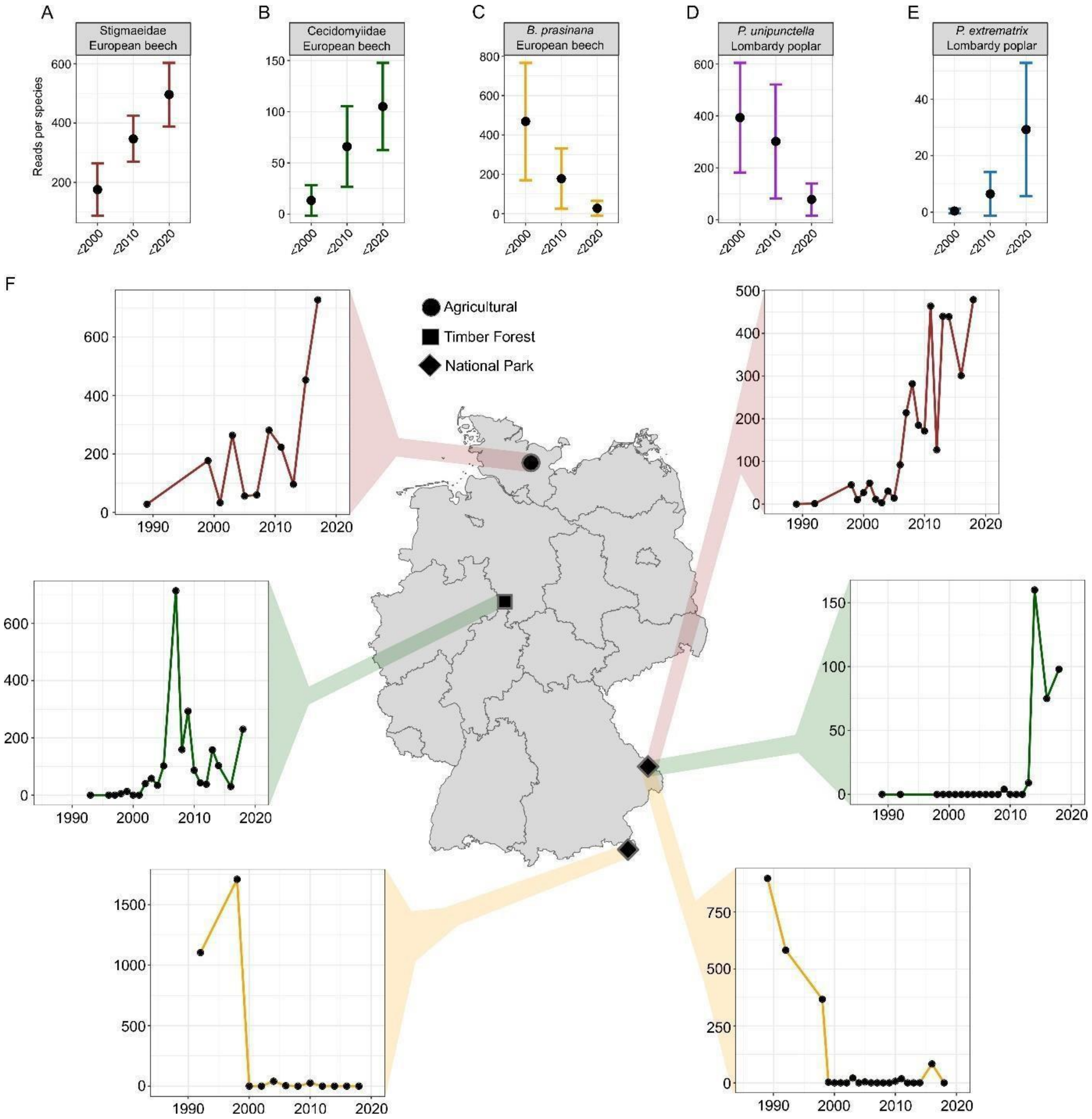
Supp. Figure 1.9: Metabarcoding-based diversity indices and quantitative PCR (qPCR)-based relative copy number of arthropod rDNA per land use type over three decades. Boxplots of (A) OTU richness, (B) copy number/biomass corrected OTU richness, (C) zOTU richness, (D) haplotype richness within OTUs, (E) relative copy number of arthropod rDNA, and (F) Jaccard dissimilarity between the given decade and the last sampling event in 2018, measured within sites. All datasets are merged by decade for clarity (before 2000, 2000–2009, and 2010–2018).



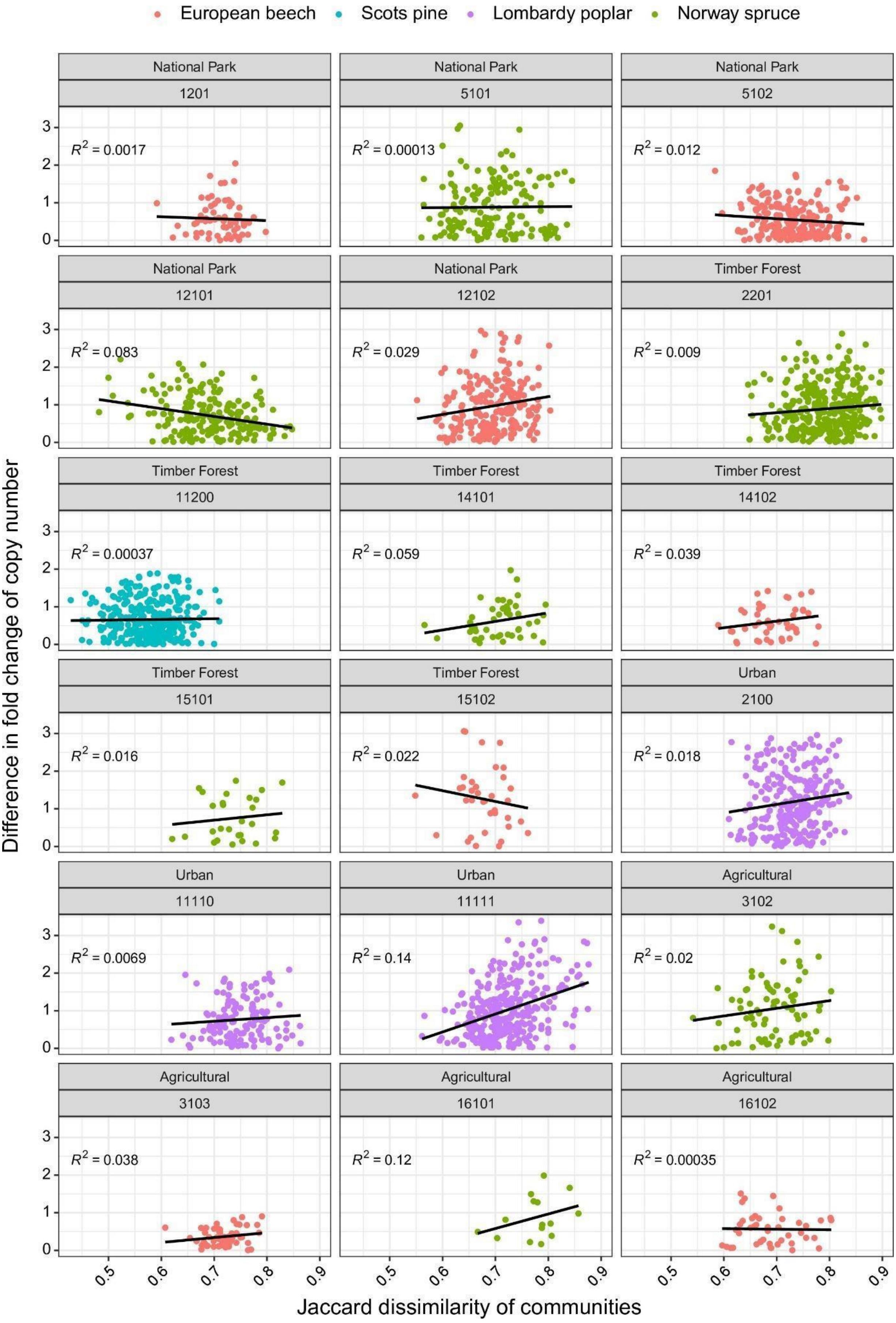
Supp. Figure 1.10: Boxplots of OTU richness by decade and land use type for the six most speciose arthropod orders in our dataset. The data are merged by decade for clarity (before 2000, 2000–2009, and 2010–2018). Richness is shown from top to bottom for Acari, Araneae, Diptera, Hemiptera, Hymenoptera, and Lepidoptera.



Supp. Figure 1.11: Arthropod diversity and relative copy number over time for all sites with time series longer than 10 years. (A) OTU richness and (B) relative arthropod DNA copy number. Colors represent tree species; symbols represent land use types. Site name is indicated above each plot.



Supp. Figure 1.12: Temporal change in relative read abundance for five exemplary arthropod OTUs at all beech and poplar sites and abundance change for three OTUs at two sites each in Germany. (A) Unidentified mite showing considerable increase over time. (B) Unidentified gall midge also showing an increase. (C) Decline in the Green Silver-Line over time. (D) Decline in Phyllocnistis unipunctella, a leaf-mining lepidopteran, at poplar sites. (E) Increase in a congeneric miner, P. extrematrix, at the same sites. The plots show mean read abundance and 95 % confidence interval. (F) Exemplary gains of the OTUs from (A) and (B) and losses of the OTU from (C) at four sites in Germany.



Supp. Figure 1.13: Correlation of dissimilarity in 18S rDNA copy number and β-diversity for all sites with time series longer than 10 years. Colors represent tree species; symbols represent land use types. Site name is indicated above each plot.

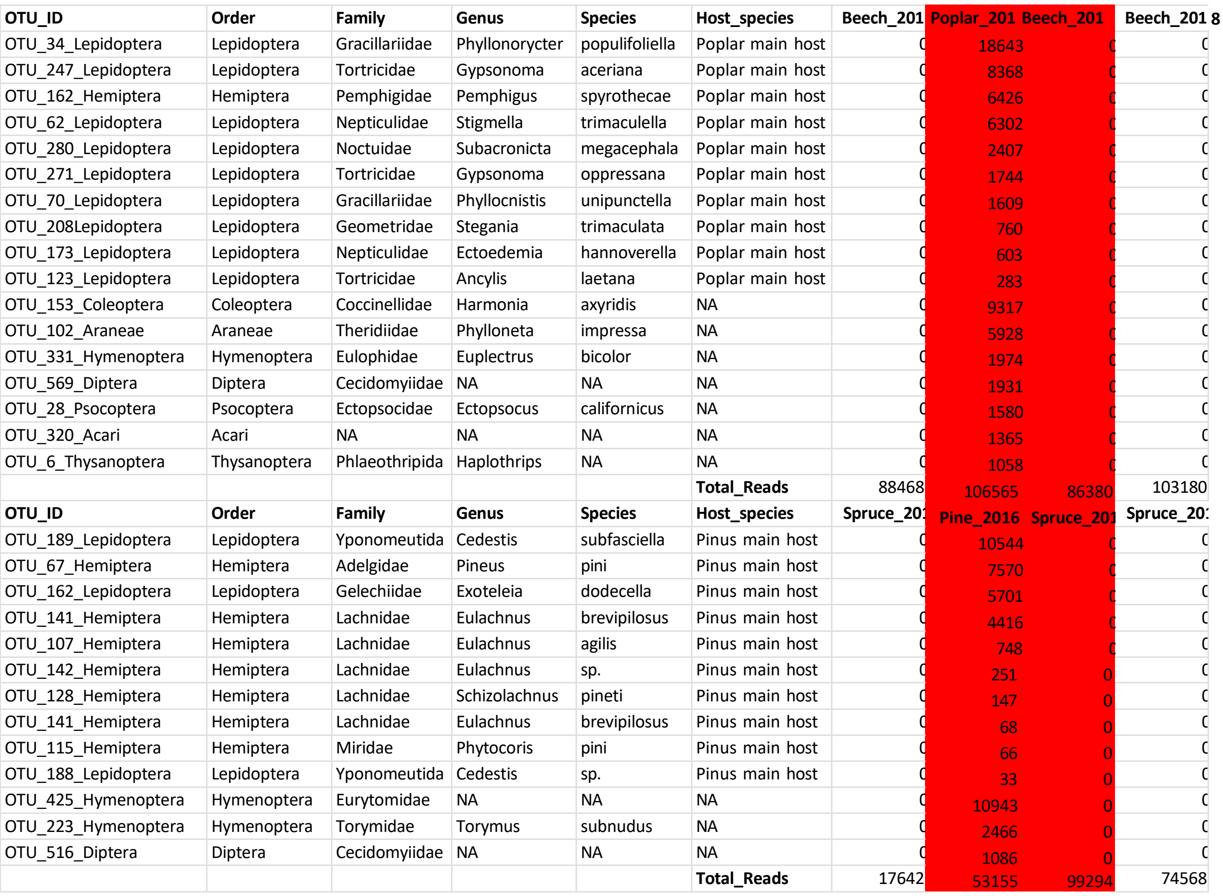
Supp. Table 1.1: Primers used in this study.

Primer Name	Sequence 5'-3' (Illumina Truseq-tails in red)	Experiment	Reference
ZBJ-ArtF1c	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGATATTGGAACWTTATATTTTATTTTTGG	Community metabarcoding	Zeale et al. 2011
ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTWACTAATCAATTWCCAAATCCTCC	Community metabarcoding	Zeale et al. 2011
NoPlantF_270	ACACTCTTTCCCTACACGACGCTCTTCCGATCTRGCHTTYCCHCGWATAAAYAAYATAAG	Community metabarcoding	Krehenwinkel et al. 2022
mICOlinR_W	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGRGGRTAWACWGTTCAWCCWGTNCC	Community metabarcoding	Krehenwinkel et al. 2022
18S_Insect_F1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGGAGGTAGTGACRAA	qPCR inner primer	This study
18S_Plant_F1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGGAGGTAGTGACAAT	qPCR inner primer	This study
18S_Insect_R1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTTTAACCGCAACAACTTT	qPCR inner primer	This study
18S_Plant_R1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTTTAACTGCAACAACTTA	qPCR inner primer	This study
18S_Insect_F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGGGAGGTAGTGACRAA	qPCR inner primer	This study
18S_Plant_F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTYGGGGAGGTAGTGACAAT	qPCR inner primer	This study
18S_Insect_R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACCGCAACAACTTTARTATACGC	qPCR inner primer	This study
18S_Plant_R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTGCAACAACTTAAATATACGC	qPCR inner primer	This study
qPCR_Tail_F2	ACACTCTTTCCCTACACGACG	nested qPCR	This study
qPCR_Tail_R2	GTGACTGGAGTTCAGACGTGT	nested qPCR	This study

Supp. Table 1.2: OTU table with metadata and quantitative PCR (qPCR) results.

The OTU table is too big for printing. The supplementary file can be downloaded via this link: Download

Supp. Table 1.3: Contamination check in the cryomill. The table shows the taxonomic annotation for the 10 most abundant host-specific OTUs, as well as all OTUs above 1000 reads for a poplar and a pine ESB sample from 2016 (highlighted in red). The read abundance for the same OTUs in a beech and spruce sample from 2016, which were processed directly after the poplar and pine sample in the mill, are highlighted in red. The OTU abundances for beech and spruce samples from the same sites and processed in consecutive years are also shown. The total read abundance for each sample is shown below the tables (Reads).



OTU_ID	Order	Family	Genus	Species	Host_species	Beech_201	Poplar_201	Beech_201	Beech_2018
OTU_34_Lepidoptera	Lepidoptera	Gracillariidae	Phyllonorycter	populifoliella	Poplar main host	0	18643	0	0
OTU_247_Lepidoptera	Lepidoptera	Tortricidae	Gypsonoma	aceriana	Poplar main host	0	8368	0	0
OTU_162_Hemiptera	Hemiptera	Pemphigidae	Pemphigus	spyrothecae	Poplar main host	0	6426	0	0
OTU_62_Lepidoptera	Lepidoptera	Nepticulidae	Stigmella	trimaculella	Poplar main host	0	6302	0	0
OTU_280_Lepidoptera	Lepidoptera	Noctuidae	Subacronicta	megacephala	Poplar main host	0	2407	0	0
OTU_271_Lepidoptera	Lepidoptera	Tortricidae	Gypsonoma	oppressana	Poplar main host	0	1744	0	0
OTU_70_Lepidoptera	Lepidoptera	Gracillariidae	Phyllocnistis	unipunctella	Poplar main host	0	1609	0	0
OTU_208Lepidoptera	Lepidoptera	Geometridae	Stegania	trimaculata	Poplar main host	0	760	0	0
OTU_173_Lepidoptera	Lepidoptera	Nepticulidae	Ectoedemia	hannoverella	Poplar main host	0	603	0	0
OTU_123_Lepidoptera	Lepidoptera	Tortricidae	Ancylis	laetana	Poplar main host	0	283	0	0
OTU_153_Coleoptera	Coleoptera	Coccinellidae	Harmonia	axyridis	NA	0	9317	0	0
OTU_102_Araneae	Araneae	Theridiidae	Phylloneta	impressa	NA	0	5928	0	0
OTU_331_Hymenoptera	Hymenoptera	Eulophidae	Euplectrus	bicolor	NA	0	1974	0	0
OTU_569_Diptera	Diptera	Cecidomyiidae	NA	NA	NA	0	1931	0	0
OTU_28_Psocoptera	Psocoptera	Ectopsocidae	Ectopsocus	californicus	NA	0	1580	0	0
OTU_320_Acari	Acari	NA	NA	NA	NA	0	1365	0	0
OTU_6_Thysanoptera	Thysanoptera	Phlaeothripida	Haplothrips	NA	NA	0	1058	0	0
					Total_Reads	88468	106565	86380	103180
OTU_ID	**Order**	**Family**	**Genus**	**Species**	**Host_species**	**Spruce_201**	**Pine_2016**	**Spruce_201**	**Spruce_201**
OTU_189_Lepidoptera	Lepidoptera	Yponomeutida	Cedestis	subfasciella	Pinus main host	0	10544	0	0
OTU_67_Hemiptera	Hemiptera	Adelgidae	Pineus	pini	Pinus main host	0	7570	0	0
OTU_162_Lepidoptera	Lepidoptera	Gelechiidae	Exoteleia	dodecella	Pinus main host	0	5701	0	0
OTU_141_Hemiptera	Hemiptera	Lachnidae	Eulachnus	brevipilosus	Pinus main host	0	4416	0	0
OTU_107_Hemiptera	Hemiptera	Lachnidae	Eulachnus	agilis	Pinus main host	0	748	0	0
OTU_142_Hemiptera	Hemiptera	Lachnidae	Eulachnus	sp.	Pinus main host	0	251	0	0
OTU_128_Hemiptera	Hemiptera	Lachnidae	Schizolachnus	pineti	Pinus main host	0	147	0	0
OTU_141_Hemiptera	Hemiptera	Lachnidae	Eulachnus	brevipilosus	Pinus main host	0	68	0	0
OTU_115_Hemiptera	Hemiptera	Miridae	Phytocoris	pini	Pinus main host	0	66	0	0
OTU_188_Lepidoptera	Lepidoptera	Yponomeutida	Cedestis	sp.	Pinus main host	0	33	0	0
OTU_425_Hymenoptera	Hymenoptera	Eurytomidae	NA	NA	NA	0	10943	0	0
OTU_223_Hymenoptera	Hymenoptera	Torymidae	Torymus	subnudus	NA	0	2466	0	0
OTU_516_Diptera	Diptera	Cecidomyiidae	NA	NA	NA	0	1086	0	0
					Total_Reads	17642	53155	99294	74568

Supplementary material

Chapter 3

Chapter three has been published in the journal *Nature Ecology & Evolution*. The data that support the findings of this study are available via the Science Data Bank repository at https://doi.org/10.57760/sciencedb.13553. Raw Illumina sequencing data are available in the European Nucleotide Archive repository under accession number PRJEB88877. Source data are provided with this paper.

Appendix

Supp. Table 2.1: List of all samples used in this study.

Species	**Location**	**Year**	**Latitude_WGS84**	**Longitude_WGS84**	**Lab_ID**
European Beech (*Fagus sylvatica*)	Bayerischer Wald	1989	48.966395	13.430375	Bu97
European Beech (*Fagus sylvatica*)	Bayerischer Wald	1992	48.966395	13.430375	Bu98
European Beech (*Fagus sylvatica*)	Bayerischer Wald	1998	48.966395	13.430375	Bu99
European Beech (*Fagus sylvatica*)	Bayerischer Wald	1999	48.966395	13.430375	Bu100
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2000	48.966395	13.430375	Bu101
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2001	48.966395	13.430375	Bu102
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2002	48.966395	13.430375	Bu103
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2003	48.966395	13.430375	Bu104
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2004	48.966395	13.430375	Bu105
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2005	48.966395	13.430375	Bu106
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2006	48.966395	13.430375	Bu107
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2007	48.966395	13.430375	Bu108
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2008	48.966395	13.430375	Bu109
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2009	48.966395	13.430375	Bu110
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2010	48.966395	13.430375	Bu111
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2011	48.966395	13.430375	Bu112
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2012	48.966395	13.430375	Bu113
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2013	48.966395	13.430375	Bu114
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2014	48.966395	13.430375	Bu115
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2016	48.966395	13.430375	Bu116
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2018	48.966395	13.430375	Bu94
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2020	48.966395	13.430375	Bu95
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2022	48.966395	13.430375	Bu96
European Beech (*Fagus sylvatica*)	Belauer See	1987	54.06061	10.15373	Bu22
European Beech (*Fagus sylvatica*)	Belauer See	1989	54.06061	10.15373	Bu23
European Beech (*Fagus sylvatica*)	Belauer See	1991	54.06061	10.15373	Bu24
European Beech (*Fagus sylvatica*)	Belauer See	1993	54.06061	10.15373	Bu25
European Beech (*Fagus sylvatica*)	Belauer See	1997	54.06061	10.15373	Bu26
European Beech (*Fagus sylvatica*)	Belauer See	1999	54.06061	10.15373	Bu27
European Beech (*Fagus sylvatica*)	Belauer See	2001	54.06061	10.15373	Bu28
European Beech (*Fagus sylvatica*)	Belauer See	2003	54.06061	10.15373	Bu29
European Beech (*Fagus sylvatica*)	Belauer See	2005	54.06061	10.15373	Bu30
European Beech (*Fagus sylvatica*)	Belauer See	2007	54.06061	10.15373	Bu31
European Beech (*Fagus sylvatica*)	Belauer See	2009	54.06061	10.15373	Bu32
European Beech (*Fagus sylvatica*)	Belauer See	2011	54.06061	10.15373	Bu33
European Beech (*Fagus sylvatica*)	Belauer See	2013	54.06061	10.15373	Bu34
European Beech (*Fagus sylvatica*)	Belauer See	2015	54.06061	10.15373	Bu35
European Beech (*Fagus sylvatica*)	Belauer See	2017	54.06061	10.15373	Bu49
European Beech (*Fagus sylvatica*)	Belauer See	2019	54.06061	10.15373	Bu50
European Beech (*Fagus sylvatica*)	Belauer See	2021	54.06061	10.15373	Bu51
European Beech (*Fagus sylvatica*)	Berchtesgaden	1990	47.56574	12.89274	Bu47
European Beech (*Fagus sylvatica*)	Berchtesgaden	1992	47.56574	12.89274	Bu48

European Beech (*Fagus sylvatica*)	Berchtesgaden	1998	47.56574	12.89274	Bu8
European Beech (*Fagus sylvatica*)	Berchtesgaden	2000	47.56574	12.89274	Bu9
European Beech (*Fagus sylvatica*)	Berchtesgaden	2002	47.56574	12.89274	Bu10
European Beech (*Fagus sylvatica*)	Berchtesgaden	2004	47.56574	12.89274	Bu11
European Beech (*Fagus sylvatica*)	Berchtesgaden	2006	47.56574	12.89274	Bu13
European Beech (*Fagus sylvatica*)	Berchtesgaden	2008	47.56574	12.89274	Bu14
European Beech (*Fagus sylvatica*)	Berchtesgaden	2010	47.56574	12.89274	Bu15
European Beech (*Fagus sylvatica*)	Berchtesgaden	2012	47.56574	12.89274	Bu16
European Beech (*Fagus sylvatica*)	Berchtesgaden	2014	47.56574	12.89274	Bu17
European Beech (*Fagus sylvatica*)	Berchtesgaden	2016	47.56574	12.89274	Bu18
European Beech (*Fagus sylvatica*)	Berchtesgaden	2018	47.56574	12.89274	Bu19
European Beech (*Fagus sylvatica*)	Berchtesgaden	2020	47.56574	12.89274	Bu20
European Beech (*Fagus sylvatica*)	Berchtesgaden	2022	47.56574	12.89274	Bu21
European Beech (*Fagus sylvatica*)	Harz	1991	51.838522	10.635239	Bu80
European Beech (*Fagus sylvatica*)	Harz	1993	51.838522	10.635239	Bu81
European Beech (*Fagus sylvatica*)	Harz	1996	51.838522	10.635239	Bu82
European Beech (*Fagus sylvatica*)	Harz	1997	51.838522	10.635239	Bu83
European Beech (*Fagus sylvatica*)	Harz	1998	51.838522	10.635239	Bu84
European Beech (*Fagus sylvatica*)	Harz	1999	51.838522	10.635239	Bu88
European Beech (*Fagus sylvatica*)	Harz	2000	51.838522	10.635239	Bu85
European Beech (*Fagus sylvatica*)	Harz	2001	51.838522	10.635239	Bu86
European Beech (*Fagus sylvatica*)	Harz	2002	51.838522	10.635239	Bu87
European Beech (*Fagus sylvatica*)	Harz	2003	51.838522	10.635239	Bu89
European Beech (*Fagus sylvatica*)	Harz	2004	51.838522	10.635239	Bu90
European Beech (*Fagus sylvatica*)	Harz	2005	51.838522	10.635239	Bu91
European Beech (*Fagus sylvatica*)	Harz	2006	51.838522	10.635239	Bu92
European Beech (*Fagus sylvatica*)	Harz	2007	51.838522	10.635239	Bu93
European Beech (*Fagus sylvatica*)	Harz	2008	51.838522	10.635239	Bu72
European Beech (*Fagus sylvatica*)	Harz	2009	51.838522	10.635239	Bu69
European Beech (*Fagus sylvatica*)	Harz	2010	51.838522	10.635239	Bu66
European Beech (*Fagus sylvatica*)	Harz	2011	51.838522	10.635239	Bu64
European Beech (*Fagus sylvatica*)	Harz	2012	51.838522	10.635239	Bu73
European Beech (*Fagus sylvatica*)	Harz	2013	51.838522	10.635239	Bu70
European Beech (*Fagus sylvatica*)	Harz	2014	51.838522	10.635239	Bu67
European Beech (*Fagus sylvatica*)	Harz	2016	51.838522	10.635239	Bu65
European Beech (*Fagus sylvatica*)	Harz	2018	51.838522	10.635239	Bu74
European Beech (*Fagus sylvatica*)	Harz	2020	51.838522	10.635239	Bu71
European Beech (*Fagus sylvatica*)	Harz	2022	51.838522	10.635239	Bu68
European Beech (*Fagus sylvatica*)	Pfälzerwald	2001	49.145482	7.713357	Bu36
European Beech (*Fagus sylvatica*)	Pfälzerwald	2003	49.145482	7.713357	Bu37
European Beech (*Fagus sylvatica*)	Pfälzerwald	2005	49.145482	7.713357	Bu38
European Beech (*Fagus sylvatica*)	Pfälzerwald	2007	49.145482	7.713357	Bu39
European Beech (*Fagus sylvatica*)	Pfälzerwald	2009	49.145482	7.713357	Bu40
European Beech (*Fagus sylvatica*)	Pfälzerwald	2011	49.145482	7.713357	Bu41
European Beech (*Fagus sylvatica*)	Pfälzerwald	2013	49.145482	7.713357	Bu42
European Beech (*Fagus sylvatica*)	Pfälzerwald	2015	49.145482	7.713357	Bu43

European Beech (*Fagus sylvatica*)	Pfälzerwald	2017	49.145482	7.713357	Bu44
European Beech (*Fagus sylvatica*)	Pfälzerwald	2019	49.145482	7.713357	Bu45
European Beech (*Fagus sylvatica*)	Pfälzerwald	2021	49.145482	7.713357	Bu46
European Beech (*Fagus sylvatica*)	Scheyern	2000	48.487405	11.428479	Bu75
European Beech (*Fagus sylvatica*)	Scheyern	2002	48.487405	11.428479	Bu76
European Beech (*Fagus sylvatica*)	Scheyern	2004	48.487405	11.428479	Bu77
European Beech (*Fagus sylvatica*)	Scheyern	2006	48.487405	11.428479	Bu78
European Beech (*Fagus sylvatica*)	Scheyern	2008	48.487405	11.428479	Bu79
European Beech (*Fagus sylvatica*)	Scheyern	2010	48.487405	11.428479	Bu1
European Beech (*Fagus sylvatica*)	Scheyern	2012	48.487405	11.428479	Bu2
European Beech (*Fagus sylvatica*)	Scheyern	2014	48.487405	11.428479	Bu3
European Beech (*Fagus sylvatica*)	Scheyern	2015	48.487405	11.428479	Bu4
European Beech (*Fagus sylvatica*)	Scheyern	2017	48.487405	11.428479	Bu5
European Beech (*Fagus sylvatica*)	Scheyern	2019	48.487405	11.428479	Bu6
European Beech (*Fagus sylvatica*)	Scheyern	2021	48.487405	11.428479	Bu7
European Beech (*Fagus sylvatica*)	Solling	1999	51.73638	9.57365	Bu52
European Beech (*Fagus sylvatica*)	Solling	2001	51.73638	9.57365	Bu53
European Beech (*Fagus sylvatica*)	Solling	2003	51.73638	9.57365	Bu54
European Beech (*Fagus sylvatica*)	Solling	2005	51.73638	9.57365	Bu55
European Beech (*Fagus sylvatica*)	Solling	2007	51.73638	9.57365	Bu56
European Beech (*Fagus sylvatica*)	Solling	2009	51.73638	9.57365	Bu57
European Beech (*Fagus sylvatica*)	Solling	2011	51.73638	9.57365	Bu58
European Beech (*Fagus sylvatica*)	Solling	2013	51.73638	9.57365	Bu59
European Beech (*Fagus sylvatica*)	Solling	2015	51.73638	9.57365	Bu60
European Beech (*Fagus sylvatica*)	Solling	2017	51.73638	9.57365	Bu61
European Beech (*Fagus sylvatica*)	Solling	2019	51.73638	9.57365	Bu62
European Beech (*Fagus sylvatica*)	Solling	2021	51.73638	9.57365	Bu63
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1985	53.519772	8.231447	Bl21
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1987	53.519772	8.231447	Bl19
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1989	53.519772	8.231447	Bl16
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1991	53.519772	8.231447	Bl3
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1993	53.519772	8.231447	Bl22
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1994	53.519772	8.231447	Bl5
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1995	53.519772	8.231447	Bl15
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1996	53.519772	8.231447	Bl24
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1997	53.519772	8.231447	Bl9
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1998	53.519772	8.231447	Bl7
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	1999	53.519772	8.231447	Bl2
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2000	53.519772	8.231447	Bl26
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2001	53.519772	8.231447	Bl29
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2002	53.519772	8.231447	Bl30
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2003	53.519772	8.231447	Bl31
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2004	53.519772	8.231447	Bl27
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2005	53.519772	8.231447	Bl34
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2006	53.519772	8.231447	Bl35
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2007	53.519772	8.231447	Bl33

Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2008	53.519772	8.231447	Bl32
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2009	53.519772	8.231447	Bl39
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2010	53.519772	8.231447	Bl38
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2011	53.519772	8.231447	Bl37
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2012	53.519772	8.231447	Bl36
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2020	53.519772	8.231447	Bl70
Bladderwrack (*Fucus vesiculosus*)	Eckwarderhörne	2021	53.519772	8.231447	BTB1
Bladderwrack (*Fucus vesiculosus*)	Ostsee	1998	54.506479	13.284812	Bl18
Bladderwrack (*Fucus vesiculosus*)	Ostsee	1999	54.506479	13.284812	Bl12
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2000	54.506479	13.284812	Bl60
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2001	54.506479	13.284812	Bl54
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2002	54.506479	13.284812	Bl66
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2003	54.506479	13.284812	Bl63
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2004	54.506479	13.284812	Bl57
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2005	54.506479	13.284812	Bl53
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2006	54.506479	13.284812	Bl67
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2007	54.506479	13.284812	Bl64
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2008	54.506479	13.284812	Bl59
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2009	54.506479	13.284812	Bl56
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2010	54.506479	13.284812	Bl68
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2011	54.506479	13.284812	Bl62
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2012	54.506479	13.284812	Bl58
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2013	54.506479	13.284812	Bl55
Bladderwrack (*Fucus vesiculosus*)	Ostsee	2021	54.506479	13.284812	BTB3
Bladderwrack (*Fucus vesiculosus*)	Sylt	1985	55.011	8.4125	Bl17
Bladderwrack (*Fucus vesiculosus*)	Sylt	1987	55.011	8.4125	Bl8
Bladderwrack (*Fucus vesiculosus*)	Sylt	1989	55.011	8.4125	Bl61
Bladderwrack (*Fucus vesiculosus*)	Sylt	1991	55.011	8.4125	Bl1
Bladderwrack (*Fucus vesiculosus*)	Sylt	1993	55.011	8.4125	Bl20
Bladderwrack (*Fucus vesiculosus*)	Sylt	1995	55.011	8.4125	Bl4
Bladderwrack (*Fucus vesiculosus*)	Sylt	1996	55.011	8.4125	Bl6
Bladderwrack (*Fucus vesiculosus*)	Sylt	1997	55.011	8.4125	Bl13
Bladderwrack (*Fucus vesiculosus*)	Sylt	1998	55.011	8.4125	Bl10
Bladderwrack (*Fucus vesiculosus*)	Sylt	1999	55.011	8.4125	Bl25
Bladderwrack (*Fucus vesiculosus*)	Sylt	2000	55.011	8.4125	Bl40
Bladderwrack (*Fucus vesiculosus*)	Sylt	2001	55.011	8.4125	Bl51
Bladderwrack (*Fucus vesiculosus*)	Sylt	2002	55.011	8.4125	Bl52
Bladderwrack (*Fucus vesiculosus*)	Sylt	2003	55.011	8.4125	Bl43
Bladderwrack (*Fucus vesiculosus*)	Sylt	2004	55.011	8.4125	Bl41
Bladderwrack (*Fucus vesiculosus*)	Sylt	2005	55.011	8.4125	Bl50
Bladderwrack (*Fucus vesiculosus*)	Sylt	2006	55.011	8.4125	Bl47
Bladderwrack (*Fucus vesiculosus*)	Sylt	2007	55.011	8.4125	Bl45
Bladderwrack (*Fucus vesiculosus*)	Sylt	2008	55.011	8.4125	Bl42
Bladderwrack (*Fucus vesiculosus*)	Sylt	2009	55.011	8.4125	Bl49
Bladderwrack (*Fucus vesiculosus*)	Sylt	2010	55.011	8.4125	Bl48
Bladderwrack (*Fucus vesiculosus*)	Sylt	2011	55.011	8.4125	Bl46

Bladderwrack (*Fucus vesiculosus*)	Sylt	2012	55.011	8.4125	Bl44
Bladderwrack (*Fucus vesiculosus*)	Sylt	2021	55.011	8.4125	BTB2
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1985	53.519772	8.231447	M2
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1986	53.519772	8.231447	M31
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1988	53.519772	8.231447	M50
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1990	53.519772	8.231447	M43
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1992	53.519772	8.231447	M46
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1993	53.519772	8.231447	M17
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1994	53.519772	8.231447	M4
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1995	53.519772	8.231447	M33
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1997	53.519772	8.231447	M39
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1998	53.519772	8.231447	M22
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	1999	53.519772	8.231447	M14
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2000	53.519772	8.231447	M1
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2001	53.519772	8.231447	M18
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2002	53.519772	8.231447	M27
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2003	53.519772	8.231447	M63
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2004	53.519772	8.231447	M45
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2005	53.519772	8.231447	M69
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2006	53.519772	8.231447	M61
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2007	53.519772	8.231447	M65
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2008	53.519772	8.231447	M67
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2009	53.519772	8.231447	M70
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2010	53.519772	8.231447	M62
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2011	53.519772	8.231447	M64
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2012	53.519772	8.231447	M66
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2013	53.519772	8.231447	M68
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2014	53.519772	8.231447	M71
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2015	53.519772	8.231447	M72
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2016	53.519772	8.231447	M73
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2017	53.519772	8.231447	M74
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2018	53.519772	8.231447	M75
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2019	53.519772	8.231447	M76
Blue Mussel (*Mytilus edulis*)	Eckwarderhörne	2020	53.519772	8.231447	M77
Blue Mussel (*Mytilus edulis*)	Ostsee	1992	54.275622	12.318046	M7
Blue Mussel (*Mytilus edulis*)	Ostsee	1993	54.275622	12.318046	M25
Blue Mussel (*Mytilus edulis*)	Ostsee	1994	54.275622	12.318046	M6
Blue Mussel (*Mytilus edulis*)	Ostsee	1995	54.275622	12.318046	M35
Blue Mussel (*Mytilus edulis*)	Ostsee	1996	54.275622	12.318046	M32
Blue Mussel (*Mytilus edulis*)	Ostsee	1998	54.275622	12.318046	M29
Blue Mussel (*Mytilus edulis*)	Ostsee	1999	54.275622	12.318046	M8
Blue Mussel (*Mytilus edulis*)	Ostsee	2000	54.275622	12.318046	M12
Blue Mussel (*Mytilus edulis*)	Ostsee	2001	54.275622	12.318046	M26
Blue Mussel (*Mytilus edulis*)	Ostsee	2002	54.275622	12.318046	M24
Blue Mussel (*Mytilus edulis*)	Ostsee	2003	54.275622	12.318046	M23
Blue Mussel (*Mytilus edulis*)	Ostsee	2004	54.275622	12.318046	M20

Blue Mussel (*Mytilus edulis*)	Ostsee	2005	54.275622	12.318046	M44
Blue Mussel (*Mytilus edulis*)	Ostsee	2006	54.275622	12.318046	M34
Blue Mussel (*Mytilus edulis*)	Ostsee	2007	54.275622	12.318046	M81
Blue Mussel (*Mytilus edulis*)	Ostsee	2008	54.275622	12.318046	M49
Blue Mussel (*Mytilus edulis*)	Ostsee	2009	54.275622	12.318046	M78
Blue Mussel (*Mytilus edulis*)	Ostsee	2010	54.275622	12.318046	M79
Blue Mussel (*Mytilus edulis*)	Ostsee	2011	54.275622	12.318046	M80
Blue Mussel (*Mytilus edulis*)	Ostsee	2012	54.275622	12.318046	M82
Blue Mussel (*Mytilus edulis*)	Ostsee	2013	54.275622	12.318046	M84
Blue Mussel (*Mytilus edulis*)	Ostsee	2014	54.275622	12.318046	M85
Blue Mussel (*Mytilus edulis*)	Ostsee	2015	54.275622	12.318046	M87
Blue Mussel (*Mytilus edulis*)	Ostsee	2016	54.275622	12.318046	M89
Blue Mussel (*Mytilus edulis*)	Ostsee	2017	54.275622	12.318046	M83
Blue Mussel (*Mytilus edulis*)	Ostsee	2018	54.275622	12.318046	M86
Blue Mussel (*Mytilus edulis*)	Ostsee	2019	54.275622	12.318046	M88
Blue Mussel (*Mytilus edulis*)	Ostsee	2020	54.275622	12.318046	M90
Blue Mussel (*Mytilus edulis*)	Sylt	1986	55.011	8.4125	M10
Blue Mussel (*Mytilus edulis*)	Sylt	1990	55.011	8.4125	M42
Blue Mussel (*Mytilus edulis*)	Sylt	1992	55.011	8.4125	M11
Blue Mussel (*Mytilus edulis*)	Sylt	1993	55.011	8.4125	M21
Blue Mussel (*Mytilus edulis*)	Sylt	1994	55.011	8.4125	M5
Blue Mussel (*Mytilus edulis*)	Sylt	1995	55.011	8.4125	M3
Blue Mussel (*Mytilus edulis*)	Sylt	1996	55.011	8.4125	M19
Blue Mussel (*Mytilus edulis*)	Sylt	1997	55.011	8.4125	M9
Blue Mussel (*Mytilus edulis*)	Sylt	1998	55.011	8.4125	M30
Blue Mussel (*Mytilus edulis*)	Sylt	1999	55.011	8.4125	M91
Blue Mussel (*Mytilus edulis*)	Sylt	2000	55.011	8.4125	M13
Blue Mussel (*Mytilus edulis*)	Sylt	2001	55.011	8.4125	M15
Blue Mussel (*Mytilus edulis*)	Sylt	2002	55.011	8.4125	M38
Blue Mussel (*Mytilus edulis*)	Sylt	2003	55.011	8.4125	M48
Blue Mussel (*Mytilus edulis*)	Sylt	2004	55.011	8.4125	M28
Blue Mussel (*Mytilus edulis*)	Sylt	2005	55.011	8.4125	M37
Blue Mussel (*Mytilus edulis*)	Sylt	2006	55.011	8.4125	M40
Blue Mussel (*Mytilus edulis*)	Sylt	2007	55.011	8.4125	M51
Blue Mussel (*Mytilus edulis*)	Sylt	2008	55.011	8.4125	M47
Blue Mussel (*Mytilus edulis*)	Sylt	2009	55.011	8.4125	M56
Blue Mussel (*Mytilus edulis*)	Sylt	2010	55.011	8.4125	M53
Blue Mussel (*Mytilus edulis*)	Sylt	2011	55.011	8.4125	M52
Blue Mussel (*Mytilus edulis*)	Sylt	2012	55.011	8.4125	M57
Blue Mussel (*Mytilus edulis*)	Sylt	2013	55.011	8.4125	M58
Blue Mussel (*Mytilus edulis*)	Sylt	2014	55.011	8.4125	M55
Blue Mussel (*Mytilus edulis*)	Sylt	2015	55.011	8.4125	M54
Blue Mussel (*Mytilus edulis*)	Sylt	2017	55.011	8.4125	M59
Blue Mussel (*Mytilus edulis*)	Sylt	2019	55.011	8.4125	M60
Lombardy Poplar (*Populus nigra*)	Leipzig	1991	51.353013	12.404477	Pa80
Lombardy Poplar (*Populus nigra*)	Leipzig	1993	51.353013	12.404477	Pa81

Lombardy Poplar (*Populus nigra*)	Leipzig	1994	51.353013	12.404477	Pa53
Lombardy Poplar (*Populus nigra*)	Leipzig	1995	51.353013	12.404477	Pa54
Lombardy Poplar (*Populus nigra*)	Leipzig	1997	51.353013	12.404477	Pa56
Lombardy Poplar (*Populus nigra*)	Leipzig	1998	51.353013	12.404477	Pa57
Lombardy Poplar (*Populus nigra*)	Leipzig	1999	51.353013	12.404477	Pa58
Lombardy Poplar (*Populus nigra*)	Leipzig	2000	51.353013	12.404477	Pa59
Lombardy Poplar (*Populus nigra*)	Leipzig	2001	51.353013	12.404477	Pa60
Lombardy Poplar (*Populus nigra*)	Leipzig	2002	51.353013	12.404477	Pa61
Lombardy Poplar (*Populus nigra*)	Leipzig	2003	51.353013	12.404477	Pa62
Lombardy Poplar (*Populus nigra*)	Leipzig	2004	51.353013	12.404477	Pa63
Lombardy Poplar (*Populus nigra*)	Leipzig	2005	51.353013	12.404477	Pa64
Lombardy Poplar (*Populus nigra*)	Leipzig	2006	51.353013	12.404477	Pa65
Lombardy Poplar (*Populus nigra*)	Leipzig	2007	51.353013	12.404477	Pa66
Lombardy Poplar (*Populus nigra*)	Leipzig	2008	51.353013	12.404477	Pa67
Lombardy Poplar (*Populus nigra*)	Leipzig	2009	51.353013	12.404477	Pa68
Lombardy Poplar (*Populus nigra*)	Leipzig	2010	51.353013	12.404477	Pa69
Lombardy Poplar (*Populus nigra*)	Leipzig	2011	51.353013	12.404477	Pa70
Lombardy Poplar (*Populus nigra*)	Leipzig	2012	51.353013	12.404477	Pa71
Lombardy Poplar (*Populus nigra*)	Leipzig	2013	51.353013	12.404477	Pa72
Lombardy Poplar (*Populus nigra*)	Leipzig	2014	51.353013	12.404477	Pa73
Lombardy Poplar (*Populus nigra*)	Leipzig	2015	51.353013	12.404477	Pa74
Lombardy Poplar (*Populus nigra*)	Leipzig	2016	51.353013	12.404477	Pa75
Lombardy Poplar (*Populus nigra*)	Leipzig	2017	51.353013	12.404477	Pa76
Lombardy Poplar (*Populus nigra*)	Leipzig	2018	51.353013	12.404477	Pa77a
Lombardy Poplar (*Populus nigra*)	Leipzig	2020	51.353013	12.404477	Pa83
Lombardy Poplar (*Populus nigra*)	Saartal	1991	49.22591	7.00576	Pa78
Lombardy Poplar (*Populus nigra*)	Saartal	1993	49.22591	7.00576	Pa2
Lombardy Poplar (*Populus nigra*)	Saartal	1994	49.22591	7.00576	Pa3
Lombardy Poplar (*Populus nigra*)	Saartal	1995	49.22591	7.00576	Pa4
Lombardy Poplar (*Populus nigra*)	Saartal	1996	49.22591	7.00576	Pa79
Lombardy Poplar (*Populus nigra*)	Saartal	1997	49.22591	7.00576	Pa6
Lombardy Poplar (*Populus nigra*)	Saartal	1998	49.22591	7.00576	Pa7
Lombardy Poplar (*Populus nigra*)	Saartal	1999	49.22591	7.00576	Pa8
Lombardy Poplar (*Populus nigra*)	Saartal	2000	49.22591	7.00576	Pa9
Lombardy Poplar (*Populus nigra*)	Saartal	2001	49.22591	7.00576	Pa10
Lombardy Poplar (*Populus nigra*)	Saartal	2002	49.22591	7.00576	Pa11
Lombardy Poplar (*Populus nigra*)	Saartal	2003	49.22591	7.00576	Pa12
Lombardy Poplar (*Populus nigra*)	Saartal	2004	49.22591	7.00576	Pa13
Lombardy Poplar (*Populus nigra*)	Saartal	2005	49.22591	7.00576	Pa14
Lombardy Poplar (*Populus nigra*)	Saartal	2006	49.22591	7.00576	Pa15
Lombardy Poplar (*Populus nigra*)	Saartal	2007	49.22591	7.00576	Pa16
Lombardy Poplar (*Populus nigra*)	Saartal	2008	49.22591	7.00576	Pa17
Lombardy Poplar (*Populus nigra*)	Saartal	2009	49.22591	7.00576	Pa18
Lombardy Poplar (*Populus nigra*)	Saartal	2010	49.22591	7.00576	Pa19
Lombardy Poplar (*Populus nigra*)	Saartal	2011	49.22591	7.00576	Pa20
Lombardy Poplar (*Populus nigra*)	Saartal	2012	49.22591	7.00576	Pa21

Lombardy Poplar (*Populus nigra*)	Saartal	2013	49.22591	7.00576	Pa22
Lombardy Poplar (*Populus nigra*)	Saartal	2014	49.22591	7.00576	Pa23
Lombardy Poplar (*Populus nigra*)	Saartal	2016	49.22591	7.00576	Pa24a
Lombardy Poplar (*Populus nigra*)	Saartal	2018	49.22591	7.00576	Pa25
Norway Spruce (*Picea abies*)	Bayerischer Wald	1998	48.966921	13.435102	Fi111
Norway Spruce (*Picea abies*)	Bayerischer Wald	1999	48.966921	13.435102	Fi112
Norway Spruce (*Picea abies*)	Bayerischer Wald	2000	48.966921	13.435102	Fi113
Norway Spruce (*Picea abies*)	Bayerischer Wald	2001	48.966921	13.435102	Fi114
Norway Spruce (*Picea abies*)	Bayerischer Wald	2002	48.966921	13.435102	Fi115
Norway Spruce (*Picea abies*)	Bayerischer Wald	2003	48.966921	13.435102	Fi116
Norway Spruce (*Picea abies*)	Bayerischer Wald	2004	48.966921	13.435102	Fi117
Norway Spruce (*Picea abies*)	Bayerischer Wald	2005	48.966921	13.435102	Fi118
Norway Spruce (*Picea abies*)	Bayerischer Wald	2006	48.966921	13.435102	Fi119
Norway Spruce (*Picea abies*)	Bayerischer Wald	2007	48.966921	13.435102	Fi120
Norway Spruce (*Picea abies*)	Bayerischer Wald	2008	48.966921	13.435102	Fi121
Norway Spruce (*Picea abies*)	Bayerischer Wald	2009	48.966921	13.435102	Fi122
Norway Spruce (*Picea abies*)	Bayerischer Wald	2010	48.966921	13.435102	Fi123
Norway Spruce (*Picea abies*)	Bayerischer Wald	2011	48.966921	13.435102	Fi124
Norway Spruce (*Picea abies*)	Bayerischer Wald	2012	48.966921	13.435102	Fi125
Norway Spruce (*Picea abies*)	Bayerischer Wald	2013	48.966921	13.435102	Fi103
Norway Spruce (*Picea abies*)	Bayerischer Wald	2014	48.966921	13.435102	Fi104
Norway Spruce (*Picea abies*)	Bayerischer Wald	2016	48.966921	13.435102	Fi105
Norway Spruce (*Picea abies*)	Bayerischer Wald	2018	48.966921	13.435102	Fi106
Norway Spruce (*Picea abies*)	Bayerischer Wald	2020	48.966921	13.435102	Fi107
Norway Spruce (*Picea abies*)	Bayerischer Wald	2022	48.966921	13.435102	Fi108
Norway Spruce (*Picea abies*)	Belauer See	1987	54.10381	10.24531	Fi64
Norway Spruce (*Picea abies*)	Belauer See	1989	54.10381	10.24531	Fi65
Norway Spruce (*Picea abies*)	Belauer See	1991	54.10381	10.24531	Fi37
Norway Spruce (*Picea abies*)	Belauer See	1993	54.10381	10.24531	Fi38
Norway Spruce (*Picea abies*)	Belauer See	1997	54.10381	10.24531	Fi39
Norway Spruce (*Picea abies*)	Belauer See	1999	54.10381	10.24531	Fi40
Norway Spruce (*Picea abies*)	Belauer See	2001	54.10381	10.24531	Fi41
Norway Spruce (*Picea abies*)	Belauer See	2003	54.10381	10.24531	Fi42
Norway Spruce (*Picea abies*)	Belauer See	2005	54.10381	10.24531	Fi43
Norway Spruce (*Picea abies*)	Belauer See	2007	54.10381	10.24531	Fi44
Norway Spruce (*Picea abies*)	Belauer See	2009	54.10381	10.24531	Fi45
Norway Spruce (*Picea abies*)	Belauer See	2011	54.10381	10.24531	Fi46
Norway Spruce (*Picea abies*)	Belauer See	2013	54.10381	10.24531	Fi47
Norway Spruce (*Picea abies*)	Belauer See	2015	54.10381	10.24531	Fi48
Norway Spruce (*Picea abies*)	Belauer See	2017	54.10381	10.24531	Fi49
Norway Spruce (*Picea abies*)	Belauer See	2019	54.10381	10.24531	Fi50
Norway Spruce (*Picea abies*)	Belauer See	2021	54.10381	10.24531	Fi51
Norway Spruce (*Picea abies*)	Berchtesgaden	1985	47.56113	12.89025	Fi30
Norway Spruce (*Picea abies*)	Berchtesgaden	1987	47.56113	12.89025	Fi31
Norway Spruce (*Picea abies*)	Berchtesgaden	1989	47.56113	12.89025	Fi32
Norway Spruce (*Picea abies*)	Berchtesgaden	1991	47.56113	12.89025	Fi33

Norway Spruce (*Picea abies*)	Berchtesgaden	1993	47.56113	12.89025	Fi34
Norway Spruce (*Picea abies*)	Berchtesgaden	1998	47.56113	12.89025	Fi35
Norway Spruce (*Picea abies*)	Berchtesgaden	2000	47.56113	12.89025	Fi36
Norway Spruce (*Picea abies*)	Berchtesgaden	2002	47.56113	12.89025	Fi19
Norway Spruce (*Picea abies*)	Berchtesgaden	2004	47.56113	12.89025	Fi20
Norway Spruce (*Picea abies*)	Berchtesgaden	2006	47.56113	12.89025	Fi21
Norway Spruce (*Picea abies*)	Berchtesgaden	2008	47.56113	12.89025	Fi22
Norway Spruce (*Picea abies*)	Berchtesgaden	2010	47.56113	12.89025	Fi23
Norway Spruce (*Picea abies*)	Berchtesgaden	2012	47.56113	12.89025	Fi24
Norway Spruce (*Picea abies*)	Berchtesgaden	2014	47.56113	12.89025	Fi25
Norway Spruce (*Picea abies*)	Berchtesgaden	2016	47.56113	12.89025	Fi26
Norway Spruce (*Picea abies*)	Berchtesgaden	2018	47.56113	12.89025	Fi27
Norway Spruce (*Picea abies*)	Berchtesgaden	2020	47.56113	12.89025	Fi28
Norway Spruce (*Picea abies*)	Berchtesgaden	2022	47.56113	12.89025	Fi29
Norway Spruce (*Picea abies*)	Harz	1996	51.792691	10.645409	Fi86
Norway Spruce (*Picea abies*)	Harz	1997	51.792691	10.645409	Fi84
Norway Spruce (*Picea abies*)	Harz	1998	51.792691	10.645409	Fi82
Norway Spruce (*Picea abies*)	Harz	1999	51.792691	10.645409	Fi80
Norway Spruce (*Picea abies*)	Harz	2000	51.792691	10.645409	Fi87
Norway Spruce (*Picea abies*)	Harz	2001	51.792691	10.645409	Fi85
Norway Spruce (*Picea abies*)	Harz	2002	51.792691	10.645409	Fi83
Norway Spruce (*Picea abies*)	Harz	2003	51.792691	10.645409	Fi81
Norway Spruce (*Picea abies*)	Harz	2004	51.792691	10.645409	Fi89
Norway Spruce (*Picea abies*)	Harz	2005	51.792691	10.645409	Fi88
Norway Spruce (*Picea abies*)	Harz	2006	51.792691	10.645409	Fi90
Norway Spruce (*Picea abies*)	Harz	2007	51.792691	10.645409	Fi91
Norway Spruce (*Picea abies*)	Harz	2008	51.792691	10.645409	Fi92
Norway Spruce (*Picea abies*)	Harz	2009	51.792691	10.645409	Fi93
Norway Spruce (*Picea abies*)	Harz	2010	51.792691	10.645409	Fi94
Norway Spruce (*Picea abies*)	Harz	2011	51.792691	10.645409	Fi95
Norway Spruce (*Picea abies*)	Harz	2012	51.792691	10.645409	Fi96
Norway Spruce (*Picea abies*)	Harz	2013	51.792691	10.645409	Fi97
Norway Spruce (*Picea abies*)	Harz	2014	51.792691	10.645409	Fi98
Norway Spruce (*Picea abies*)	Harz	2016	51.792691	10.645409	Fi99
Norway Spruce (*Picea abies*)	Harz	2018	51.792691	10.645409	Fi100
Norway Spruce (*Picea abies*)	Harz	2020	51.792691	10.645409	Fi101
Norway Spruce (*Picea abies*)	Harz	2022	51.792691	10.645409	Fi102
Norway Spruce (*Picea abies*)	Pfälzerwald	1993	49.0902	7.43575	Fi52
Norway Spruce (*Picea abies*)	Pfälzerwald	2001	49.0902	7.43575	Fi53
Norway Spruce (*Picea abies*)	Pfälzerwald	2003	49.0902	7.43575	Fi54
Norway Spruce (*Picea abies*)	Pfälzerwald	2005	49.0902	7.43575	Fi55
Norway Spruce (*Picea abies*)	Pfälzerwald	2007	49.0902	7.43575	Fi56
Norway Spruce (*Picea abies*)	Pfälzerwald	2009	49.0902	7.43575	Fi57
Norway Spruce (*Picea abies*)	Pfälzerwald	2011	49.0902	7.43575	Fi58
Norway Spruce (*Picea abies*)	Pfälzerwald	2013	49.0902	7.43575	Fi59
Norway Spruce (*Picea abies*)	Pfälzerwald	2015	49.0902	7.43575	Fi60

Norway Spruce (*Picea abies*)	Pfälzerwald	2017	49.0902	7.43575	Fi61
Norway Spruce (*Picea abies*)	Pfälzerwald	2019	49.0902	7.43575	Fi62
Norway Spruce (*Picea abies*)	Pfälzerwald	2021	49.0902	7.43575	Fi63
Norway Spruce (*Picea abies*)	Scheyern	2000	48.487405	11.428479	Fi8
Norway Spruce (*Picea abies*)	Scheyern	2002	48.487405	11.428479	Fi9
Norway Spruce (*Picea abies*)	Scheyern	2004	48.487405	11.428479	Fi10
Norway Spruce (*Picea abies*)	Scheyern	2006	48.487405	11.428479	Fi11
Norway Spruce (*Picea abies*)	Scheyern	2008	48.487405	11.428479	Fi12
Norway Spruce (*Picea abies*)	Scheyern	2010	48.487405	11.428479	Fi150
Norway Spruce (*Picea abies*)	Scheyern	2012	48.487405	11.428479	Fi13
Norway Spruce (*Picea abies*)	Scheyern	2014	48.487405	11.428479	Fi14
Norway Spruce (*Picea abies*)	Scheyern	2015	48.487405	11.428479	Fi15
Norway Spruce (*Picea abies*)	Scheyern	2017	48.487405	11.428479	Fi16
Norway Spruce (*Picea abies*)	Scheyern	2019	48.487405	11.428479	Fi17
Norway Spruce (*Picea abies*)	Scheyern	2021	48.487405	11.428479	Fi18
Norway Spruce (*Picea abies*)	Solling	1999	51.787381	9.610741	Fi72
Norway Spruce (*Picea abies*)	Solling	2001	51.787381	9.610741	Fi73
Norway Spruce (*Picea abies*)	Solling	2003	51.787381	9.610741	Fi74
Norway Spruce (*Picea abies*)	Solling	2005	51.787381	9.610741	Fi75
Norway Spruce (*Picea abies*)	Solling	2007	51.787381	9.610741	Fi76
Norway Spruce (*Picea abies*)	Solling	2009	51.787381	9.610741	Fi126
Norway Spruce (*Picea abies*)	Solling	2011	51.787381	9.610741	Fi77
Norway Spruce (*Picea abies*)	Solling	2013	51.787381	9.610741	Fi78
Norway Spruce (*Picea abies*)	Solling	2015	51.787381	9.610741	Fi67
Norway Spruce (*Picea abies*)	Solling	2017	51.787381	9.610741	Fi68
Norway Spruce (*Picea abies*)	Solling	2019	51.787381	9.610741	Fi69
Norway Spruce (*Picea abies*)	Solling	2021	51.787381	9.610741	Fi70
Zebramussel (*Dreissena polymorpha*)	Bimmen	1995	51.858614	6.073054	Z37
Zebramussel (*Dreissena polymorpha*)	Bimmen	1996	51.858614	6.073054	Z38
Zebramussel (*Dreissena polymorpha*)	Bimmen	1999	51.858614	6.073054	Z40
Zebramussel (*Dreissena polymorpha*)	Bimmen	2001	51.858614	6.073054	Z42
Zebramussel (*Dreissena polymorpha*)	Bimmen	2004	51.858614	6.073054	Z43
Zebramussel (*Dreissena polymorpha*)	Bimmen	2016	51.858614	6.073054	Z44
Zebramussel (*Dreissena polymorpha*)	Bimmen	2017	51.858614	6.073054	Z45
Zebramussel (*Dreissena polymorpha*)	Blankenese	1995	53.556886	9.80897	Z119
Zebramussel (*Dreissena polymorpha*)	Blankenese	1996	53.556886	9.80897	Z120
Zebramussel (*Dreissena polymorpha*)	Blankenese	1997	53.556886	9.80897	Z121
Zebramussel (*Dreissena polymorpha*)	Blankenese	1998	53.556886	9.80897	Z122
Zebramussel (*Dreissena polymorpha*)	Blankenese	1999	53.556886	9.80897	Z123
Zebramussel (*Dreissena polymorpha*)	Blankenese	2003	53.556886	9.80897	Z125
Zebramussel (*Dreissena polymorpha*)	Blankenese	2004	53.556886	9.80897	Z126
Zebramussel (*Dreissena polymorpha*)	Blankenese	2006	53.556886	9.80897	Z128
Zebramussel (*Dreissena polymorpha*)	Blankenese	2007	53.556886	9.80897	Z129
Zebramussel (*Dreissena polymorpha*)	Blankenese	2008	53.556886	9.80897	Z130
Zebramussel (*Dreissena polymorpha*)	Blankenese	2009	53.556886	9.80897	Z131
Zebramussel (*Dreissena polymorpha*)	Blankenese	2016	53.556886	9.80897	Z132

Zebramussel (*Dreissena polymorpha*)	Blankenese	2017	53.556886	9.80897	Z133
Zebramussel (*Dreissena polymorpha*)	Blankenese	2018	53.556886	9.80897	Z134
Zebramussel (*Dreissena polymorpha*)	Cumlosen	1998	53.03962009	11.63766977	Z107
Zebramussel (*Dreissena polymorpha*)	Cumlosen	1999	53.03962009	11.63766977	Z108
Zebramussel (*Dreissena polymorpha*)	Cumlosen	2000	53.03962009	11.63766977	Z109
Zebramussel (*Dreissena polymorpha*)	Cumlosen	2001	53.03962009	11.63766977	Z110
Zebramussel (*Dreissena polymorpha*)	Cumlosen	2002	53.03962009	11.63766977	Z111
Zebramussel (*Dreissena polymorpha*)	Cumlosen	2005	53.03962009	11.63766977	Z114
Zebramussel (*Dreissena polymorpha*)	Cumlosen	2006	53.03962009	11.63766977	Z115
Zebramussel (*Dreissena polymorpha*)	Cumlosen	2013	53.03962009	11.63766977	Z116
Zebramussel (*Dreissena polymorpha*)	Cumlosen	2014	53.03962009	11.63766977	Z117
Zebramussel (*Dreissena polymorpha*)	Cumlosen	2016	53.03962009	11.63766977	Z118
Zebramussel (*Dreissena polymorpha*)	Jochenstein	2004	48.566659	13.60556	Z86
Zebramussel (*Dreissena polymorpha*)	Jochenstein	2006	48.566659	13.60556	Z88
Zebramussel (*Dreissena polymorpha*)	Jochenstein	2007	48.566659	13.60556	Z89
Zebramussel (*Dreissena polymorpha*)	Jochenstein	2008	48.566659	13.60556	Z90
Zebramussel (*Dreissena polymorpha*)	Jochenstein	2017	48.566659	13.60556	Z91
Zebramussel (*Dreissena polymorpha*)	Jochenstein	2019	48.566659	13.60556	Z92
Zebramussel (*Dreissena polymorpha*)	Koblenz	1995	50.34718	7.60211	Z25
Zebramussel (*Dreissena polymorpha*)	Koblenz	1996	50.34718	7.60211	Z26
Zebramussel (*Dreissena polymorpha*)	Koblenz	2001	50.34718	7.60211	Z30
Zebramussel (*Dreissena polymorpha*)	Koblenz	2015	50.34718	7.60211	Z31
Zebramussel (*Dreissena polymorpha*)	Koblenz	2016	50.34718	7.60211	Z32
Zebramussel (*Dreissena polymorpha*)	Koblenz	2017	50.34718	7.60211	Z33
Zebramussel (*Dreissena polymorpha*)	Koblenz	2018	50.34718	7.60211	Z34
Zebramussel (*Dreissena polymorpha*)	Koblenz	2020	50.34718	7.60211	Z36
Zebramussel (*Dreissena polymorpha*)	Prossen	1999	50.92708	14.11624	Z1
Zebramussel (*Dreissena polymorpha*)	Prossen	2000	50.92708	14.11624	Z2
Zebramussel (*Dreissena polymorpha*)	Prossen	2001	50.92708	14.11624	Z3
Zebramussel (*Dreissena polymorpha*)	Prossen	2002	50.92708	14.11624	Z4
Zebramussel (*Dreissena polymorpha*)	Prossen	2003	50.92708	14.11624	Z5
Zebramussel (*Dreissena polymorpha*)	Prossen	2004	50.92708	14.11624	Z6
Zebramussel (*Dreissena polymorpha*)	Prossen	2005	50.92708	14.11624	Z7
Zebramussel (*Dreissena polymorpha*)	Prossen	2006	50.92708	14.11624	Z8
Zebramussel (*Dreissena polymorpha*)	Prossen	2007	50.92708	14.11624	Z9
Zebramussel (*Dreissena polymorpha*)	Prossen	2008	50.92708	14.11624	Z10
Zebramussel (*Dreissena polymorpha*)	Prossen	2009	50.92708	14.11624	Z11
Zebramussel (*Dreissena polymorpha*)	Prossen	2010	50.92708	14.11624	Z12
Zebramussel (*Dreissena polymorpha*)	Prossen	2011	50.92708	14.11624	Z13
Zebramussel (*Dreissena polymorpha*)	Prossen	2012	50.92708	14.11624	Z14
Zebramussel (*Dreissena polymorpha*)	Prossen	2013	50.92708	14.11624	Z15
Zebramussel (*Dreissena polymorpha*)	Prossen	2014	50.92708	14.11624	Z16
Zebramussel (*Dreissena polymorpha*)	Prossen	2015	50.92708	14.11624	Z17
Zebramussel (*Dreissena polymorpha*)	Prossen	2016	50.92708	14.11624	Z18
Zebramussel (*Dreissena polymorpha*)	Prossen	2017	50.92708	14.11624	Z19
Zebramussel (*Dreissena polymorpha*)	Prossen	2018	50.92708	14.11624	Z20

Zebramussel (*Dreissena polymorpha*)	Rehlingen	1994	49.371943	6.69865	Z50
Zebramussel (*Dreissena polymorpha*)	Rehlingen	1995	49.371943	6.69865	Z51
Zebramussel (*Dreissena polymorpha*)	Rehlingen	1996	49.371943	6.69865	Z52
Zebramussel (*Dreissena polymorpha*)	Rehlingen	1997	49.371943	6.69865	Z53
Zebramussel (*Dreissena polymorpha*)	Rehlingen	1998	49.371943	6.69865	Z54
Zebramussel (*Dreissena polymorpha*)	Rehlingen	1999	49.371943	6.69865	Z55
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2000	49.371943	6.69865	Z56
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2001	49.371943	6.69865	Z57
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2002	49.371943	6.69865	Z58
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2003	49.371943	6.69865	Z59
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2004	49.371943	6.69865	Z60
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2005	49.371943	6.69865	Z61
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2006	49.371943	6.69865	Z62
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2007	49.371943	6.69865	Z63
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2008	49.371943	6.69865	Z64
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2009	49.371943	6.69865	Z65
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2010	49.371943	6.69865	Z66
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2011	49.371943	6.69865	Z67
Zebramussel (*Dreissena polymorpha*)	Rehlingen	2012	49.371943	6.69865	Z68
Zebramussel (*Dreissena polymorpha*)	Ulm	2003	48.33667	9.93357	Z69
Zebramussel (*Dreissena polymorpha*)	Ulm	2004	48.33667	9.93357	Z70
Zebramussel (*Dreissena polymorpha*)	Ulm	2005	48.33667	9.93357	Z71
Zebramussel (*Dreissena polymorpha*)	Ulm	2006	48.33667	9.93357	Z72
Zebramussel (*Dreissena polymorpha*)	Ulm	2007	48.33667	9.93357	Z73
Zebramussel (*Dreissena polymorpha*)	Ulm	2008	48.33667	9.93357	Z74
Zebramussel (*Dreissena polymorpha*)	Ulm	2009	48.33667	9.93357	Z75
Zebramussel (*Dreissena polymorpha*)	Ulm	2010	48.33667	9.93357	Z76
Zebramussel (*Dreissena polymorpha*)	Ulm	2011	48.33667	9.93357	Z77
Zebramussel (*Dreissena polymorpha*)	Ulm	2012	48.33667	9.93357	Z78
Zebramussel (*Dreissena polymorpha*)	Ulm	2013	48.33667	9.93357	Z79
Zebramussel (*Dreissena polymorpha*)	Ulm	2014	48.33667	9.93357	Z80
Zebramussel (*Dreissena polymorpha*)	Ulm	2016	48.33667	9.93357	Z81
Zebramussel (*Dreissena polymorpha*)	Zehren	2000	51.26445	13.4026	Z21
Zebramussel (*Dreissena polymorpha*)	Zehren	2001	51.26445	13.4026	Z22
Zebramussel (*Dreissena polymorpha*)	Zehren	2002	51.26445	13.4026	Z23
Zebramussel (*Dreissena polymorpha*)	Zehren	2003	51.26445	13.4026	Z24
Zebramussel (*Dreissena polymorpha*)	Zehren	2004	51.26445	13.4026	Z94
Zebramussel (*Dreissena polymorpha*)	Zehren	2005	51.26445	13.4026	Z95
Zebramussel (*Dreissena polymorpha*)	Zehren	2006	51.26445	13.4026	Z96
Zebramussel (*Dreissena polymorpha*)	Zehren	2007	51.26445	13.4026	Z97
Zebramussel (*Dreissena polymorpha*)	Zehren	2008	51.26445	13.4026	Z98
Zebramussel (*Dreissena polymorpha*)	Zehren	2009	51.26445	13.4026	Z99
Zebramussel (*Dreissena polymorpha*)	Zehren	2010	51.26445	13.4026	Z100
Zebramussel (*Dreissena polymorpha*)	Zehren	2011	51.26445	13.4026	Z101
Zebramussel (*Dreissena polymorpha*)	Zehren	2012	51.26445	13.4026	Z102
Zebramussel (*Dreissena polymorpha*)	Zehren	2013	51.26445	13.4026	Z103

Zebramussel (*Dreissena polymorpha*)	Zehren	2014	51.26445	13.4026	Z104
Zebramussel (*Dreissena polymorpha*)	Zehren	2016	51.26445	13.4026	Z105

Supp. Table 2.2: Number of samples, OTU count, as well as number of phyla and orders of each taxonomic group per sampled species.

Sampling Species	**Barcode Marker**	**Resulting Datasets**	**Sample count**	**OTU count**	**No. of phyla**	**No. of orders**
			cleaned dataset and **filtered dataset (model)**			
Tree leaves European Beech (*Fagus sylvatica*) Norway Spruce (*Picea abies*) Pyramid Poplar (*Populus nigra*)	COI mtDNA	"Metazoa"	282 254	3,275 2,040	1 1	24 19
	ITS1	"Fungi"	282 273	6,250 3,208	5 4	113 86
	16S rDNA	"Bacteria"	149 122	5,183 4,195	16 14	94 82
Zebra Mussel *(Dreissena polymorpha)*	18S rDNA	"Algae/Protozoa"	113 109	5,587 3,043	20 17	173 145
	18S rDNA	"Metazoa"	113 110	523 320	16 15	71 56
	16S rDNA	"Bacteria"	109 102	14,292 5,891	34 28	184 144
Bladderwrack (*Fucus vesiculosus*)	18S rDNA	"Metazoa"	67 60	787 399	18 14	78 56
	16S rDNA	"Bacteria"	66 60	5,474 3,865	25 23	101 82
Blue mussel (*Mytilus edulis*)	18S rDNA	"Algae/Protozoa"	88 84	3,551 2,207	20 17	160 149
	16S rDNA	"Bacteria"	88 84	21,266 6,724	47 33	180 125

Supp. Table 2.3: List of all OTUs that match the reference sequence to 100 % over at least 85 % of the amplicon length.

The list of all OTUs matching the reference sequence to 100% over at least 85% of the amplicon length is too big for printing. The supplementary file can be downloaded via this link:
Download

Supp. Table 2.4: List of functionally annotated fungi found in and on tree leaves and respective trends in occurrence.

The list of functionally annotated fungi is too big for printing. The supplementary file can be downloaded via this link:
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Supp. Table 2.5: Details of the laboratory workflow.

The list of the laboratory workflow is too big for printing. The supplementary file can be downloaded via this link:
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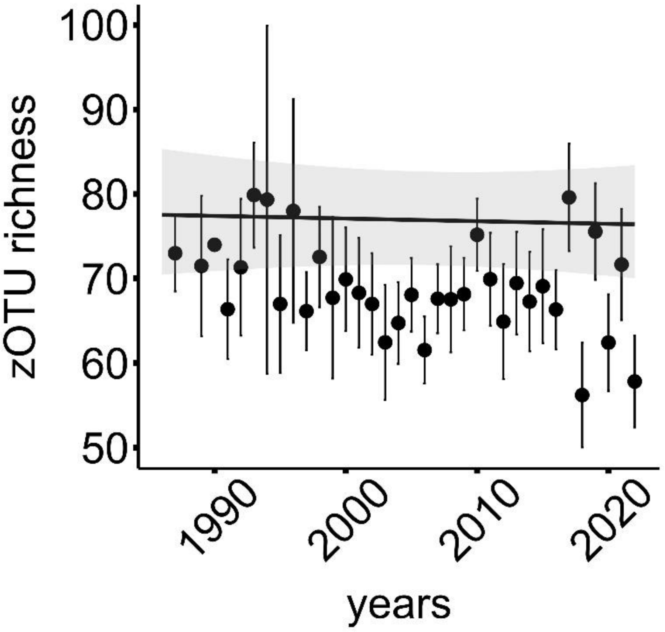
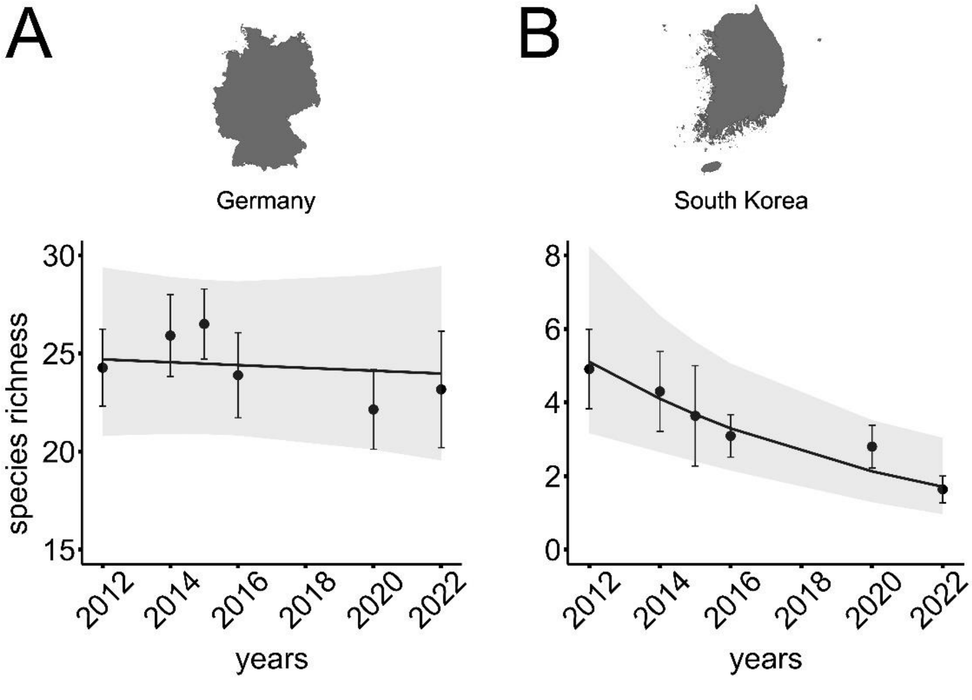
Supplementary material

Chapter 4

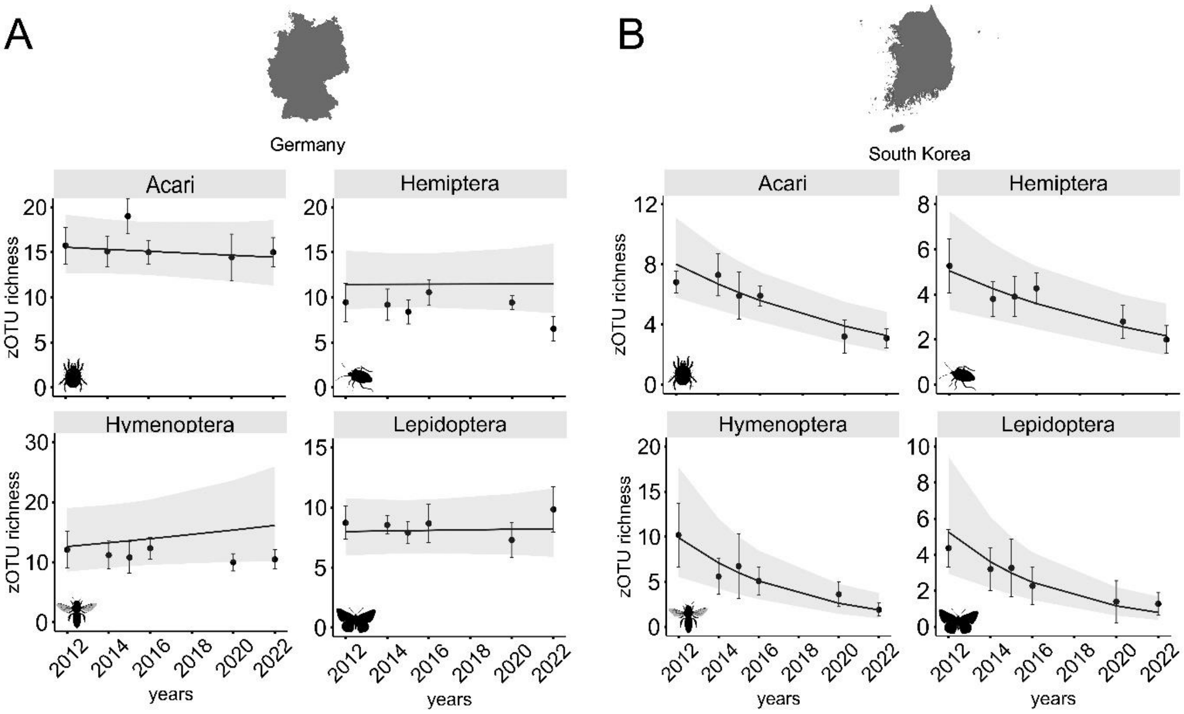
Chapter four has been published in the journal *Insect Conservation and Diversity*. The data that support the findings of this study are openly available in Science Data Bank at https://doi.org/10.57760/sciencedb.15787.



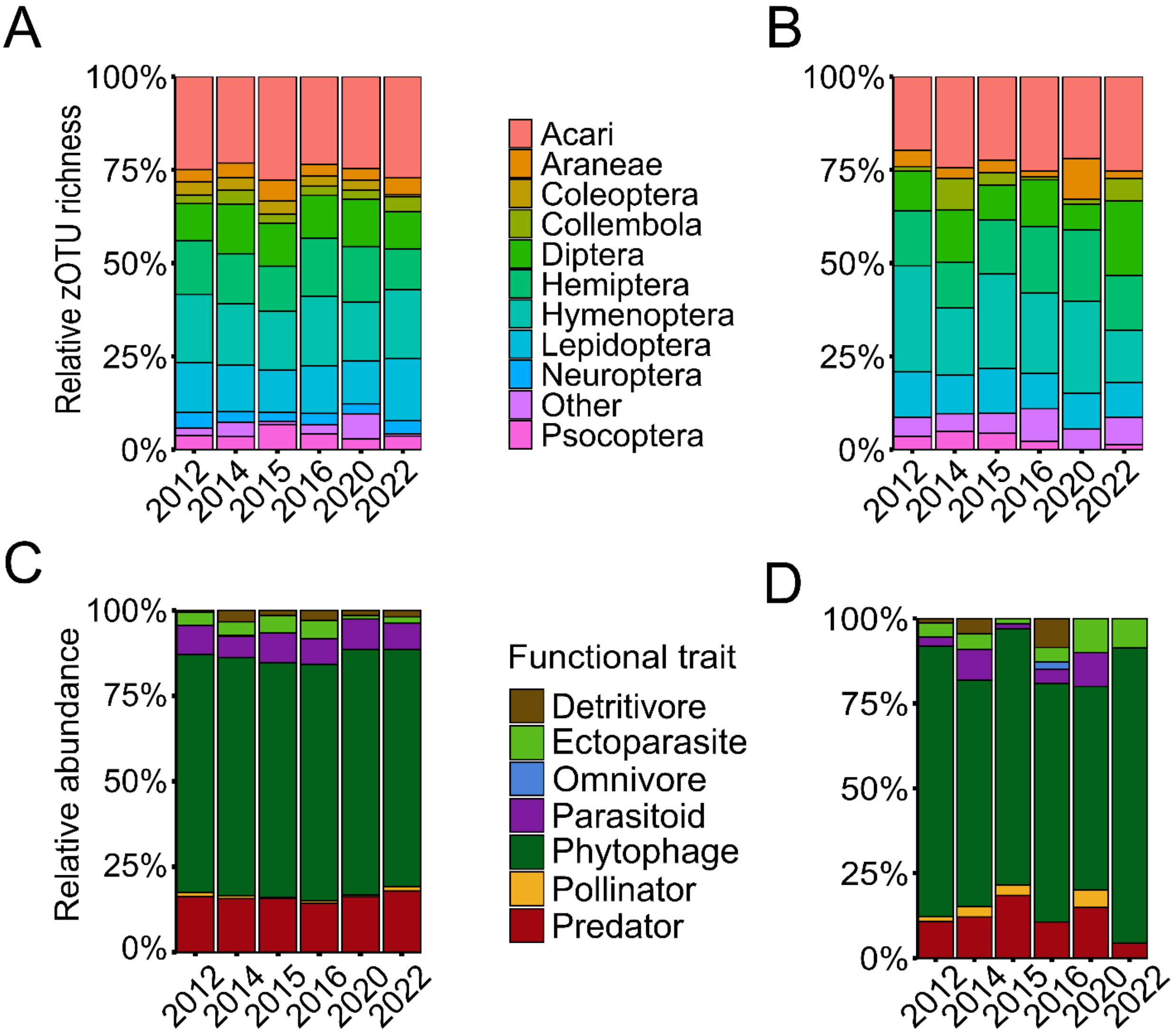
Supp. Figure 3.1: Trends of zOTU richness of forest arthropod communities over three decades in German ESB samples. The solid dots represent predicted means of summarised zOTU richness values across sampling locations and time windows, and vertical lines indicate 95% confidence intervals. The data were analysed using a generalised linear model (GLM).



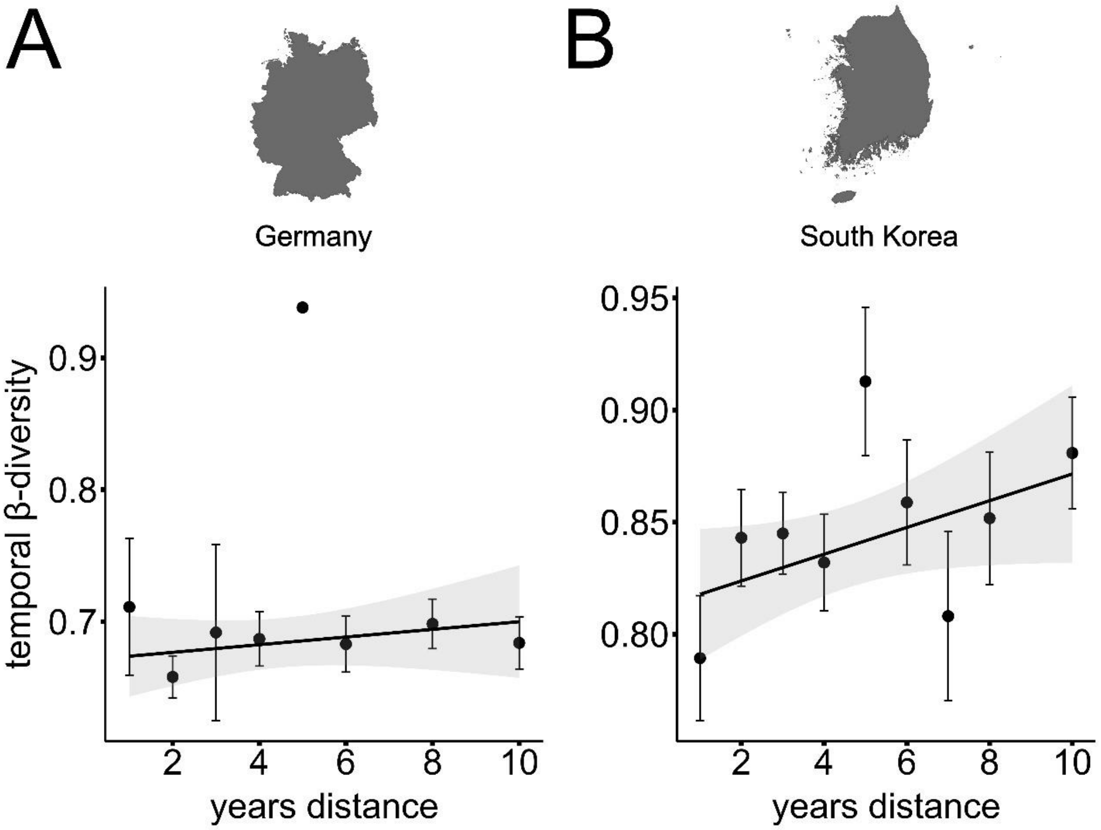
Supp. Figure 3.2: Trends of species richness of forest arthropod communities over 11 years in (a) German ESB samples and (b) Korean ESB samples. The solid dots represent predicted means of summarised species richness values across sampling locations and time windows, and vertical lines indicate 95% confidence intervals. The data were analysed using a generalised linear model (GLM).



Supp. Figure 3.3: Trends of zOTU richness of the four most abundant orders in forest arthropod communities over 11 years in German ESB and Korean ESB samples. The solid dots represent predicted means of summarised zOTU richness values across sampling locations and time windows, and vertical lines indicate 95% confidence intervals. The data were analysed using a generalised linear model (GLM). Icons refer to analysed arthropod order.



Supp. Figure 3.4: Taxonomic and functional trait composition of canopy-associated arthropod communities in German and Korean ESB samples. (a,b) Barplots showing recovered taxon composition of zOTUs across time in samples from (a) the German ESB and (b) the Korean ESB. Taxa amounting to less than 2% of the total zOTU number are merged as ‘Other’. (c,d) Barplots showing percent composition of taxa representing different functional traits across time, for (c) German ESB samples and (d) Korean ESB samples.



Supp. Figure 3.5: Temporal changes in community compositions (β-diversity measured using Jaccard distance) as a function of the time interval (in years) between samples from the same sampling site. The solid dots represent predicted means of summarised Jaccard diversity indices across sampling locations and time windows, and vertical lines indicate 95% confidence intervals.

Supp. Table 3.1: Sampling locations and associated tree species of the South Korean and German Environmental Specimen Banks (ESB).

Location	**Tree species**	**Land cover within 10 km radius**	**Environmental Specimen Bank**
GwanakSan (Seoul)	*Pinus densiflora* (Red pine)	Urban	South Korean ESB
SeorakSan (Gangwon do)	*Pinus densiflora* (Red pine)	National park	South Korean ESB
WorakSan (Chungcheongbuk do)	*Pinus densiflora* (Red pine)	National park	South Korean ESB
ChiakSan (Gangwon do)	*Pinus koraiensis* (Korean pine)	National park	South Korean ESB
ChungnyeongSan (Gyeonggi do)	*Pinus koraiensis* (Korean pine)	Agricultural/ Urban	South Korean ESB
TaehwaSan (Gyeonggi do)	*Pinus koraiensis* (Korean pine)	Agricultural/ Urban	South Korean ESB
GwanakSan (Seoul)	*Quercus mongolica* (Mongolian oak)	Urban	South Korean ESB
JiriSan (Jeollanam do)	*Quercus mongolica* (Mongolian oak)	National park	South Korean ESB
YeoninSan (Gyeonggi do)	*Quercus mongolica* (Mongolian oak)	Agricultural/ Urban	South Korean ESB
Yeosu (Jeollanam do)	*Zelkova serrata* (Japanese zelkova)	Urban	South Korean ESB
JiriSan (Jeollanam do)	*Zelkova serrata* (Japanese zelkova)	National park	South Korean ESB
Bavarian forest	*Fagus sylvatica* (Beech)	National park	German ESB
Lake Belau	*Fagus sylvatica* (Beech)	Agricultural	German ESB
Berchtesgaden	*Fagus sylvatica* (Beech)	National park	German ESB
Harz	*Fagus sylvatica* (Beech)	National park	German ESB
Palatinate forest	*Fagus sylvatica* (Beech)	Timber forest	German ESB
Scheyern	*Fagus sylvatica* (Beech)	Agricultural	German ESB
Solling	*Fagus sylvatica* (Beech)	Timber forest	German ESB
Bavarian forest	*Picea abies* (Spruce)	National park	German ESB
Lake Belau	*Picea abies* (Spruce)	Agricultural	German ESB
Berchtesgaden	*Picea abies* (Spruce)	National park	German ESB
Harz	*Picea abies* (Spruce)	National park	German ESB
Palatinate forest	*Picea abies* (Spruce)	Timber forest	German ESB
Scheyern	*Picea abies* (Spruce)	Agricultural	German ESB
Solling	*Picea abies* (Spruce)	Timber forest	German ESB
Düben Heath	*Pinus sylvestris* (Pine)	Timber forest	German ESB
Leipzig	*Populus nigra* (Poplar)	Urban	German ESB
Saar valley	*Populus nigra* (Poplar)	Urban	German ESB

Supp. Table 3. 2: List of all samples including sampling location, year, respective species, latitude and longitude of sampling location, month of sampling and mean annual temperature if accessible.

The list of all samples including meta data is too big for printing. The supplementary file can be downloaded via this link: Download.

Supp. Table 3.3: Details of the laboratory workflow.

The list of the laboratory workflow is too big for printing. The supplementary file can be downloaded via this link: Download.

Supp. Table 3.4: Values for testing of overdispersion.

The list of the values for testing of overdispersion is too big for printing. The supplementary file can be downloaded via this link: Download.

Supp. Table 3.5: List of all zOTUs and arthropod species that are exclusively found in near-natural and anthropogenic landscapes.

The list of all zOTUs matching the reference sequence to 100% over 100% of the amplicon length is too big for printing. The supplementary file can be downloaded via this link: Download.

Supp. Table 3.6: List of all zOTUs and arthropod species that are exclusively found in near-natural and anthropogenic landscapes.

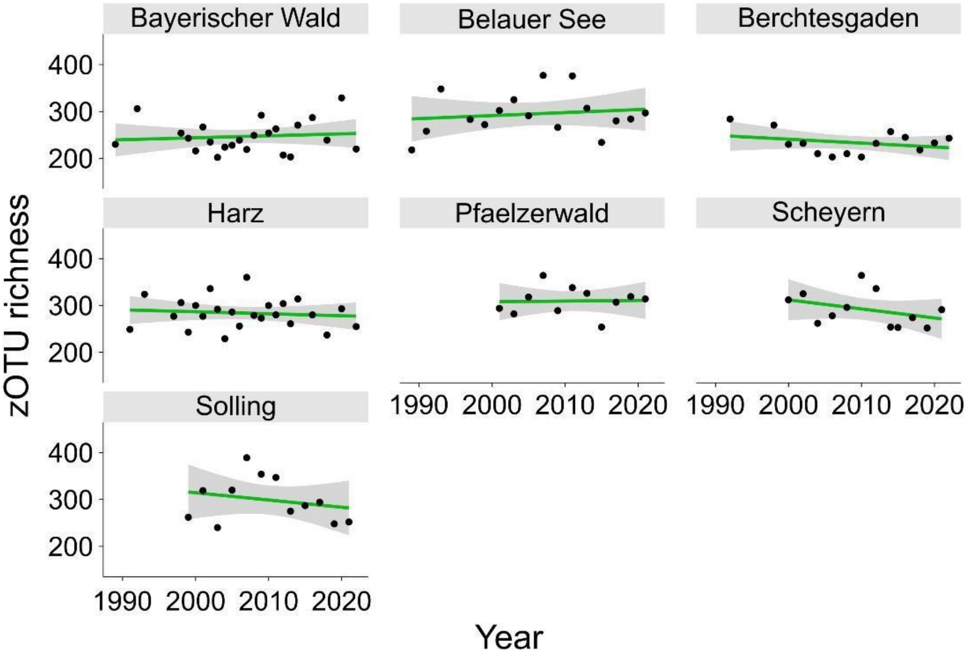
The list of zOTUs and arthropod species exclusively found in near-natural and anthropogenic landscapes is too big for printing. The supplementary file can be downloaded via this link: Download.

Supplementary material

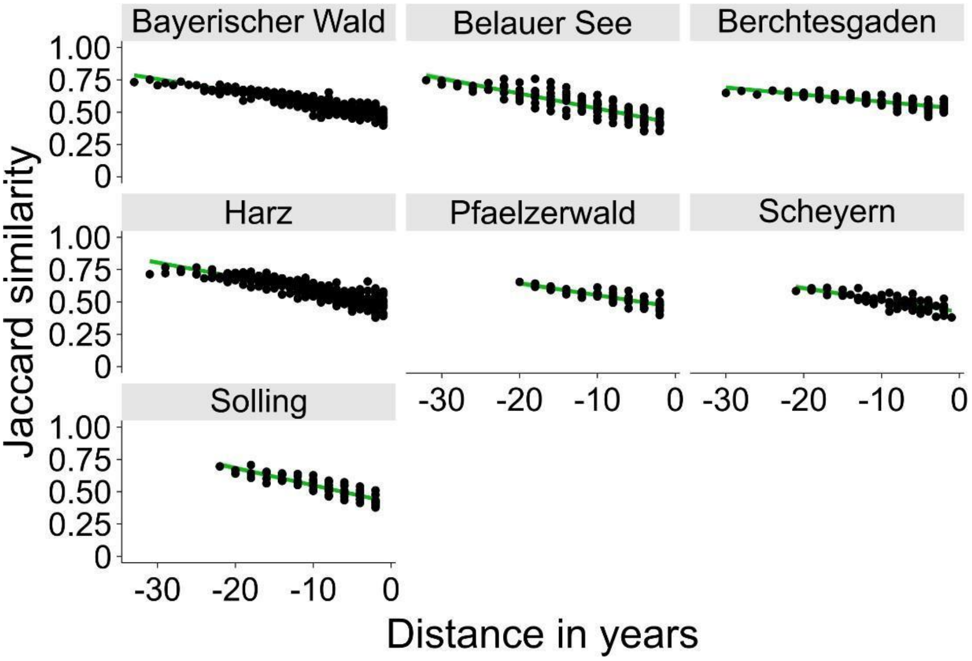
Chapter 5

Chapter five will be submitted to the journal Forest Ecosystems. All data, on which the study is based, has been uploaded to the Science Data Bank Repository including raw sequencing files, the OTU tables and the R script (https://doi.org/10.57760/sciencedb.23683).

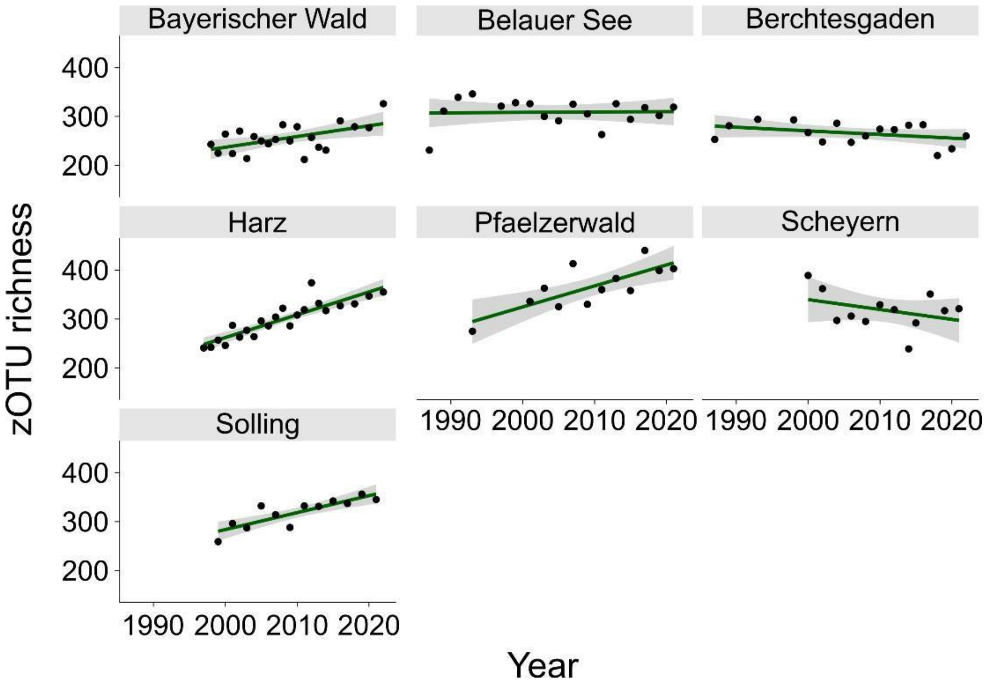
Appendix



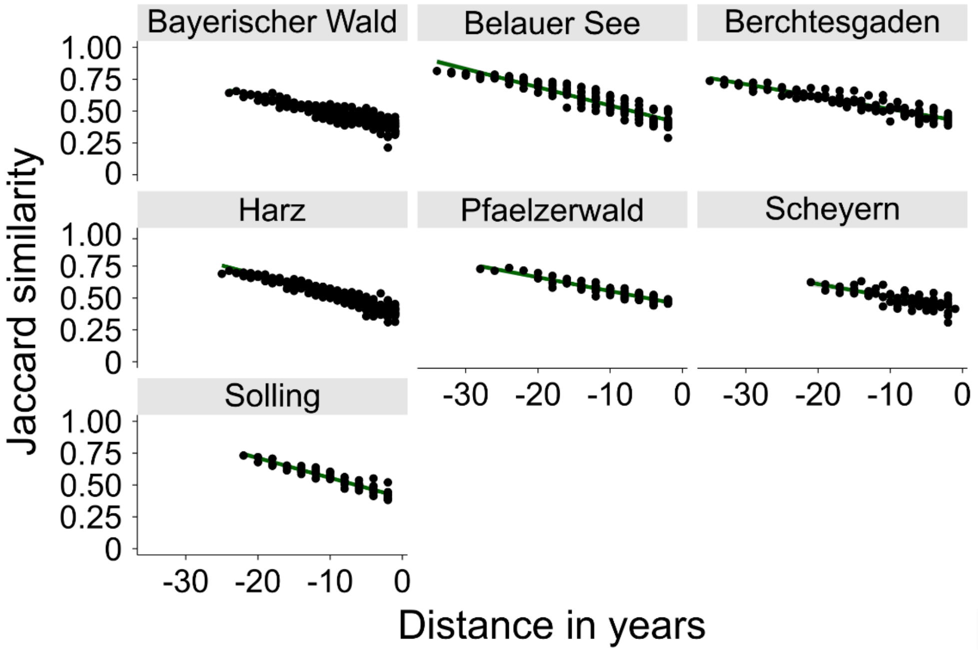
Supp. Figure 4.1: Temporal changes of fungal communities associated with beech with each sampling site shown separately. Trends of zOTU richness (α-diversity) of the associated communities from 1987 to 2022.



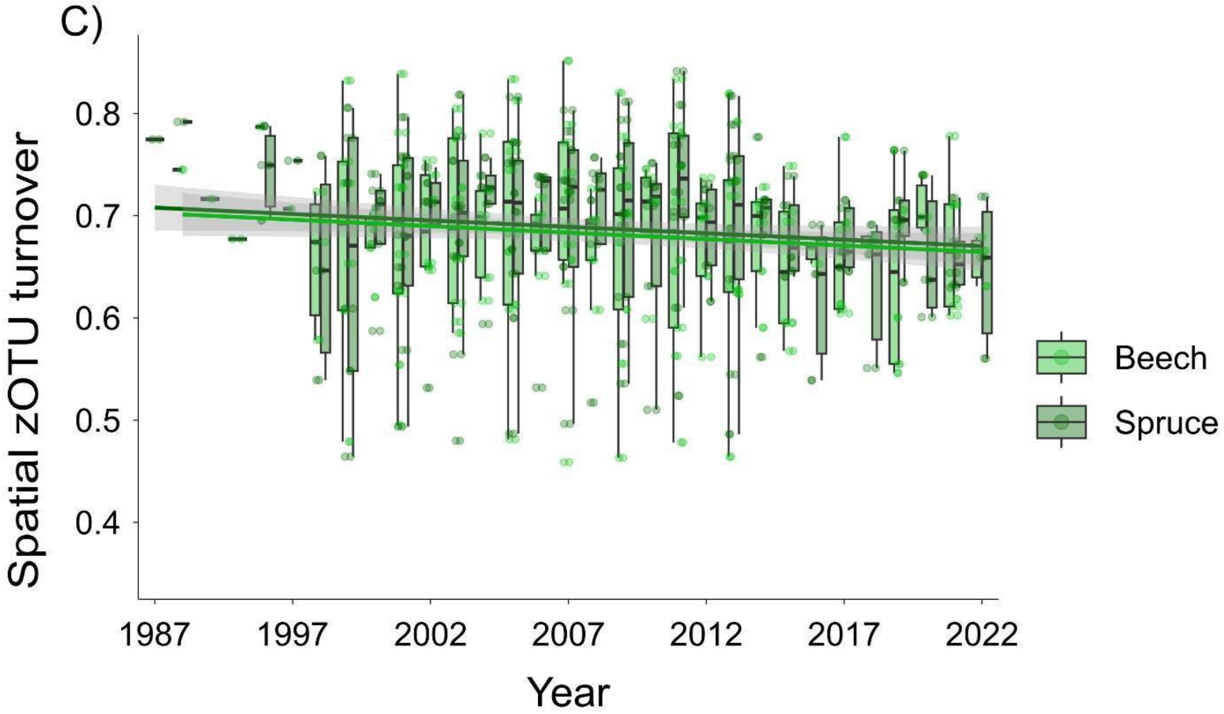
Supp. Figure 4.2: Temporal changes of fungal communities associated with sprucewith each sampling site shown separately. Trends of zOTU richness (α-diversity) of the associated communities from 1987 to 2022.



Supp. Figure 4.3: Temporal changes of fungal communities associated with beech with each sampling site shown separately. Temporal changes in community composition (β-diversity) as a function of the time interval (in years) between samples from the same sampling site.



Supp. Figure 4.4: Temporal changes of fungal communities associated with spruce with each sampling site shown separately. Temporal changes in community composition (β-diversity) as a function of the time interval (in years) between samples from the same sampling site.



Supp. Figure 4.5: Temporal changes of fungal communities associated with beech and spruce. Trends in spatial β-diversity (degree of dissimilarity in community composition between different sampling locations, measured using Jaccard distance) of the associated communities from 1987 to 2022.

Supp. Table 4. 1: List of all samples including sampling location, year, respective species, latitude and longitude of sampling location.

Species	**Location**	**Year**	**Latitude_WGS84**	**Longitude_WGS84**	**Lab_ID**
European Beech (*Fagus sylvatica*)	Bayerischer Wald	1989	48.966395	13.430375	Bu97
European Beech (*Fagus sylvatica*)	Bayerischer Wald	1992	48.966395	13.430375	Bu98
European Beech (*Fagus sylvatica*)	Bayerischer Wald	1998	48.966395	13.430375	Bu99
European Beech (*Fagus sylvatica*)	Bayerischer Wald	1999	48.966395	13.430375	Bu100
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2000	48.966395	13.430375	Bu101
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2001	48.966395	13.430375	Bu102
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2002	48.966395	13.430375	Bu103
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2003	48.966395	13.430375	Bu104
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2004	48.966395	13.430375	Bu105
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2005	48.966395	13.430375	Bu106
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2006	48.966395	13.430375	Bu107
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2007	48.966395	13.430375	Bu108
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2008	48.966395	13.430375	Bu109
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2009	48.966395	13.430375	Bu110
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2010	48.966395	13.430375	Bu111
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2011	48.966395	13.430375	Bu112
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2012	48.966395	13.430375	Bu113
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2013	48.966395	13.430375	Bu114
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2014	48.966395	13.430375	Bu115
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2016	48.966395	13.430375	Bu116
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2018	48.966395	13.430375	Bu94
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2020	48.966395	13.430375	Bu95
European Beech (*Fagus sylvatica*)	Bayerischer Wald	2022	48.966395	13.430375	Bu96
European Beech (*Fagus sylvatica*)	Belauer See	1987	54.06061	10.15373	Bu22
European Beech (*Fagus sylvatica*)	Belauer See	1989	54.06061	10.15373	Bu23
European Beech (*Fagus sylvatica*)	Belauer See	1991	54.06061	10.15373	Bu24
European Beech (*Fagus sylvatica*)	Belauer See	1993	54.06061	10.15373	Bu25
European Beech (*Fagus sylvatica*)	Belauer See	1997	54.06061	10.15373	Bu26
European Beech (*Fagus sylvatica*)	Belauer See	1999	54.06061	10.15373	Bu27
European Beech (*Fagus sylvatica*)	Belauer See	2001	54.06061	10.15373	Bu28
European Beech (*Fagus sylvatica*)	Belauer See	2003	54.06061	10.15373	Bu29
European Beech (*Fagus sylvatica*)	Belauer See	2005	54.06061	10.15373	Bu30
European Beech (*Fagus sylvatica*)	Belauer See	2007	54.06061	10.15373	Bu31
European Beech (*Fagus sylvatica*)	Belauer See	2009	54.06061	10.15373	Bu32
European Beech (*Fagus sylvatica*)	Belauer See	2011	54.06061	10.15373	Bu33
European Beech (*Fagus sylvatica*)	Belauer See	2013	54.06061	10.15373	Bu34
European Beech (*Fagus sylvatica*)	Belauer See	2015	54.06061	10.15373	Bu35
European Beech (*Fagus sylvatica*)	Belauer See	2017	54.06061	10.15373	Bu49
European Beech (*Fagus sylvatica*)	Belauer See	2019	54.06061	10.15373	Bu50
European Beech (*Fagus sylvatica*)	Belauer See	2021	54.06061	10.15373	Bu51
European Beech (*Fagus sylvatica*)	Berchtesgaden	1990	47.56574	12.89274	Bu47
European Beech (*Fagus sylvatica*)	Berchtesgaden	1992	47.56574	12.89274	Bu48
European Beech (*Fagus sylvatica*)	Berchtesgaden	1998	47.56574	12.89274	Bu8

European Beech (*Fagus sylvatica*)	Berchtesgaden	2000	47.56574	12.89274	Bu9
European Beech (*Fagus sylvatica*)	Berchtesgaden	2002	47.56574	12.89274	Bu10
European Beech (*Fagus sylvatica*)	Berchtesgaden	2004	47.56574	12.89274	Bu11
European Beech (*Fagus sylvatica*)	Berchtesgaden	2006	47.56574	12.89274	Bu13
European Beech (*Fagus sylvatica*)	Berchtesgaden	2008	47.56574	12.89274	Bu14
European Beech (*Fagus sylvatica*)	Berchtesgaden	2010	47.56574	12.89274	Bu15
European Beech (*Fagus sylvatica*)	Berchtesgaden	2012	47.56574	12.89274	Bu16
European Beech (*Fagus sylvatica*)	Berchtesgaden	2014	47.56574	12.89274	Bu17
European Beech (*Fagus sylvatica*)	Berchtesgaden	2016	47.56574	12.89274	Bu18
European Beech (*Fagus sylvatica*)	Berchtesgaden	2018	47.56574	12.89274	Bu19
European Beech (*Fagus sylvatica*)	Berchtesgaden	2020	47.56574	12.89274	Bu20
European Beech (*Fagus sylvatica*)	Berchtesgaden	2022	47.56574	12.89274	Bu21
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European Beech (*Fagus sylvatica*)	Harz	1993	51.838522	10.635239	Bu81
European Beech (*Fagus sylvatica*)	Harz	1996	51.838522	10.635239	Bu82
European Beech (*Fagus sylvatica*)	Harz	1997	51.838522	10.635239	Bu83
European Beech (*Fagus sylvatica*)	Harz	1998	51.838522	10.635239	Bu84
European Beech (*Fagus sylvatica*)	Harz	1999	51.838522	10.635239	Bu88
European Beech (*Fagus sylvatica*)	Harz	2000	51.838522	10.635239	Bu85
European Beech (*Fagus sylvatica*)	Harz	2001	51.838522	10.635239	Bu86
European Beech (*Fagus sylvatica*)	Harz	2002	51.838522	10.635239	Bu87
European Beech (*Fagus sylvatica*)	Harz	2003	51.838522	10.635239	Bu89
European Beech (*Fagus sylvatica*)	Harz	2004	51.838522	10.635239	Bu90
European Beech (*Fagus sylvatica*)	Harz	2005	51.838522	10.635239	Bu91
European Beech (*Fagus sylvatica*)	Harz	2006	51.838522	10.635239	Bu92
European Beech (*Fagus sylvatica*)	Harz	2007	51.838522	10.635239	Bu93
European Beech (*Fagus sylvatica*)	Harz	2008	51.838522	10.635239	Bu72
European Beech (*Fagus sylvatica*)	Harz	2009	51.838522	10.635239	Bu69
European Beech (*Fagus sylvatica*)	Harz	2010	51.838522	10.635239	Bu66
European Beech (*Fagus sylvatica*)	Harz	2011	51.838522	10.635239	Bu64
European Beech (*Fagus sylvatica*)	Harz	2012	51.838522	10.635239	Bu73
European Beech (*Fagus sylvatica*)	Harz	2013	51.838522	10.635239	Bu70
European Beech (*Fagus sylvatica*)	Harz	2014	51.838522	10.635239	Bu67
European Beech (*Fagus sylvatica*)	Harz	2016	51.838522	10.635239	Bu65
European Beech (*Fagus sylvatica*)	Harz	2018	51.838522	10.635239	Bu74
European Beech (*Fagus sylvatica*)	Harz	2020	51.838522	10.635239	Bu71
European Beech (*Fagus sylvatica*)	Harz	2022	51.838522	10.635239	Bu68
European Beech (*Fagus sylvatica*)	Pfälzerwald	2001	49.145482	7.713357	Bu36
European Beech (*Fagus sylvatica*)	Pfälzerwald	2003	49.145482	7.713357	Bu37
European Beech (*Fagus sylvatica*)	Pfälzerwald	2005	49.145482	7.713357	Bu38
European Beech (*Fagus sylvatica*)	Pfälzerwald	2007	49.145482	7.713357	Bu39
European Beech (*Fagus sylvatica*)	Pfälzerwald	2009	49.145482	7.713357	Bu40
European Beech (*Fagus sylvatica*)	Pfälzerwald	2011	49.145482	7.713357	Bu41
European Beech (*Fagus sylvatica*)	Pfälzerwald	2013	49.145482	7.713357	Bu42
European Beech (*Fagus sylvatica*)	Pfälzerwald	2015	49.145482	7.713357	Bu43
European Beech (*Fagus sylvatica*)	Pfälzerwald	2017	49.145482	7.713357	Bu44

European Beech (*Fagus sylvatica*)	Pfälzerwald	2019	49.145482	7.713357	Bu45
European Beech (*Fagus sylvatica*)	Pfälzerwald	2021	49.145482	7.713357	Bu46
European Beech (*Fagus sylvatica*)	Scheyern	2000	48.487405	11.428479	Bu75
European Beech (*Fagus sylvatica*)	Scheyern	2002	48.487405	11.428479	Bu76
European Beech (*Fagus sylvatica*)	Scheyern	2004	48.487405	11.428479	Bu77
European Beech (*Fagus sylvatica*)	Scheyern	2006	48.487405	11.428479	Bu78
European Beech (*Fagus sylvatica*)	Scheyern	2008	48.487405	11.428479	Bu79
European Beech (*Fagus sylvatica*)	Scheyern	2010	48.487405	11.428479	Bu1
European Beech (*Fagus sylvatica*)	Scheyern	2012	48.487405	11.428479	Bu2
European Beech (*Fagus sylvatica*)	Scheyern	2014	48.487405	11.428479	Bu3
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European Beech (*Fagus sylvatica*)	Scheyern	2017	48.487405	11.428479	Bu5
European Beech (*Fagus sylvatica*)	Scheyern	2019	48.487405	11.428479	Bu6
European Beech (*Fagus sylvatica*)	Scheyern	2021	48.487405	11.428479	Bu7
European Beech (*Fagus sylvatica*)	Solling	1999	51.73638	9.57365	Bu52
European Beech (*Fagus sylvatica*)	Solling	2001	51.73638	9.57365	Bu53
European Beech (*Fagus sylvatica*)	Solling	2003	51.73638	9.57365	Bu54
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European Beech (*Fagus sylvatica*)	Solling	2013	51.73638	9.57365	Bu59
European Beech (*Fagus sylvatica*)	Solling	2015	51.73638	9.57365	Bu60
European Beech (*Fagus sylvatica*)	Solling	2017	51.73638	9.57365	Bu61
European Beech (*Fagus sylvatica*)	Solling	2019	51.73638	9.57365	Bu62
European Beech (*Fagus sylvatica*)	Solling	2021	51.73638	9.57365	Bu63
Norway Spruce (*Picea abies*)	Bayerischer Wald	1998	48.966921	13.435102	Fi111
Norway Spruce (*Picea abies*)	Bayerischer Wald	1999	48.966921	13.435102	Fi112
Norway Spruce (*Picea abies*)	Bayerischer Wald	2000	48.966921	13.435102	Fi113
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Norway Spruce (*Picea abies*)	Bayerischer Wald	2004	48.966921	13.435102	Fi117
Norway Spruce (*Picea abies*)	Bayerischer Wald	2005	48.966921	13.435102	Fi118
Norway Spruce (*Picea abies*)	Bayerischer Wald	2006	48.966921	13.435102	Fi119
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Norway Spruce (*Picea abies*)	Bayerischer Wald	2009	48.966921	13.435102	Fi122
Norway Spruce (*Picea abies*)	Bayerischer Wald	2010	48.966921	13.435102	Fi123
Norway Spruce (*Picea abies*)	Bayerischer Wald	2011	48.966921	13.435102	Fi124
Norway Spruce (*Picea abies*)	Bayerischer Wald	2012	48.966921	13.435102	Fi125
Norway Spruce (*Picea abies*)	Bayerischer Wald	2013	48.966921	13.435102	Fi103
Norway Spruce (*Picea abies*)	Bayerischer Wald	2014	48.966921	13.435102	Fi104
Norway Spruce (*Picea abies*)	Bayerischer Wald	2016	48.966921	13.435102	Fi105
Norway Spruce (*Picea abies*)	Bayerischer Wald	2018	48.966921	13.435102	Fi106
Norway Spruce (*Picea abies*)	Bayerischer Wald	2020	48.966921	13.435102	Fi107

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Norway Spruce (*Picea abies*)	Belauer See	1987	54.10381	10.24531	Fi64
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Norway Spruce (*Picea abies*)	Belauer See	2005	54.10381	10.24531	Fi43
Norway Spruce (*Picea abies*)	Belauer See	2007	54.10381	10.24531	Fi44
Norway Spruce (*Picea abies*)	Belauer See	2009	54.10381	10.24531	Fi45
Norway Spruce (*Picea abies*)	Belauer See	2011	54.10381	10.24531	Fi46
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Norway Spruce (*Picea abies*)	Belauer See	2015	54.10381	10.24531	Fi48
Norway Spruce (*Picea abies*)	Belauer See	2017	54.10381	10.24531	Fi49
Norway Spruce (*Picea abies*)	Belauer See	2019	54.10381	10.24531	Fi50
Norway Spruce (*Picea abies*)	Belauer See	2021	54.10381	10.24531	Fi51
Norway Spruce (*Picea abies*)	Berchtesgaden	1985	47.56113	12.89025	Fi30
Norway Spruce (*Picea abies*)	Berchtesgaden	1987	47.56113	12.89025	Fi31
Norway Spruce (*Picea abies*)	Berchtesgaden	1989	47.56113	12.89025	Fi32
Norway Spruce (*Picea abies*)	Berchtesgaden	1991	47.56113	12.89025	Fi33
Norway Spruce (*Picea abies*)	Berchtesgaden	1993	47.56113	12.89025	Fi34
Norway Spruce (*Picea abies*)	Berchtesgaden	1998	47.56113	12.89025	Fi35
Norway Spruce (*Picea abies*)	Berchtesgaden	2000	47.56113	12.89025	Fi36
Norway Spruce (*Picea abies*)	Berchtesgaden	2002	47.56113	12.89025	Fi19
Norway Spruce (*Picea abies*)	Berchtesgaden	2004	47.56113	12.89025	Fi20
Norway Spruce (*Picea abies*)	Berchtesgaden	2006	47.56113	12.89025	Fi21
Norway Spruce (*Picea abies*)	Berchtesgaden	2008	47.56113	12.89025	Fi22
Norway Spruce (*Picea abies*)	Berchtesgaden	2010	47.56113	12.89025	Fi23
Norway Spruce (*Picea abies*)	Berchtesgaden	2012	47.56113	12.89025	Fi24
Norway Spruce (*Picea abies*)	Berchtesgaden	2014	47.56113	12.89025	Fi25
Norway Spruce (*Picea abies*)	Berchtesgaden	2016	47.56113	12.89025	Fi26
Norway Spruce (*Picea abies*)	Berchtesgaden	2018	47.56113	12.89025	Fi27
Norway Spruce (*Picea abies*)	Berchtesgaden	2020	47.56113	12.89025	Fi28
Norway Spruce (*Picea abies*)	Berchtesgaden	2022	47.56113	12.89025	Fi29
Norway Spruce (*Picea abies*)	Harz	1996	51.792691	10.645409	Fi86
Norway Spruce (*Picea abies*)	Harz	1997	51.792691	10.645409	Fi84
Norway Spruce (*Picea abies*)	Harz	1998	51.792691	10.645409	Fi82
Norway Spruce (*Picea abies*)	Harz	1999	51.792691	10.645409	Fi80
Norway Spruce (*Picea abies*)	Harz	2000	51.792691	10.645409	Fi87
Norway Spruce (*Picea abies*)	Harz	2001	51.792691	10.645409	Fi85
Norway Spruce (*Picea abies*)	Harz	2002	51.792691	10.645409	Fi83
Norway Spruce (*Picea abies*)	Harz	2003	51.792691	10.645409	Fi81
Norway Spruce (*Picea abies*)	Harz	2004	51.792691	10.645409	Fi89
Norway Spruce (*Picea abies*)	Harz	2005	51.792691	10.645409	Fi88

Norway Spruce (*Picea abies*)	Harz	2006	51.792691	10.645409	Fi90
Norway Spruce (*Picea abies*)	Harz	2007	51.792691	10.645409	Fi91
Norway Spruce (*Picea abies*)	Harz	2008	51.792691	10.645409	Fi92
Norway Spruce (*Picea abies*)	Harz	2009	51.792691	10.645409	Fi93
Norway Spruce (*Picea abies*)	Harz	2010	51.792691	10.645409	Fi94
Norway Spruce (*Picea abies*)	Harz	2011	51.792691	10.645409	Fi95
Norway Spruce (*Picea abies*)	Harz	2012	51.792691	10.645409	Fi96
Norway Spruce (*Picea abies*)	Harz	2013	51.792691	10.645409	Fi97
Norway Spruce (*Picea abies*)	Harz	2014	51.792691	10.645409	Fi98
Norway Spruce (*Picea abies*)	Harz	2016	51.792691	10.645409	Fi99
Norway Spruce (*Picea abies*)	Harz	2018	51.792691	10.645409	Fi100
Norway Spruce (*Picea abies*)	Harz	2020	51.792691	10.645409	Fi101
Norway Spruce (*Picea abies*)	Harz	2022	51.792691	10.645409	Fi102
Norway Spruce (*Picea abies*)	Pfälzerwald	1993	49.0902	7.43575	Fi52
Norway Spruce (*Picea abies*)	Pfälzerwald	2001	49.0902	7.43575	Fi53
Norway Spruce (*Picea abies*)	Pfälzerwald	2003	49.0902	7.43575	Fi54
Norway Spruce (*Picea abies*)	Pfälzerwald	2005	49.0902	7.43575	Fi55
Norway Spruce (*Picea abies*)	Pfälzerwald	2007	49.0902	7.43575	Fi56
Norway Spruce (*Picea abies*)	Pfälzerwald	2009	49.0902	7.43575	Fi57
Norway Spruce (*Picea abies*)	Pfälzerwald	2011	49.0902	7.43575	Fi58
Norway Spruce (*Picea abies*)	Pfälzerwald	2013	49.0902	7.43575	Fi59
Norway Spruce (*Picea abies*)	Pfälzerwald	2015	49.0902	7.43575	Fi60
Norway Spruce (*Picea abies*)	Pfälzerwald	2017	49.0902	7.43575	Fi61
Norway Spruce (*Picea abies*)	Pfälzerwald	2019	49.0902	7.43575	Fi62
Norway Spruce (*Picea abies*)	Pfälzerwald	2021	49.0902	7.43575	Fi63
Norway Spruce (*Picea abies*)	Scheyern	2000	48.487405	11.428479	Fi8
Norway Spruce (*Picea abies*)	Scheyern	2002	48.487405	11.428479	Fi9
Norway Spruce (*Picea abies*)	Scheyern	2004	48.487405	11.428479	Fi10
Norway Spruce (*Picea abies*)	Scheyern	2006	48.487405	11.428479	Fi11
Norway Spruce (*Picea abies*)	Scheyern	2008	48.487405	11.428479	Fi12
Norway Spruce (*Picea abies*)	Scheyern	2010	48.487405	11.428479	Fi150
Norway Spruce (*Picea abies*)	Scheyern	2012	48.487405	11.428479	Fi13
Norway Spruce (*Picea abies*)	Scheyern	2014	48.487405	11.428479	Fi14
Norway Spruce (*Picea abies*)	Scheyern	2015	48.487405	11.428479	Fi15
Norway Spruce (*Picea abies*)	Scheyern	2017	48.487405	11.428479	Fi16
Norway Spruce (*Picea abies*)	Scheyern	2019	48.487405	11.428479	Fi17
Norway Spruce (*Picea abies*)	Scheyern	2021	48.487405	11.428479	Fi18
Norway Spruce (*Picea abies*)	Solling	1999	51.787381	9.610741	Fi72
Norway Spruce (*Picea abies*)	Solling	2001	51.787381	9.610741	Fi73
Norway Spruce (*Picea abies*)	Solling	2003	51.787381	9.610741	Fi74
Norway Spruce (*Picea abies*)	Solling	2005	51.787381	9.610741	Fi75
Norway Spruce (*Picea abies*)	Solling	2007	51.787381	9.610741	Fi76
Norway Spruce (*Picea abies*)	Solling	2009	51.787381	9.610741	Fi126
Norway Spruce (*Picea abies*)	Solling	2011	51.787381	9.610741	Fi77
Norway Spruce (*Picea abies*)	Solling	2013	51.787381	9.610741	Fi78
Norway Spruce (*Picea abies*)	Solling	2015	51.787381	9.610741	Fi67

Norway Spruce (*Picea abies*)	Solling	2017	51.787381	9.610741	Fi68
Norway Spruce (*Picea abies*)	Solling	2019	51.787381	9.610741	Fi69
Norway Spruce (*Picea abies*)	Solling	2021	51.787381	9.610741	Fi70

Supp. Table 4. 2: Details of the laboratory workflow.

The list of the laboratory workflow is too big for printing. The supplementary file can be downloaded via this link:
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Curriculum vitae

Name	Julian Maximilian Josef HANS
Date of birth	02.09.1994
Place of residence	Am Deimelberg 19, 54295, Trier
E-Mail	jmjhans94@web.de

07.2020 – 03.2021 07.2019 – 03.2020	Research assistant at the Environmental Specimen Bank Project Group Trier, Trier University
11.2017 – 02.2018 03.2017 – 08.2017	Research assistant at the Department of Environmental Toxicology, Trier University

Since 01.04.2021	Dissertation at the Department of Biogeography
	Name of the Dissertation: Biodiversity in a bottle – metabarcoding environmental samples from long-term monitoring archives for retrospective assessment of changes in biotic communities in the Anthropocene
10.2017 – 03.2021	Trier University: Umweltbiowissenschaften (M. Sc.) Specialising in ecology and biodiversity
Master's thesis:	Tell me what you ate and I know where you're from – A food network reconstruction of spider diet from species of the Pacific
10.2014 – 09.2017	Trier University: Umweltbiowissenschaften (B. Sc.)
2005 – 2014	Gymnasium Saarburg: Abitur

03.2019 – 04.2019	Bundesamt für Infrastruktur, Umweltschutz und Dienstleistungen der Bundeswehr
09.2018 – 09.2018 09.2016 – 09.2016 08.2015 – 09.2015	Verein Jordsand zum Schutze der Seevögel und Natur e. V.

PC-Programs	R Studio (good), ArcGIS (good), MS-Office Program (very good)
Foreign languages	English (C1), French (B2), Spanish (A2)
Species expertise	Plants (good), Birds (good), Native freshwater fish (good)
Drivers license	B, BE, C1, C1E

Since 05.2018	Member of the animal welfare organisation Trier und Umgebung e. V.
Since 08.2018	Member of the organisation Jordsand zum Schutze der Seevögel und Natur e. V.
Hobbies	Reading and Sport (Endurance sport, Ballroom dance, Hip-Hop, Bouldering)

Affidavit

I, Julian Hans, declare that I have written this doctoral thesis independently. I further declare that I have not used any sources or aids other than those indicated and that I have marked as such any thoughts taken directly or indirectly from outside sources. I have not yet submitted the thesis to any other faculty of any other University in the same or a comparable form. It has not yet been published. I agree that my dissertation will be checked with a relevant plagiarism detection software.

Date and Signature