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Environmental DNA for management and ecosystem biomonitoring: Applications and methodological advances

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Environmental DNA for management and ecosystem biomonitoring: Applications and methodological advances

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par

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Summary

In the context of climate change and biodiversity crisis, there is an urgent need to understand biological communities from a whole ecosystem perspective, so that adequate conservation policies can be implemented. Conventional monitoring approaches, mostly relying on direct observation of organisms, prevent the study of multitrophic biodiversity as they are costly and time-consuming. Over the past two decades, the field of environmental DNA, based on the detection of genetic DNA traces that organisms release into their environment, has greatly expanded and enabled rapid and reliable detection of biological communities. Thanks to methodological advances, we are now starting to use eDNA to answer complex ecological questions from a whole ecosystem approach. However, the intricacy of interpreting the retrieved eDNA data has compromised its application for such a goal, and the boundaries of its ecological applications are still to be established. In this thesis, we have used eDNA techniques to study biological communities and species interactions in a great range of environments, from terrestrial to aquatic communities.

In the first part of this thesis, we assessed how management decisions shaped the trophic interactions of large herbivores to understand ecosystem dynamics. In the first chapter, we studied the effects of large-scale culling of herbivores during four years on their dietary niche partitioning in a previously unregulated dense herbivore population in the Oostvaardersplassen (Netherlands). We found that the diet of the main species being culled was altered as a consequence of the reduction in the total herbivore density. In chapter two, we studied how niche overlap in the two most abundant ungulates in the Bialowieza forest (Poland) responds to different forest management. For that purpose, we categorised the forest based on habitat quality, depending on logging, and on predation risk, depending on wolf presence or absence. We found that niche overlap within Red deer but not within Bison was reduced with increasing predation risk, suggesting their feeding behaviour is affected by wolf presence.

In the second part of this thesis, we studied how environmental disturbances can modify biological communities and which ecological drivers predict their shifts. In the third chapter, we studied how conventional and organic agricultural practices influence biological communities in a Swiss vineyard landscape. We compared soil arthropod communities between the two agricultural approaches and identified which pesticides had the greatest influence on these communities. In the fourth chapter, we studied the seasonality and spatial heterogeneity of the macroinvertebrate community in an alpine river system in Switzerland, in the framework of sediment management in hydroelectric areas, while comparing eDNA and traditional kick-net sampling techniques. We sampled water along a whole year and found the macroinvertebrates respond to the sediment rise from the glacier during the melting season. This suggests offering sediment-free conditions for macroinvertebrates during the melting season in managed rivers as a mitigating solution to alpine hydropower exploitations.

Taken together, these studies show the potential of eDNA as a robust and reliable method to answer complex ecological questions; and constitute a toolkit for future studies in a great diversity of research fields.

Résumé

Dans le contexte du changement climatique et de la crise globale de la biodiversité, il est urgent de comprendre les communautés biologiques du point de vue de l'ensemble de l'écosystème, afin de pouvoir mettre en œuvre des politiques de conservation pertinentes. Les méthodes de suivi conventionnels, qui reposent principalement sur l'observation directe des organismes, rendent difficile l'étude de la biodiversité multitrophique, car elles sont coûteuses en termes de temps et d'argent. Au cours des deux dernières décennies, le domaine d'étude de l'ADN environnemental (eDNA), basé sur la détection des traces génétiques d'ADN que les organismes laissent dans leur environnement, s'est considérablement développé et a permis une caractérisation rapide et fiable des communautés biologiques. Ces progrès méthodologiques permettent de commencer à utiliser l'eDNA pour répondre à des questions écologiques complexes dans le cadre d'une approche globale de l'écosystème. Cependant, la complexité de l'interprétation des données d'eDNA a restreint son application pour un tel objectif, et les limites de ses applications écologiques restent à établir. Dans cette thèse, nous avons utilisé les techniques d'eDNA pour étudier les communautés biologiques et les interactions entre les espèces dans un large éventail d'environnements, des communautés terrestres aux communautés aquatiques.

Dans la première partie de cette thèse, nous avons évalué comment les décisions de gestion ont façonné les interactions trophiques des grands herbivores, ce qui nous a permis de contribuer à la compréhension de la dynamique des écosystèmes. Dans le premier chapitre, nous avons étudié les effets de l'abattage à grande échelle d'herbivores pendant quatre ans sur la répartition de leur niche alimentaire dans une population dense d'herbivores non régulée dans l'Oostvaardersplassen (Pays-Bas). Nous avons constaté que le régime alimentaire des principales espèces abattues se voyait altéré suite la réduction de la densité totale des herbivores. Dans le deuxième chapitre, nous avons étudié comment le chevauchement des niches dans les deux espèces d'ongulés les plus abondants dans la forêt de Bialowieza (Pologne) réagit aux différentes stratégies de gestion forestière, en classant la forêt en fonction de la qualité de l'habitat, à la suite de l'exploitation forestière, et en fonction du risque de prédation, selon la présence de loups. Nous avons constaté que le chevauchement des niches du cerf élaphe, mais pas du bison, était réduit avec l'augmentation du risque de prédation, ce qui suggère que leur comportement alimentaire est affecté par la présence du loup.

Dans la deuxième partie de cette thèse, nous avons étudié comment les perturbations environnementales peuvent modifier les communautés biologiques et quels facteurs écologiques sont déterminants. Dans le troisième chapitre, nous avons étudié l'influence des pratiques agricoles conventionnelles et écologiques sur les communautés biologiques dans un paysage viticole suisse. Nous avons comparé les communautés d'arthropodes du sol entre les deux approches agricoles et identifié quels pesticides avaient la plus grande influence sur ces communautés. Dans le quatrième chapitre, nous avons étudié la saisonnalité et l'hétérogénéité spatiale de la communauté de macroinvertébrés dans un système fluvial alpin en Suisse, dans le cadre de la gestion des sédiments dans les zones hydroélectriques, tout en comparant l'eDNA et les techniques traditionnelles d'échantillonnage à la nasse. Nous avons échantillonné l'eau tout au long de l'année et constaté que les macroinvertébrés réagissent à l'incrément des sédiments du glacier pendant la saison de fonte, ce qui plaide en faveur de conditions exemptes

de sédiments pour les macroinvertébrés pendant la saison de fonte dans les rivières gérées, comme solution d'atténuation des exploitations hydroélectriques alpines.

Dans l'ensemble, cette recherche a montré le potentiel de l'ADN environnemental en tant que méthode robuste et fiable pour répondre à des questions écologiques complexes.

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Introduction

Environmental DNA (eDNA) is a rapidly evolving tool that has gained attention in recent years as a non-invasive and cost-effective technique for biomonitoring and ecosystem management (Taberlet et al., 2018). eDNA is defined as the genetic material shed into the environment by organisms through mucus, urine, faeces and other biological materials. This genetic material can then be collected and extracted from environmental samples (e.g., soil (Calderón-Sanou et al., 2021), water (Keck et al., 2022), or air (Clare et al., 2021)). This eDNA can then be sequenced and assigned to a taxon using a reference database. In other words, this technique allows for the identification of the different species present in the environment without prior isolation of target organisms. The analysis of eDNA has become a key component of ecological studies, and represents a crucial research domain for future biodiversity monitoring and management (Taberlet et al., 2018).

First applied to microorganisms (Ogram et al., 1987), eDNA research has expanded to macroorganisms and ecosystems as a whole, providing a new approach to how we study and monitor ecosystem dynamics. Furthermore, it has led to the realisation that traces left by organisms on the environment contain valuable information about their ecosystem and the interactions occurring between species (Pinakhina & Chekunova, 2020). While early studies were mostly based on Polymerase Chain Reaction (PCR) followed by Sanger sequencing or on quantitative PCR (qPCR) assays, the advent of high-throughput sequencing enabled the simultaneous sequencing of complex DNA mixtures present in the same environment, which is known as DNA metabarcoding (Taberlet et al., 2012). The name originates from the meta-barcodes present in the environmental sample. Each barcode corresponds to the DNA sequence which is used to identify the species where it came from, which is unique to each taxon. Metabarcoding relies on PCR amplification of DNA extracted using barcoded primers, i.e. primers with an attachment DNA which allows to link each amplicon to its sample origin. The development of such sets of primers targeting a broad taxonomic range of species has facilitated the amplification of many species within the same PCR reaction (Taberlet et al., 2018).

The technique enables the detection of whole ecological communities, but its reliability for quantitative assessments has been discussed since the beginning (Harper et al., 2020) and remains to date an ongoing and unresolved debate. Indeed, only a relative abundance based on the proportion of sequences retrieved in the samples is measurable using the metabarcoding approach (Harper et al., 2018). However, the number of reads per sequence is highly dependent on the DNA extraction protocol and the PCR conditions (Nagler et al., 2022). For example: the amplification cycles can lead to an overrepresentation of abundant sequences, primer specificity can have higher affinity for particular species. Even a single nucleotide polymorphism (SNP) can already have an impact (Bellemain et al., 2010). Furthermore, co-extracted molecules can inhibit the PCR reaction and complicate cross-PCR quantitative comparisons. Altogether, PCR-introduced biases in DNA metabarcoding are considered the main constraint for a reliable quantification (Schenekar et al., 2020). To overcome such limitations, new protocols have emerged to either skip the PCR by using hybridization capture protocols (i.e., using probes to enrich the DNA sample and sequence directly, e.g. (Mariac et

al., 2018; Seeber et al., 2019)), or to divide taxa in the PCR amplification process through multiplexing techniques (i.e. combination of primers to detect exclusively certain taxonomic groups, e.g. (De Barba et al., 2014; Wood et al., 2019)). Research efforts are also dedicated to the question of quantification, screening the influence of different variables on metabarcoding results (e.g. (Dowle et al., 2016)).
Traditional survey methods such as stomach content analysis, direct observations, kick-net sampling, stomach and faecal content analysis or pitfall traps, come with several constraints for researchers, such as: the physical requirements needed to collect the data (Dulsat-Masvidal et al., 2021), the limitations to correctly identify species (Menezes et al., 2010) or other technical difficulties to obtain data (Ruiz-Gutierrez et al., 2016). eDNA offers a robust and sensitive alternative to former methods. An illustrative example is the early detection of an invasive species. When the species is at an early stage of invasion, few individuals are present and they can be difficult to observe using conventional survey methods, whereas using the eDNA approach allows them to be detected, as they shed DNA in the environment, which can easily be collected (Harper et al., 2018). This environmental footprint is left by all individuals, regardless of their size. Even at a microscopic level species can be detected without the need of being observed, although in that case, instead of collecting biological material shed by individuals, the whole microorganism is sampled by extracting its DNA from the environmental sample to detect it.

Despite the versatility of eDNA techniques, the resolution and reliability of the data collected represents, still today, a major challenge for researchers (Cordier et al., 2018; Dufresnes et al., 2019; Zinger et al., 2020). As an emerging multidisciplinary technique, further research on experimental design is needed to improve eDNA metabarcoding accuracy and depth of detection (Klymus et al., 2019). The goal is to minimise and control artefacts and biases potentially produced during the eDNA workflow, from field sampling to molecular protocols and bioinformatic pipelines (Zinger et al., 2019). Indeed, DNA in the environment might be released, degraded and/or transported in different manners depending on the characteristics of the environment. Therefore, to successfully retrieve information from the DNA collected, a number of parameters related to the experimental design, such as the type of the sample collected (i.e., water, soil, scats), the location or the scale of the study must be considered. For instance, the physicochemical properties of the environmental sample can reduce the quantity of DNA extracted or inhibit the PCR reaction. Early eDNA studies exposed its limitations, as they struggled to provide the resolution or reliability to answer ecological questions (Beng & Corlett, 2020; Burian et al., 2021; Jerde et al., 2011; Kestel et al., 2022) or even questions which had been successfully addressed using pre-eDNA techniques (Qu & Stewart, 2019; Roussel et al., 2015). First, the ecological information contained in an environmental sample is limited to a certain area of ecological influence, i.e. the area for which the environmental factors of interest are affecting the sampling location. This surface varies depending on the focus of study, so research questions need to be accommodated around this limitation to ensure the feasibility of the project. The larger the area of study and the amount of species accounted for are, the trickier the sampling and the interpretation of the results becomes (Chalmandrier et al., 2019). Certainly, it is not the same to attempt to detect the presence of a single species than to study the community response to an ecological disturbance. Furthermore, DNA can be

subject to degradation and dilution, which can affect the accuracy of species identification and abundance estimates (Holman et al., 2021). Additionally, it may be necessary to optimise eDNA extraction and analysis protocols for different taxa. Second, the processing of the environmental sample to obtain DNA sequences involves many fieldwork, laboratory and bioinformatic steps which require transversal expertise in order to ensure the quality of the information retrieved (Zinger et al., 2021). All these necessary steps currently add noise to the data produced and biases biological interpretations. For all these reasons, eDNA is a complex domain of research. The experimental design has to account for the ecological limitations of the methodology and evaluate the resolutive capacity of the data beforehand. However, it also has great potential for biological research because of its fundamental principle: it targets species within their environment rather than within their physical bodies. This is the conceptual breakthrough of eDNA that we have just started to explore.

Overall, eDNA has revolutionised the way we monitor and manage ecosystems. It can provide a powerful tool for conservationists and ecosystem managers to identify species of concern and take appropriate actions to protect and conserve them. As eDNA technology continues to advance and become more widely adopted, it is likely to play an increasingly important role in our efforts to preserve and manage the biodiversity of our planet.

eDNA as a tool for ecosystem management

Efforts in conservation management aim at preserving natural areas, its ecosystem and its biodiversity. Management organisations have to balance conservation of the ecosystem with the challenges caused to some extent by human and wildlife interactions (Redpath et al., 2013). These include damage to crops, disturbances with inhabited areas, monitoring of invasive or endangered species and landscape management, among others. Such decisions require a proper understanding of the current status of the target species and the ecosystem involved, which usually demands a large investment on biomonitoring.

Traditional techniques for biomonitoring are based on the direct observation or capture of individuals, such as camera trapping (Nasanbat et al., 2021; Trolliet et al., 2014), GPS collaring (Odden et al., 2014) or transects (Basset et al., 2013). They involve intensive fieldwork labour, so efforts need to be adequately allocated to obtaining reliable data on, for example, population trends, species ecology or interactions, while accounting for the sampling effort and costs. Traditional techniques require physical endurance, a large expertise to identify species and a good knowledge of the ecology or the environment. From an ecological perspective, these limitations (Taberlet et al., 2018) have brought biomonitoring research to focus mainly on the presence/absence of a species or their absolute abundance rather than on the interactions between species (i.e. trophic interactions, competition, predation). Such constraints usually lead to a selection of target species based on their potential to answer the ecological questions as indicators of the whole ecosystem (e.g. (Hegland et al., 2010)).

During the last years, eDNA techniques have proven their potential to contribute to ecosystem management, providing a powerful alternative tool to traditional survey methods to answer ecological questions of interest in many ecosystems (Pawlowski et al., 2021). The main advantages of eDNA are its accuracy and the amount of information that can be extracted from a single sample, while being non-invasive. Also, it can detect changes in an ecosystem at an early stage, because it can detect the presence of species from a very low abundance (Harper et al., 2018), allowing managers to take action before changes settle and the impact to the ecosystem worsens. It is a sensitive and cost-effective method to target practical demands from landscape, agricultural or wildlife management. The information contained in each sample spans back in time, from days to months depending on the type of sample, which can be both an advantage or a disadvantage depending on the goal of the research (Beentjes et al., 2019; Troth et al., 2021). It can also contain information which covers a large area of the ecosystem; for example, when studying the diet of a carnivore, the information contained in the scats of the carnivore informs on all the species it has fed on and confirms their presence within the carnivore's territory (Franklin et al., 2019). All the research done on this topic has given eDNA techniques a solid background to expand beyond biomonitoring and study ecosystem dynamics in the light of management and conservation. By providing more detailed and accurate information about ecosystem health, eDNA analysis can help managers make more informed decisions about how to manage natural and agricultural areas. This leads to more effective conservation and management strategies that benefit both wildlife and human populations (Beng & Corlett, 2020).

Currently, eDNA applications to evaluate ecosystem management can be grouped in the following categories:

1. *Biomonitoring:* identify the presence and abundance of different species in an ecosystem, providing valuable information about the overall biodiversity of a natural area and the impact of management practices on wildlife populations. Moreover, it can provide a more accurate and comprehensive picture of ecosystem health over time, allowing managers to track changes and evaluate the effectiveness of management strategies. This can be useful for identifying emerging threats, measuring the success of restoration efforts, and adapting management practices to changing environmental conditions.

2. *Invasive species detection:* detect the presence of invasive species, which can be a major threat to both natural and agricultural ecosystems. By identifying and monitoring invasive species using eDNA, specially at an early invasion stage when eDNA is more reliable than traditional methods, managers can take steps to control or eradicate them before they cause significant damage.

3. *Soil health assessment:* provide information about the biological communities present in soil (e.g. micro- or macroscopic communities). This can help farmers or land managers make more informed decisions about fertilisation, crop rotation, and pest control. It can detect the presence of pathogens and pests in agricultural systems and

contribute to developing more targeted and effective control measures to reduce the need for broad-spectrum pesticides and other chemical treatments. In turn, this can lead to more sustainable and environmentally friendly agricultural practices. This can be important for both natural and agricultural ecosystems, as healthy soil is essential for plant growth and ecosystem functioning.

4. *Water quality monitoring:* assess the quality of water in natural and agricultural ecosystems by detecting the presence of pathogens or reference species which are used as water quality indicators. This can be useful for identifying sources of contamination, such as agricultural runoff or sewage discharge. This information can be used to develop more effective management strategies to protect water quality and ensure the availability of clean water for both human and ecosystem use.

From an applied point of view, implementing eDNA tools brings managers a more comprehensive understanding of their actions on the ecosystem. eDNA can be particularly useful in several aspects of ecosystem management (Banerjee et al., 2021). Nevertheless, using eDNA to evaluate the consequences of management decisions on the ecosystem as a whole, rather than putting the focus on a single or few species, is still at an early stage, as few researchers have used the technique for such an ambitious purpose (e.g. (Harper et al., 2019; Mauvisseau et al., 2020)), so the true potential of this technique is yet to be unravelled.

Sampling trophic interactions

Understanding the implications of changes in species interactions for the functioning and adaptive potential of a biological system is a major challenge. Ecosystems are shaped by environmental changes, which are often accelerated in the current human-dominated world due to habitat loss, climate change or species extinction among others. Such increasing pressure on ecosystems requires studying current ecosystem adaptive potentials in the light of conservation. Loss or introduction of some species can cause changes in the ecological niche of other species in the ecosystem or even lead to cascading losses of species (De Visser et al., 2011; Srinivasan et al., 2007). New evolutionary opportunities can arise while others are brought down, forcing species to evolve and/or adapt through phenotypic and behavioural adjustments (Piersma, T. J.A. Van Gils, 2012).

Trophic interactions are defined as the transfer of energy from individuals of one species to those of another species. The sum of trophic interactions of an ecosystem is known as trophic network, or more colloquially, as food chain (Bascompte, 2010; Melian & Bascompte, 2002). These networks become increasingly complex with each species added into the network, and require a multivariate approach to be studied in a comprehensive way (Stouffer & Bascompte, 2010; Thompson et al., 2012). In order to quantify the role a particular species or interaction is having and relate it to the functioning of the ecosystem, researchers require a complete coverage of the trophic interactions of that species on the whole network to retrieve reliable information to be used for conservation purposes (Duffy et al., 2007). Focusing on key

structural trophic interactions in the ecological network or target species, as biological indicators, is a way to provide meaningful results on the ecological dynamics of the studied system (Kissling & Schleuning, 2015). This approach is followed both by traditional and eDNA techniques, optimising the fieldwork and/or the DNA sequencing effort. Overall, trophic interactions can be used as proxies to inform on the effects of management policies in the ecosystem (Svenning et al., 2019). However, several species might be selected for sampling trophic interactions, as the results obtained from a single interaction might not be sufficient to accurately provide guidelines for ecosystem management. This is because when using eDNA, the path between the trophic interaction data and its ecological significance is not straightforward (Beng & Corlett, 2020; Clare, 2014). We have to first quantify trophic interactions and then link them to the management policies enforced on the ecosystem, adding noise to the data along the way. Since eDNA allows for the assessment of multiple parts of the trophic network from a single sample, encompassing multiple species within the sample from a single species, the targeted trophic interactions need to be chosen according to prior knowledge of the area, so as to aim for a strong and sensitive enough ecological signal to draw robust conclusions about the research or management question of interest.

Traditional methods for studying trophic interactions, such as stomach content analysis (Hyslop, 1980; Rohner et al., 2013), faecal analysis (Anthony & Smith, 1974) or direct observation (Paley & Kerley, 1998) can be time-consuming and labour-intensive. In contrast, eDNA metabarcoding has been increasingly used to assess trophic interactions among species, which can be extracted from environmental samples such as scats or soil (Sheppard & Harwood, 2005). For example, the diet of a herbivore can be studied by sampling the DNA present in the scat samples, given the digestion of the plants ingested is never complete (e.g. (van Beeck Calkoen et al., 2019). This DNA can be extracted, amplified and sequenced so as to quantify the diet of a species. Once their diet is quantified, we can address ecological questions related to diet selection (Wanniarachchi et al., 2022), species competition (Serite et al., 2021) or niche partitioning (Schure et al., 2020) within an ecosystem. However, trophic interactions are not restricted to dietary studies, they can be studied in various ways, which are highly dependent on the goal of each particular research (Abdala-Roberts et al., 2019). To exemplify the range of study of trophic interactions, a recent research project studied the effects of tree defoliation due to a moth outbreak, which was monitored sampling soil eDNA (Calderón-Sanou et al., 2021). They detected an increased belowground diversity at different trophic levels, tracing the cascading effects of the outbreak into the soil ecosystem and relating it to the belowground trophic interactions. Hence, trophic interactions can be studied beyond scats and diet. In this case, eDNA can also be used to identify the presence, abundance and distribution of different plant or animal species, which can inform beyond the dietary preferences of the studied species compared to traditional methods. For example, eDNA can detect plant or animal species that may be difficult to identify through traditional sampling methods, such as rare or cryptic species (Groen et al., 2022). Altogether, using eDNA to study trophic interactions can provide a more complete understanding of a species diet and the ecological processes driving their dietary choices.

Regarding diet analyses, they enable more precise taxonomic identification of species than previous microhistological techniques (Khanam et al., 2016). However, there are also some potential limitations to using eDNA for studying diets and trophic interactions (Banerjee et al., 2021; Yoccoz, 2012). For instance, it requires complete reference databases with the DNA sequence for all the species within all diets of the ecosystem (discussed in (Schenekar et al., 2020)). In this line, there is still an ongoing debate to decide which are the best DNA regions to amplify for each taxonomic group (Ficetola et al., 2021), and reference databases are being enlarged in parallel instead of developing a consensus across taxa on which DNA region best fulfils metabarcoding requirements (e.g. COI vs 16S, (Zhan et al., 2014)). Also, the cost to tackle all trophic interactions at ecosystem level remains high because of the fieldwork and sequencing efforts required. Moreover, eDNA can be subject to degradation and dilution, which can affect the accuracy of species identification and abundance estimates (Troth et al., 2021). Additionally, eDNA may not be able to distinguish between live and dead organisms, which could result in false positives or overestimation of species abundance (Kamoroff & Goldberg, 2018). Overall, using eDNA to study trophic interactions between herbivores and their food sources has several potential advantages over traditional methods. However, it is important to account for the limitations and challenges associated, and to integrate other complementary methods to better understand trophic dynamics in ecosystems.

Ecosystem management, trophic interactions and eDNA

Temperate grazing ecosystems are well-studied and have a relatively low herbivore and plant diversity, which makes them technically easier to study (Frank et al., 1998). In terms of taxonomy, most plant species have already been sequenced and thus, the effort to complete the reference databases for eDNA studies is low compared to other ecosystems (Fahner et al., 2016; Pansu et al., 2019). Sampling in such environments does not require expensive or time-consuming fieldwork, and the results can be easily compared to similar ecosystems across Europe.

Some of these ecosystems undergo herbivore-related management policies, which provides a unique opportunity to study how these affect the trophic interactions within, making them large natural experimental setups (Kowalczyk et al., 2011; Niedziałkowski et al., 2019; Theunissen, 2019). The majority of natural areas are managed, but few are strict reserves, which usually implies a conflict between the preservation of the ecosystem and the exploitation of its natural resources (e.g. (Chapman et al., 2003)). Balancing the two is a difficult task, especially when both are to be maximised to obtain the most of the ecosystem, i.e. biodiversity- and economy-wise (Essington et al., 2018). Decision-making translates into particular disturbances in the ecosystem, expecting to steer the dynamics of the ecological network towards a desired status (Fisher et al., 2009). Species interactions are usually the cause or the consequence of the disturbance aimed to tackle, and measuring trophic interactions is a reliable way to quantify them. Such disturbances need to be supported by research evidence, but this is usually limited or biassed by the sampling methodology, as explained above.

In addition, changes in management can trigger unexpected shifts in the flow of energy and nutrients in such herbivore-driven ecosystems (Veldhuis et al., 2014). This promotes resource

partitioning among herbivore species, where smaller herbivores require higher quality resources (Olff et al., 2009) and larger herbivores require greater amounts of resources but handle lower quality (Danell, 2006; Olff et al., 2002). This has various implications for nutrient flow, food web structure and the overall ecosystem functioning. The stoichiometric axis of food-web organisation in food webs implies that variation in resource types produced by plants promotes coexistence of different-sized herbivores (Ruifrok et al., 2015). Not only does this result in alternative energy channels compared to a single food chain, thus promoting robustness of the ecosystem, but it also has important consequences for the interplay of herbivores with higher trophic levels (e.g. McCary et al., 2016). When herbivores change their behaviour and diet, their competitive and facilitative interactions can change. As such, management policies have an impact on the trophic interactions between herbivores. However, these adaptive changes in herbivore assemblages are still poorly documented and understood.

In Chapter 1 and 2, we studied the suitability of eDNA techniques as a quick and cost-efficient method to provide detailed scientific evidence on the effects of management policies applied in natural areas by quantifying herbivores' trophic interactions.
We selected two study systems (the Oostvaardersplassen, NL; and the Bialowieza forest, PL) with distinctive and well documented herbivore assemblages to evaluate the effects of management policies on the ecosystem using eDNA (Kowalczyk et al., 2011; Lorimer & Driessen, 2014). We used trophic interactions of the main herbivore species as our response variable, inferred from their diet composition. We collected faeces from selected herbivore species to test the effect of management policies on the ecosystem network dynamics by comparing their diet compositions using eDNA metabarcoding. Both systems have undergone different changes due to anthropogenic effects through specific management decisions in the recent past, described below in detail. Such changes have altered the ecosystem, modifying the interaction between species, which makes them great systems to study the relationship between management, i.e. anthropogenic effects, and species trophic interactions. The data collected aimed at drawing a clear answer on the impact management had on herbivore interactions and thus on the ecosystem as a whole. Ultimately, we aimed at integrating the results obtained into the current decision-making of management policies in the studied areas.

Assessing agricultural management using eDNA

Soil is the foundation of agricultural systems and hence, of our society (Brevik & Hartemink, 2010; Kibblewhite et al., 2008). Soil has been cultivated for centuries, and its management has aimed at improving crop yield, in recent years through the use of pesticides and fertilisers (Carvalho, 2017; Liu et al., 2015). Reliance on these two products to ensure crop viability has led to underlook soil biology, reducing soil to its physicochemical expression. Only recently, agricultural management has started to shift to account for the importance of the biological component of the soil, and has recovered the interest for the benefits the organisms living in it can provide to agriculture. We are now experiencing a rise in sustainable agriculture, i.e. partially or totally accounting for the soil biological community to maximise the potential benefits it can provide (Velten et al., 2015). Hence, sustainable agriculture, also known as

biological or organic, has upscaled the variables at stake beyond crop yield, balancing productivity with the soil properties, accounting for its physical, chemical and biological parts and the interplay between the three (Bünemann et al., 2006).

Despite this new trend, most agricultural land is still managed under a conventional approach, i.e., maximising the yield of the crops disregarding the potential negative effects to the soil ecosystem (Gomiero et al., 2008; Morgan & Murdoch, 2000). These practices are characterised by the addition of high amounts of pesticides, fertilisers, usually synthetic, and herbicides, such as Glyphosate (Duke, 2018). Combined, they can lead to soil degradation resulting in reduced crop yields and increased environmental impacts (De Ponti et al., 2012). As such, from an environmental perspective, the link between agriculture, fertilisers and pesticides is of utmost importance for the productivity of the crops, but the impact on the soil biodiversity is generally overlooked (Bünemann et al., 2006; Prashar & Shah, 2016). Fertilisers are designed to provide plants with the necessary nutrients to grow and produce healthy crops. Pesticides are chemicals that are used to control pests, including insects, weeds, and fungi, that can damage crops and reduce yields. Both are widely used in agriculture to increase crop productivity and protect them from pests and diseases. However, the use of these chemicals can have significant impacts on soil health, which can affect the long-term sustainability of agricultural systems. While they have been used in agriculture for decades, they have come under increasing scrutiny in recent years due to their proven damage to the environment (Mahmood et al., 2016; Rahman & Zhang, 2018). One of the primary concerns when using pesticides is their potential to harm non-target species, such as beneficial microorganisms, insects, birds, and mammals (Stanley & Preetha, 2016). Another concern is their potential to contribute to the development of pesticide-resistant pests, which can lead to increased use of pesticides and pose a greater risk to the environment and human health (Gould et al., 2018). Parallely, excessive or improper use of fertilisers can lead to nutrient runoff and contamination of water sources, leading to negative impacts on aquatic ecosystems and drinking water (Khan et al., 2018). This contamination can persist for years or even decades, posing long-term risks to the environment and human health.

To address these environmental concerns, there has been increasing interest in sustainable agriculture practices to minimise the use of pesticides and thus, the damage to the environment and human health (El Chami et al., 2020). However, this approach has not yet taken over in conventional methods for pest control, which still focus on maximising crop yield overlooking the environmental impacts they might cause the soil ecosystem. As such, there is a need for sustainable agriculture practices that promote healthy ecosystems but without compromising the productivity of the crops (Lankoski & Thiem, 2020). Overall, there are two main factors which currently endanger the functioning of soil ecosystems: excessive use of pesticides and fertilisers and difficulties to assess the status of the soil ecosystem to monitor its health.
The soil ecosystem constitutes a complex community with multiple interactions that together contribute to nutrient cycling (Tully & Ryals, 2017), soil structure (Bronick & Lal, 2005) and plant-soil feedback (Mariotte et al., 2018). Within, soil arthropods, such as mites, springtails, and beetles, are important components of this ecosystem and provide many ecological services which can be very beneficial to the agricultural goals, such as organic matter recycling, soil bioturbation, etc., or detrimental, i.e. pests (Noriega et al., 2018). Moreover, they have been

used as ecosystem indicators, because they are relatively easy to sample and can be macroscopically identified. However, the soil arthropod community as a whole is usually neglected by agricultural managers despite its many benefits for farmers. The use of pesticides, herbicides and fertilisers is known to have a negative impact on their biodiversity (Menta et al., 2020; Pérez-Bote & Romero, 2012), which can in turn affect soil health and agricultural productivity. However, new advances on pesticides and herbicides have improved the specificity of their active compounds against particular groups of organisms, reducing the damage on the crops while maintaining the soil biodiversity and the benefits it brings for the productivity of the crops (Tilman et al., 2002). Regarding fertilisers, research is now addressing their potential runoff of nutrients which contaminates the soil and the water below the crops (Sharpley, 2016).
In this line, eDNA can be a great method to assess the status of the soil ecosystem. It can be used to assess the impact of pesticide and fertiliser use on the soil ecosystem by detecting and quantifying the DNA of different micro- or macroscopic species in soil samples (Kestel et al., 2022). For example, by comparing soil samples from areas with different levels of pesticide use, it is possible to determine how pesticide use affects the abundance and diversity of soil arthropods (e.g. (Agerbo Rasmussen et al., 2021).

Again, the main advantages of using eDNA for this purpose is that it can be a non-invasive and cost-effective method. Traditional methods for assessing soil arthropod biodiversity, such as pitfall trapping (Hohbein & Conway, 2018) or soil arthropod extraction (Parisi et al., 2005), are time-consuming, labour-intensive and invasive (i.e. they may require the sacrifice of the collected individuals). In contrast, eDNA can be extracted from soil samples, which can be collected relatively easily (Allen et al., 2021). This approach was first used to study the soil microbial community (Ogram et al., 1987), but has now expanded to all groups present in the soil ecosystem. Another advantage of using eDNA is that it can provide a more comprehensive assessment of soil arthropod biodiversity than traditional methods. eDNA can detect arthropod species that may be difficult to identify taxonomically or to capture. This can provide a more complete understanding of the impacts that pesticide, herbicide and fertiliser use have on soil arthropod communities.
However, there are also some potential limitations to using eDNA for studying the soil arthropod community. Besides the general eDNA limitations already discussed above, the sampling of soil for eDNA analysis can bring additional challenges. For example, the humic acids present in the soil inhibit the process of DNA amplification and might require advanced laboratory expertise to adjust DNA extraction protocol, dilution fold or PCR parameters to increase the DNA yield (Schrader et al., 2012). Moreover, the soil ecosystem expands belowground, and there are multiple functional layers with unique functions. As such, this implies that the layer of soil sampled will only represent that particular depth of the soil, and prior knowledge on soil ecology is advised in order to select the layer of interest, i.e., the one responsible for the ecological service under study.

Assessing aquatic ecosystems using eDNA

Freshwater ecosystems are among the most diverse ecosystems on earth (Dudgeon et al., 2006). They include lakes, rivers, wetlands, ponds, puddles and all the inbetweens. The diversity hosted in these environments plays a key role in determining the physicochemical conditions of the water, but the opposite also occurs (Früh et al., 2012). Biological activity interplays with physicochemical conditions and drives the community of each water body, favouring the growth of certain species while excluding others. This creates a dynamic system that can be either beneficial or detrimental to human interests. For example, an algae bloom due to an excess of nutrients in an inland fish farm can asphyxiate the stocked fish (Platt et al., 2003), while the introduction of a predator in a pond can reduce the presence of an invasive species (Louette, 2012). Such variability brings unique challenges to biomonitoring efforts aiming at assessing the biodiversity present in a water body. Traditional methods such as electric fishing (Growns et al., 1996), kick-net sampling (Svensson et al., 2018), microscopic observation (Neu & Lawrence, 2006), acoustic monitoring (Measey et al., 2017) or camera trapping (Colyn et al., 2017) all share the need to see or hear the individuals aimed to sample. This requirement and potential limiting factor has been the driver behind the rise of eDNA metabarcoding for freshwater biomonitoring, a growing research field that also has many industrial applications (reviewed in Coble et al., 2019). eDNA is naturally shed by all the individuals that get in contact with it. This physical interaction allows for the detection of species present in a water body without the need to observe them. When these are very rare or cryptic, the benefits of eDNA compared to traditional methods become clear (Biggs et al., 2015; Valentini et al., 2016). However, eDNA analyses need to be adjusted to the nature and intrinsic variability of the freshwater ecosystems under study to maximise the resolutive power of the samples collected. To begin with, eDNA concentration is highly variable depending on which body of water is being sampled (Barnes et al., 2014). For example, ponds, because of their still and shallow nature, contain higher concentrations of eDNA than rivers in equal climatic conditions (reviewed in Rees et al., 2014). This is because rivers flush downstream the eDNA being released by organisms, reducing its concentration and confounding the true location of its source (Burian et al., 2021).

Another factor is related to the amount of water needed per sample. eDNA concentration is highly variable between species (Lacoursière-Roussel et al., 2016). This concentration is dependent on the degree of interaction that the target species has had with the water body (Mas-Carrió et al., 2022). For example, we will probably require less amount of water from a pond, collected or filtered, to detect the presence of algae than to detect a bird species using the pond to wash and to drink. This illustrates the complexity of studying aquatic ecosystems using eDNA. Researchers have to account for the relative DNA concentration of the target species and the dynamics of its DNA once released in the system prior to sampling. This is necessary to adjust the laboratory work protocols (i.e. dilution fold and PCR conditions) so as to avoid false negatives (Burian et al., 2021). These can mainly occur due to the low DNA concentration of the target species or because of its low relative concentration, as most abundant DNA can mask the amplification of the target species when using generalistic primers. To overcome such problems, it is advised to perform amplification tests through qPCR analysis during the preparatory work at the laboratory or consider eDNA capture enrichment protocols (Wilcox et al., 2018).

Besides the species composition in the water, researchers also need to take in account other parameters of the water being sampled, such as turbidity, pH, velocity and depth (McCartin et al., 2022). Turbidity, for example, can have a huge impact on the sampling efforts, especially if the samples are collected through filtration rather than collection, as it can clog the filters used for eDNA water sampling and compromise the volume of water required for each sample (Kumar et al., 2022). Following up, depth will determine which part of the aquatic ecosystem is being recorded and pH or oxygen levels can inform on the degree of inhibition expected during PCR amplification, due to the co-extracted acid or basic molecules present in the water (Hunter et al., 2019).

As mentioned, eDNA can be used to monitor both dynamic and static freshwater ecosystems, i.e. rivers and lakes. However, there are some particularities to consider when using this tool in these two types of aquatic environments (Schenekar, 2023). First, the movement of water makes it more challenging to detect species in rivers. eDNA may be diluted and transported over long distances, making it more difficult to pinpoint the exact location of a particular species (Shogren et al., 2017). Second, rivers are generally more connected to other ecosystems (such as to other rivers or terrestrial ecosystems) than lakes because of their greater catchment area. This means that species can move more easily between different parts of the ecosystem, which can make it more challenging to assign the detected eDNA from a particular species with the sampling location (Burian et al., 2021). Third, some species in rivers may be more elusive or difficult to detect using eDNA, such as species that are primarily nocturnal, burrowed in sediments, or have low population densities (Rees et al., 2014). In contrast, lakes are easier to navigate and access more locations to sample eDNA, which can facilitate the detection of all species present. Finally, sediment deposition, typical of rivers steep or glacial rivers, can alter the distribution of eDNA in the water column. Sediment can contribute to sink eDNA, reducing the amount of DNA available in the water column (Kumar et al., 2022). This can make it more difficult to detect rare or low-abundance species in rivers. In contrast, lakes often have less sediment deposition and a more homogenous water column, which can make it easier to detect species.

In summary, while eDNA can be a valuable tool for monitoring both river and lake ecosystems, there are a variety of factors to consider when designing and implementing eDNA monitoring programs in these environments. Understanding the particularities of each ecosystem, such as water flow, habitat complexity, connectivity, sediment deposition, and species richness can help researchers and managers make informed decisions about how to optimise eDNA monitoring efforts in freshwater ecosystems.

Thesis outline

The main objective of this thesis is to apply eDNA techniques to answer complex ecological questions linked to management policies, while identifying its strengths and limitations of using it from a whole ecosystem approach. To date, research using eDNA for ecosystem management has avoided targeting complex ecological questions due to the intricacy to

interpret the retrieved data. However, there is little evidence of the limits and boundaries of this methodology for such a goal, as it is very case-dependent. In this thesis, we assess the reliability of eDNA techniques to provide guidelines to improve management policies for the studied ecosystems and its transferability to new ones. We have studied its suitability and feasibility from a multivariate approach, sampling scats, water and soil in a variety of temperate ecosystems. As such, we have upscaled eDNA techniques to tackle complex ecological questions expanding the range of species interactions occurring within the ecosystem. In parallel, we have advanced on methodological aspects of eDNA detection to strengthen the use of eDNA for biomonitoring purposes and certify its benefits compared to traditional techniques. Altogether, we have advanced on the understanding of the dynamics of eDNA in natural ecosystems and shed light on the ambitious goal to use eDNA as a tool to assess ecosystem dynamics and management policies altogether. To summarise, this thesis is divided in five chapters:

Chapter 1: In this chapter, we study the effects of culling on the diet of a previously unregulated herbivore assembly using eDNA. The Oostvaardersplassen is an artificially created reserve in the Netherlands which hosts a dense population of herbivores. Their population reached carrying capacity in 2011 and since then, herbivore numbers have been oscillating increasingly, leading to peaks of mortality and a public campaign to actively regulate the population, which was resolved with the start of a culling programme in late 2018. We monitored the diet of the four main herbivore species since culling began by sampling their scats during four years. We study how population shifts of each herbivore species relate to their dietary choices and the associated niche overlap, within and between species. We then put in perspective eDNA methodology to assess the status of dense populations in temperate ecosystems and the suitability of the technique to monitor herbivore interactions.

Chapter 2: The Bialowieza forest in Poland is known for being one of the last intact primaeval forests in Europe. However, the forest is subdivided in different management regimes: some areas are under strict protection while others allow logging and hunting/culling. This has created an ecologically diverse setup as a consequence of the management differences within the forest. In this chapter, we compare the effect of management on the dietary interaction between the two most abundant ungulates of the forest, the European bison and the Red deer, across the Bialowieza forest using eDNA. We sampled their scats and studied how the niche overlap between these two species changes across the different management regimes within the forest, associated with habitat quality as a consequence of logging, and with predation risk due to the wolf populations naturally present.

Chapter 3: The soil ecosystem is a complex network of species interacting with the physicochemical properties, which are constantly modified through the addition of pesticides, fertilisers and herbicides. In vineyards, there is a growing interest to reduce the addition of such products in the soil in order to improve soil biodiversity and exploit the ecological services it can provide. However, little is known about the influence agricultural management and the products applied have on the soil community of the vineyards. In this chapter, we study the soil ecosystem from conventional and organic vineyards in Switzerland and the potential effect the different products applied have on the soil arthropod community. We reconstruct the arthropod

soil community using eDNA and test how the two management approaches drive the arthropod community. We identify which products are related to community differences and provide guidelines for improving biological quality of soils in vineyards.

Chapter 4: In this chapter, we study a pristine alpine river system at 2000m in Switzerland. We monitored the macroinvertebrate benthic community every two months during a whole year using eDNA and traditional kick-net sampling techniques. We compare the two methodologies and study the dynamics of the macroinvertebrate community along the year, putting these shifts in perspective of the physicochemical characteristics of each particular stream, to disentangle the role tributary rivers play in a glacier fed river system for the benthic macroinvertebrates. We assess both the changes of the community in the floodplain over the year and the role of the glacier and glacial lake in defining seasonal biotic patterns. We also put our results in perspective to regulated river systems, where dams regulate the flow of water, nutrients and sediments, to draw guidelines to improve water management to protect benthic macroinvertebrates and improve biomonitoring efforts.

Chapter 5: In remote desert areas where scattered water bodies attract terrestrial species, eDNA could answer the need for optimising biomonitoring efforts. In this chapter, we compare traditional camera trapping and eDNA metabarcoding methods in water bodies from two desert ecosystems, the Trans-Altai Gobi in Mongolia and the Kalahari in Botswana. We recorded the visiting patterns of wildlife using camera traps and studied the correlation with the biodiversity captured with the eDNA approach. We investigate how well waterborne eDNA captures signals of terrestrial and avian fauna in remote desert environments and compare the two approaches in such extreme environments while drawing recommendations for future eDNA-based biomonitoring.

Contribution to chapters

I contributed to the experimental design of chapter 1-2-3-4. I contributed to the fieldwork data collection for chapters 1-2-3-4. I was responsible for the DNA extraction, amplification, library preparation and sequencing for chapters 1-2-3-4. I contributed to the generation of the datasets for chapters 1-2-3-4-5. I performed sequencing and statistical analyses for all chapters. I contributed to the interpretation of the results for all chapters. I wrote the first draft of chapter 1-2-3-4-5.

Chapter 1 - Density-dependent resource partitioning of temperate large herbivore populations under rewilding

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Author contribution
EM-C, HO and LF designed the study and supervised all the analyses. PC conducted the plant survey and supervised herbivore countings. EM-C conducted the fieldwork, laboratory work, bioinformatics, data analyses and prepared the figures. EM-C wrote the first draft of the manuscript, with input from all other authors.

Abstract

In diverse tropical grazer assemblies with abundant large predators, smaller herbivores have been shown to be limited by predation and food quality, while the largest species are regulated by food abundance. Much less is known on large herbivore resource partitioning in temperate grazing ecosystems, where humans typically regulate their numbers, avoid multispecies assemblages, and restrict natural predators. In the unique 5000ha Oostvaardersplassen rewilding ecosystem in The Netherlands, a multispecies assemblage of cattle, horses, red deer and geese developed after initial introduction of a few individuals in 1983, without any human population regulation in the next 35 years. Starting from 2010, only animals that unlikely would survive the winter were culled from an animal welfare perspective, without interfering much in long-term population dynamics. Carrying capacity was reached around 2008, after which numbers started fluctuating depending on winter conditions. A population crash, especially of red deer, in late winter 2018 led to heavy societal debate around animal welfare, after which active population regulation by culling and translocation was introduced but with still relatively little impact on total population numbers in the next 3 years. During these first 35 years, this herbivore assemblage without predation regulation gradually changed into increasing dominance of the smaller herbivore species and increasingly strong episodic winter mortality (associated with density-dependent low body reserves) followed by rapid population recovery. This suggests strong niche overlap and competition between these very different-sized herbivores at assembly-level carrying capacity possibly due to their homogenising effect on vegetation composition and structure at high densities. We used eDNA metabarcoding of dung to quantify the diet composition of cattle, horse, red deer and geese, annually in early winter from 2018-2021 and calculated their niche overlap in relation to their population changes. Overall, we found strong diet overlap between them as expected from their long-term population dynamics. The diet of horse and cattle remained mostly unaltered and it was the one of red deer that changed the most across the years. Between the four years, niche overlap decreased with red deer population size, the most abundant species. This relationship was strongest in species interactions which included red deer. When calculated as total energy expenditure, we also found that our results in niche overlap are more linked to the shifts in red deer than to the total herbivore energy fluctuation. We suggest red deer changed their diet in response to their own population size, reducing their niche overlap with increasing red deer population. In this case, resource competition translated into shorter vegetation height, reducing resource availability and forcing herbivores to consume different plant taxon. We conclude that in this temperate ecosystem without strong population regulation by predators or humans, inter- and intraspecific resource competition are key factors structuring community composition and dynamics from small to large herbivores, with a competitive advantage of the smaller species, but with also several opportunities for resource partitioning.

Introduction

Terrestrial ecosystems with a dominant role of large herbivores are still widely found across the tropics as in savannahs, but become increasingly restricted to smaller, often fenced areas due to the growing human population and associated land use changes (Olff et al., 2002). In Europe in contrast, 'grazing ecosystems' are being increasingly enlarged through 'rewilding' initiatives, although often still incomplete with respect to top predators (Svenning et al., 2016). Management interventions in these ecosystems usually involve removal, reintroduction, culling or supplementary feeding, which potentially redefines their ecological role such as through diet selection, with potential cascading effects on other species groups (Pansu et al., 2019). Yet, the ecosystem-wide consequences of different management interventions in grazing ecosystems is still poorly understood. In large herbivore populations, management includes controlled stocking rates, culling, relocation or sterilisation that can change the behaviour of herbivore species, readjusting their feeding strategy, competitive and/or facilitative interactions (Danell, 2006). However, these adaptive changes in herbivore assemblages are still poorly documented and understood while also their cascading consequences for biodiversity are unclear.

Studying this role of herbivores and their interactions in ecological networks can be done in several ways. One of them is the study of the diet composition of each herbivore species, so the preference or avoidance of different plant taxon and the resulting diet overlap between different species. This provides insights on intra- and interspecific interactions beyond studying population dynamics, as it can inform on (density-dependent) competition versus resource partitioning (Kartzinel et al., 2015) or landscape usage (Abbas et al., 2011) among others. The Oostvaardersplassen ecosystem (OVP), the Netherlands, has become a benchmark for rewilding in northern Europe (Gordon et al., 2021; ICMO2, 2010; Jepson, 2016; Lorimer & Driessen, 2014) and has served as a model grazing ecosystem (Smit et al., 2015). The area, which is fully fenced, has limited resources and no top predators, making herbivore populations bottom-up regulated (Frank et al., 2018). As such, population growth is regulated by environmental conditions, resource availability and interspecific competition. Since the reintroduction of red deer in 1992, this species has experienced a sustained population growth compared to the other large herbivores at the OVP (i.e. horse and cattle, Figure 1A), which has steadily reduced the community weighted biomass (i.e., the mean weight of each herbivore; Fig 1D). This raised the concern of several conservation organisations (M. Buurmans, 2021; Theunissen, 2019), arguing the carrying capacity of the reserve had been reached and hence population number should be reduced by human intervention (Figure 1C, 1E). After two harsh winters (2016 and 2017), the area was severely exposed to outcries in the media on the increasing number of mortalities (especially horses, drawing public attention). Severe climatic conditions of the area, lack of sheltering landscape, resource limitation or herbivore competition were some of the hypotheses to explain such mortality. After increasing presence on the news, public debates and protests (discussed in (Theunissen, 2019)), a Dutch court approved the culling of approximately 80% of the red deer population (~4000 individuals) within the reserve in order to reduce their competitive pressure on horses. This provided a unique opportunity to study the effects population size has on the diet composition and niche

overlap as a result of culling but also to test the suitability of the competition hypothesis. The culling was initially planned to last for a few months, but due to the technical difficulties, the task was never achieved and culling on red deer kept on going at lower intensity until present time. In the meantime, part of the horse population was also culled or relocated. A maximum capacity of 1100 mammalian large herbivores was set for the reserve, and population size of the three major herbivore species was planned to be reduced accordingly, making birds the main focus of the conservation efforts (Begeleidingscommissie Beheer Oostvaardersplassen, 2018). This is still an ongoing task and the final numbers cannot be provided in this study (but see Figure 1A-C).
Due to the absence of predators in the OVP, trophic interactions are restricted to plant-herbivore interactions, i.e. diet quantification; and herbivore-herbivore interactions, i.e. niche overlap. Both metrics are based on the quantification of plant composition in each individual diet and in the environment. The OVP is a well-studied ecosystem and has a relatively low herbivore and plant diversity, which makes it technically easy to study. Sampling in such environments is not expensive nor time consuming, and the results can be compared to similar ecosystems across Europe.

In this project, we use environmental DNA (eDNA) metabarcoding to unravel density-dependent niche overlap between the four main vertebrate herbivore species in the Oostvaardersplassen rewilding area. Metabarcoding of plant DNA present in the herbivores' dung allows for the estimation of the relative contribution of each plant taxon to their diet. This method enables more precise taxonomic identification of plant taxa than previous microhistological techniques (Valentini et al., 2009), unlocking a new approach in community ecology to study diet composition, species competition and niche partitioning (Kowalczyk et al., 2011; Pegard et al., 2009; Rayé et al., 2011; Soininen et al., 2009). In the OVP, we collected dung of cattle, horses, red deer and geese. Dung was sampled in November 2018, before the culling, and three times after the culling had started (November 2019, 2020 and 2021) to follow-up potential associated changes in diet in response to both this management change and to inter-annual population fluctuations. Overall, the management intervention on the OVP was motivated by societal pressure on improving animal welfare by preventing food shortages. This led to the management change in 2018 from reactive culling (only individuals that would not survive the winter anyway) to proactive culling, aiming at a target maximum density. Such proactive herbivore culling can change species interactions and therefore potentially have a strong impact on biodiversity, productivity, nutrient cycling and soil health (Ripple & Beschta, 2012; Thoresen et al., 2021). We explored if improved food availability due to lower densities (due to culling or winter conditions) was associated with species-specific diet shifts, and hence changes in niche overlap. In ecosystems with a large role of big predators, smaller herbivores have been found to be more limited by predation than larger species (Sinclair et al., 2003), reducing their competition for food with large herbivores, where smaller species are more limited by food quality and larger species are limited by food quantity, linked to grass height (Hopcraft et al., 2010, 2012). In the OVP rewilding regime, without a strong role of large predators, previous studies found that increasing population sizes of all herbivore species lead to shorter grass but of higher quality (Cornelissen, 2017), leading to an expected competitive advantage of smaller herbivores and potential higher niche overlap between all species.

However, these smaller species have less body reserves, making them potentially more sensitive to winter mortality, leading to stronger population fluctuations close to carrying capacity than in larger species, potentially relaxing competitive interactions (Coulson et al., 2001). Moreover, the different digestive strategies within the present herbivores, i.e. ruminants (Heck cattle and Red deer) and non-ruminants (Konik horse and Geese), determine how plant material is fermented and energy extracted and could also drive such competitive interactions (Clauss et al., 2003). Different hypotheses are possible for how interspecific niche overlap of different-sized herbivores changes with densities of an assemblage with species of different body size close to carrying capacity. On the one hand, increasing density may increase competition for preferred plants. Smaller species may then be superior competitors (e.g. by being able to graze preferred plants the shortest) which forces larger species to select other resources. This may indicate that smaller species are more limited by food quality and large species by food quantity. In that case higher total densities, especially of smaller species, are expected to reduce niche overlap. On the other hand, high population densities of all herbivores can reduce vegetation heterogeneity, and change heterogeneous vegetation to homogeneous, high quality sward that is beneficial of all species (facilitation effects). In that case, increasing density is expected to promote resource overlap. The net consequences of density variation for resource partitioning within a large herbivore assemblage close to carrying capacity is therefore yet unclear and the subject of investigation in this study.

Materials and Methods

Study area

The Oostvaardersplassen is a nature reserve in the centre of the Netherlands (56 km^2) (Suppl. Figure 1A). The area is situated at 4m below the water level of the surrounding Lake IJsselmeer, and emerged in 1968 as part of Zuidelijk Flevoland, a large land reclamation polder of 430 km2. The Oostvaardersplassen part of the polder was initially designated for industrial and agricultural purposes but became rapidly recognized as a key important breeding location for birds that were rare or absent at that moment in the Netherlands, such as greylag goose, spoonbill and bearded reedling (Smit et al., 2015). In fact, greylag geese started playing a key role in keeping the young developing reed marsh open, promoting the diversity of other wetland birds. In 1973-75, agricultural and industrial development was put on hold and nature-oriented water management was started to preserve and stimulate the marshes ecological value. Also neighbouring grasslands that were initially drained and developed for agriculture were added to the protected areas to support moulting greylag geese that played such a key role in the marsh part of the ecosystem. This led to an ecosystem configuration of the Oostvaardersplassen of approximately half marshes dominated by *Phragmites australis* and half grasslands. To keep these grasslands open and suitable for the geese, large herbivores were introduced 8 years later under a free-ranging management without interventions in their population development. In 1983, 32 Heck cattle were introduced, followed by 20 Konik horses in 1984 and 44 Red deer in 1992. By May 2017, these populations had grown to 180 cattle, 864 horses and 2650 red deer. These large herbivores are restricted to stay in the boundaries of the protected area within a fence and mostly use the grasslands, although some of the red deer also spend time in the

marshes. In addition, the area saw a growing visitation by greylag geese, breeding, foraging and moulting in the marsh part, while also foraging in the grassland part; and barnacle geese, which visit the grasslands for foraging. There is some predation of red fox on juvenile greylag geese, but without a large role in population regulation, while red deer, horses and cattle are not subject to predation. As for top predators, after an absence of 120 years, wolves returned to the Netherlands in 2018 but due to landscape fragmentation and lack of corridors this species has not reached the Oostvaardersplassen yet.

Sample collection

Dung samples were collected across the grassland part of the OVP, with collections divided in three blocks (see Suppl. Figure 1A) during November 2018, 2019, 2020 and 2021 for the four main herbivore species, i.e., cattle, horse, red deer and geese (Barnacle geese and Greylag geese combined as the species were not identified from the dung shape). Per species and year, 15 scat samples were collected (5 per block, Suppl. Figure 1A), leading to a total of 60 scat per year. Samples were spaced by at least 10 m to reduce the chance of re-sampling the same individual, and GPS coordinates were taken for each sample. Only freshly deposited dung samples were collected, and samples were then stored in dried silica beads at room temperature, in order to dry and preserve them, without need for freezing, until DNA extraction could be done at the University of Lausanne, Switzerland.

DNA extraction

We used between 0.5 and 1 g of dry dung as the starting point for the extraction. Extractions were performed using the NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany) following the manufacturer protocol. A subset of the extractions was tested for inhibitors with quantitative real-time PCR (qPCR) applying different dilutions (2x, 10x and 50x) in triplicates. qPCR reagents and conditions were the same as in DNA metabarcoding PCR reactions (see below), with the addition of 10,000-fold diluted SybrGreen (Thermo Fisher Scientific, USA). Following these analyses, all samples were diluted 5-fold before PCR amplification. All extractions were performed in a laboratory restricted to low DNA-content analyses.

DNA metabarcoding

DNA extracts were amplified using a generalist plant primer pair (Sper01, (Taberlet et al., 2018)), targeting all vascular plant taxon (Spermatophyta). Sper01 targets a 10-220 bp gene fragment of the P6 loop of trnL intron, chloroplast DNA. To assign the DNA sequences to each sample, primers were tagged with eight variable nucleotides added to their 5'-end with at least five differences between tags. The PCR reactions were performed in a final volume of 20 µL. The mixture contained 1 U AmpliTaq® Gold 360 mix (Thermo Fisher Scientific, USA), 0.04 µg of bovine serum albumin (Roche Diagnostics, Basel, Switzerland), 0.2 µM of tagged forward and reverse primers and 2 µL of 5-fold diluted template DNA. PCR cycling conditions were denaturation for 10 minutes at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 52 °C and 1 min at 72 °C, with a final elongation step of 7 min at 72 °C. Amplifications were performed separately for each species and in replicates (4 per sample) in PCR plates each containing 60 DNA extracts, 12 blanks as well as 8 extraction, 8 negative and 8 positive PCR controls (DNA assembly of 10 plant species with increasing relative concentrations). The use

of blanks allows estimating the proportion of tag switches (i.e., false combination of tags, generating chimeric sequences) during library preparation (Schnell et al., 2015). Amplification success and fragment sizes were confirmed on agarose gel. PCR products were subsequently pooled per PCR plate. Amplicons were purified using a MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Purified pools were quantified using a Qubit® 2.0 Fluorometer (Life Technology Corporation, USA). Library preparation was done following the TagSteady Protocol (Carøe & Bohmann, 2020). After adapter ligation, libraries were validated on a fragment analyzer (Advanced Analytical Technologies, USA). Final libraries were quantified, normalised and pooled before 150 paired-end sequencing on an Illumina MiniSeq sequencing system with a Mid Output Kit (Illumina, San Diego, CA, USA).

Bioinformatic data analyses

The bioinformatic processing of the raw sequence output was performed using the OBITools package (Boyer et al., 2016). Initially, forward and reverse reads were assembled with a minimum quality score of 40. The joined sequences were assigned to samples based on unique tags combinations. Assigned sequences were then de-replicated, retaining only unique sequences. All sequences with less than 100 reads per library were discarded as well as those not fitting the range of metabarcode lengths. This was followed by two different clustering methods. First, pairwise dissimilarities between reads were computed and lesser abundant sequences with single nucleotide dissimilarity were clustered into the most abundant ones. Second, we used the Sumaclust algorithm (Mercier C, 2013) to further refine the resulting clusters based on a sequence similarity of 97 %. It uses the same clustering algorithm as UCLUST (Prasad, D.V., 2015) and it is mainly used to identify erroneous sequences produced during amplification and sequencing, derived from its main (centroid) sequence. Remaining sequences were assigned to taxa using a reference database. We built a database for Sper01 by running an *in silico* PCR based on all the plant sequences available in the EMBL database (European Molecular Biology Laboratory). We kept a single sequence per taxonomic id that was annotated at least to genus level.

Further data cleaning and filtering was done in R (version 4.0.2) using the metabaR package (Zinger et al., 2021). Sequences that were more abundant in extraction and PCR controls than in samples were considered as contamination and removed. Operational taxonomic units (OTUs) with similarity to the reference sequence lower than 97 % were also eliminated from the dataset. Removal of tag-leaked sequences was done independently for each library. This approach allowed us to discard single OTUs instead of whole PCR replicates. However, PCR replicates with too small reads count were also discarded. Remaining PCR replicates were merged by individual, keeping the mean relative read abundance (RRA), frequency of occurrence (FOO) and presence-absence. For studying plant selectivity, we used the relative read abundance (RRA) instead of using frequency of occurrence (FOO) or presence/absence because we were interested in the relative consumption of each plant taxon rather than which species are consumed.

Plant surveys

The plant composition in the Oostvaardersplassen was determined using Braun-Blanquet abundance categories (Braun-Blanquet, J., 1932). This classification method groups plant

taxon in 9 different categories based on coverage ranges. We measured plant coverage this way using 45 2x2 metre quadrants on the grassland. This vegetation survey was conducted in August 2018, before the sampling began (Suppl. Table 1). Braun-Balnquet categories were transformed to relative abundance keeping the median value of the range of abundance for each category. We used the same plant composition of the Oostvaardersplassen for the four sampled years because the plant composition of the grassland remained stable (Cornelissen, 2017).

Herbivore census

Herbivore populations have been counted on a yearly basis since 1983 (Suppl. Table 2). Populations were estimated by the management authority rangers through whole-area ground counts from vehicles, complemented by aerial counts since 2011. Herbivores were counted each October and May and separated into adults and juveniles to estimate the winter mortality and reproductive rates for each species. Geese populations (both barnacle and greylag) are calculated as the average number of wintering geese per month, counted from december to april.

Herbivore biomass and energy expenditure

To be able to compare species with different body mass, herbivore counts were transformed to herbivore biomass and daily energy expenditure (DEE). Geese values used for the total energy expenditure correspond to the October counts. As individual average body mass of each herbivore we used: heck cattle (420kg), konik horse (375kg), red deer (120kg), greylag geese (3.3kg) and barnacle geese (1.9kg). To calculate their population-level energy expenditure, we use the allometric relation between individual body mass and metabolic rate. For this we used *Equation 1* for large herbivores (Demment & Van Soest, 1985) and *Equation 2* for geese (Mooij, J.H., 1992):

$$DEE = 140 * (\text{live body weight})^{0.75} \qquad \text{Equation 1}$$

$$DEE = 2.55 * 417 * (\text{live body weight}^{0.71}) / 2 \qquad \text{Equation 2}$$

Calculations for geese were done for barnacle and greylag geese independently. For the two geese species, the result was divided by two because they were estimated to spend approximately half their time foraging in the Oostvaardersplassen on an annual basis, compared to the large grazers that are there every day.

Statistical analysis

All downstream analyses were carried out using R software (Version 4.0.2). Firstly, we calculated the dissimilarity matrix (Bray-Curtis distance) for each individual dung sample based on the final OTU table (for RRA, FOO and presence-absence) and visualised the variation in OTU composition between individual dung samples using non-metrical dissimilarity scaling (NMDS).

Then, we calculated the selectivity of the different herbivore species for each plant taxon by comparing the selected diets to the available diet (i.e. plant relative abundances in the field). We used Jacobs' *D* index (Jacobs, 1974) to measure plant selectivity, as follows:

$$D = \frac{r - p}{r + p - 2rp} \qquad \textit{Equation 3}$$

r indicates the relative abundance of a plant taxon in the diet of an individual and p indicates the relative abundance of that same plant taxon in the environment. *D* values range from -1 to 1. Negative values indicate avoidance and positive values indicate selection. Values close to 0 indicate similar utilisation to availability. We calculated Jacobs' *D* index and categorised each plant taxon as selected or not using only the RRA dataset, this is the case for all selectivity related calculations. When visualising the Jacobs' *D* selectivity index, we also calculated the number of individuals for each year (up to 15) that ate each plant taxon. This indicates the spread of consumption of a plant taxon within each species. We also visualised the overall selectivity by multiplying the relative abundance of each plant taxon present in the OVP with the sum of RRA obtained per herbivore species per year. This value served as a proxy to visualise the relative surface of the OVP being exploited by each herbivore species, and the differences across years.

To obtain a general view on the selectivity of each species, we performed a linear model (Model 1) on the RRA dataset using the relative abundance of plant species as explanatory variable, as follows:

RRA ~ Herbivore/year/plant abundance *Model 1*

We used the estimates for each level (i.e. one estimate per herbivore species and year) to investigate the link with the herbivore population assembly in terms of total energy expenditure.

Then, we calculated Pianka's niche overlap index (Equation 4, (Pianka, 1973)) using the *spaa* package to investigate niche overlap within and between species as follows:

$$Pianka_{\square\square} = \frac{\Sigma_i^n p_{ij} p_{ik}}{\sqrt{\Sigma_i^n p_{ij}^2 \Sigma_i^n p_{ik}^2}} \qquad \textit{Equation 4}$$

where p_{ij} and p_{ik} are the proportion of plant OTU *i* by individual *j* and *k*, respectively, and *n* is the total number of plant OTU categories. Values close to 0 indicate no overlap, close to 1 indicates full overlap, i.e. same diets. All niche analyses were carried using the FOO dataset.

Finally, we used generalised linear mixed models (GLMM), with the *glmmTMB* package, to investigate the significance of niche partitioning for each species interactions across the sampled years. Data distribution was assessed using the *performance* package. We first explored the observed niche overlap for the ten types of species interactions (i.e., all combinations of cattle, red deer, horse and geese) for each different year. We used the *Year* variable instead of the separate counts of herbivores or their energy expenditure because *Year* condenses the fluctuations in herbivore population as a categorical variable. The model was built using a beta distribution and with the *glmmTMB* R package, as follows:

Niche overlap ~ Species interaction / Year *Model 2*

We also modelled niche overlap between species against herbivore energy expenditure (for all species together and separately) in order to scale the relative consumption of resources by each species:

Niche overlap ~ Species interaction / Total energy consumption + (1|year) *Model 3*

We further compared the Akaike information criterion (AIC), i.e. relative quality of statistical models for a given set of data, of each energy expenditure model (following the structure of *Model 3*) for all herbivores grouped (Total) and for each herbivore species individually.

Results

Herbivore population dynamics

We visualised herbivore population dynamics in Figure 1A-D. They show ungulate populations in May (after winter mortality, before recruitment as the best stable estimate of long-term population dynamics), but the analysis of the relations between density and diet were done using herbivore population sizes estimated in October (Suppl. Figure 1B), as they are more relevant for our diet analysis based on dung collected each November. Geese populations displayed in Figure 1 correspond to the wintering average. The long-term population trends show a continuous increase of red deer population (Figure 1A) until the reserve reached its carrying capacity (around 2011, Figure 1A, E). After this point, the total herbivore population started to fluctuate around this carrying capacity, but without a clear sign of decline after 2018, despite the change in management in that year

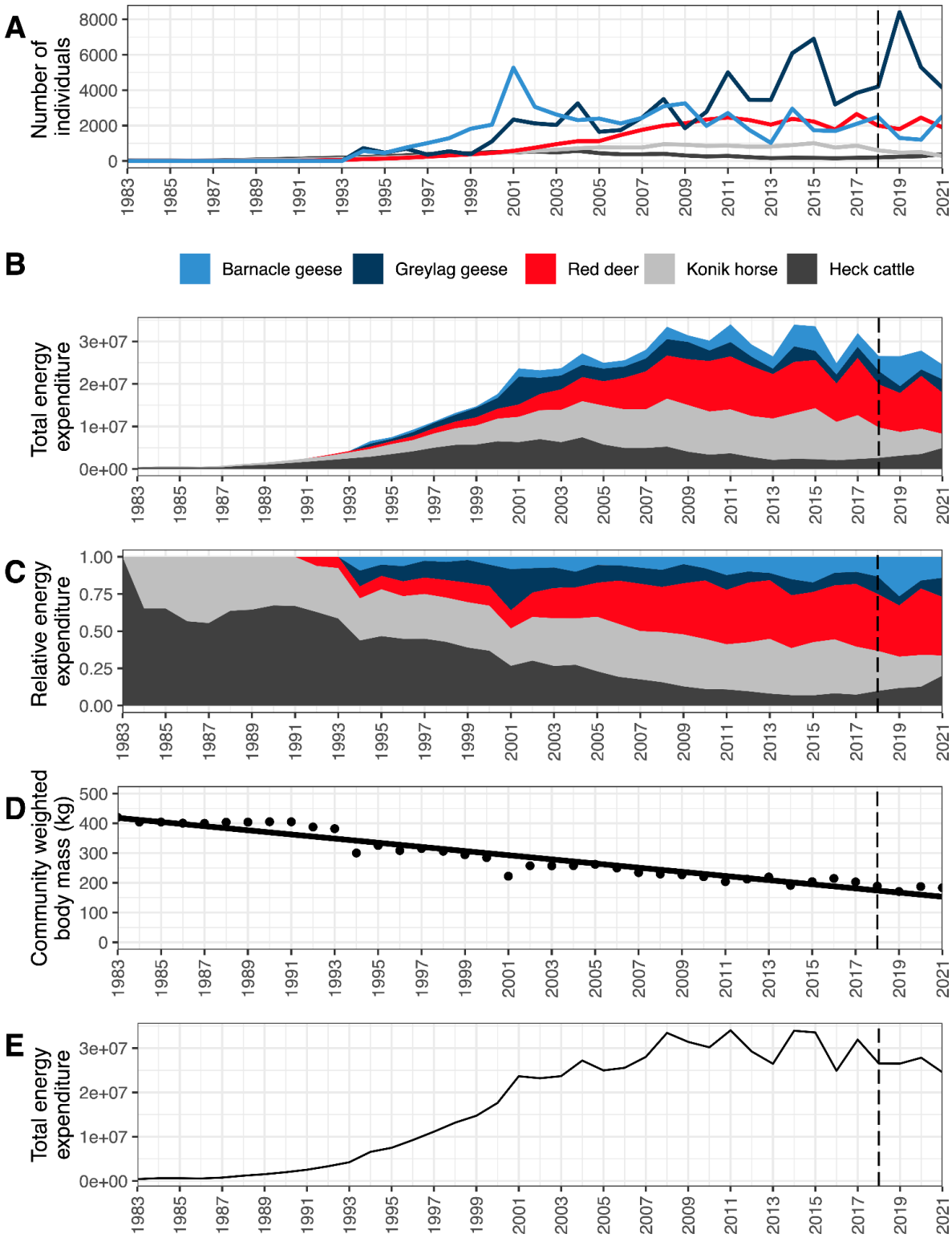

Figure 1. A) Land herbivore population size since 1983. B) Total energy expenditure expressed as kcal per day. See Materials and methods for how energy expenditure per species is calculated. C) Relative energy expenditure, calculated as the percentage of the total energy expenditure shown in B). D) Average body weight of one herbivore per year in the reserve. Notice the continuous decrease towards smaller herbivores throughout the years ($R^2 = 0.92$, p-value<0.001). E) Total energy expenditure combining all the species. Maximum was reached

in 2008 and the energy has been fluctuating around that maximum since then. Raw data available in Suppl. Table 2.

Diet composition in relation to vegetation dominance

The grasslands of the Oostvaardersplassen were dominated by the graminoids *Agrostis stolonifera* and *Lolium perenne*, and the forbs *Cirsium arvense* and *Plantago lanceolata* (Figure 2A). The diet analyses showed substantial deviations from this, showing clear selection by the herbivores for a wider range of plant taxon.

After quality filtering, we kept 3,472,674 reads of 284 different OTUs (Operational Taxonomic Units) for the Sper01 assay that were assigned to 98 different plant taxa. We visualised the diet by plant genus for each species and year using the FOO data (Figure 2B, Suppl. Figure 2 using RRA). The dominant group was the *Poales* order (graminoids) for all herbivores, with *Agrostis stolonifera, Dactylis glomerata* and *Lolium perenne* as dominant species. This was expected given the Oostvaardersplassen grasslands and marshes are mainly covered by graminoids. The other plant orders are clearly less represented in all species, but we observe Asterales and Lamiales plant taxon are more present in red deer and cattle than in horse. We also identified *Trifolium* sp. (Fabales) and *Plantago lanceolata* (Lamiales), as an important diet component which are also relatively abundant in the study (see Figure 2A and Suppl. Table 1 for the relative abundance of each plant taxon in the OVP).

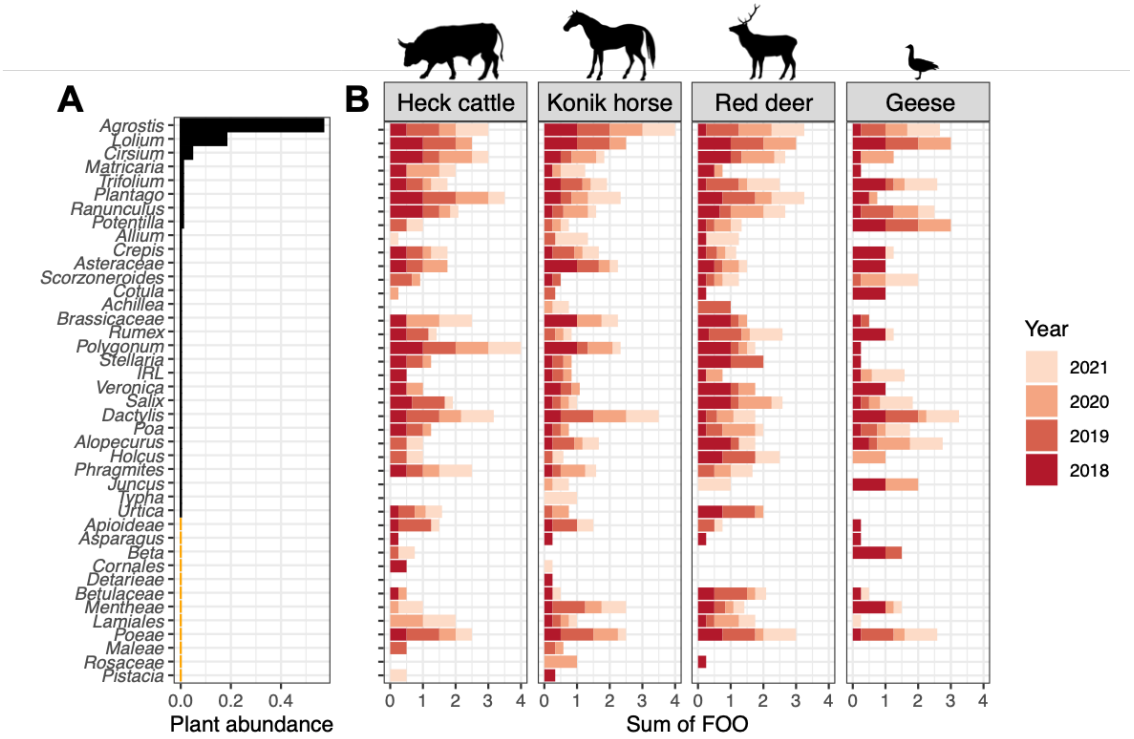

Figure 2. A) Relative abundance in the OVP per plant taxon, and in the diet of the four herbivore species. Abundances shown in orange correspond to plant sequences which were not assigned to an existing species found in the Oostvaardersplassen. IRL stands for a subgroup of the *Fabaceae* family. B) Sum of FOO for each species and year. (See Suppl. Figure 2 for the Sum of RRA for each species and year).

Herbivore diet composition

We computed a non-metric multidimensional scaling (NMDS) to visualise the diet composition differences between the four species and years using a Bray-Curtis distance matrix using the frequency of occurrence (FOO) dataset (Figure 3A, Suppl. Figure 3 for a comparison between RRA, FOO and p/a). The NMDS was calculated including all species and years together to compare across species. Horse diets clustered more than the other species across the sampled years. Cattle and red deer diets mostly overlapped in the multivariate space, while geese clustered the furthest from the other herbivores (Figure 3A). Horse multivariate space was in any case fully within the space of red deer and/or cattle, suggesting strong niche overlap. But also, substantial year-to-year differences were found in diet between herbivores, and with an interaction between year and species. When visualising the dissimilarity in diets within species, we calculated the NMDS for each species separately. We observed that the diet of cattle and red deer clearly overlap and have a similar range of variance. Horse diet is less diverse and the one of geese shows the greatest dissimilarity (Figure 3A). By species, we observed in 2020 the diets for each species were the most dissimilar, except for geese (Figure 3B-E).

Plant selectivity

We further quantified the selectivity of each herbivore species by comparing their diet composition to the relative abundance of each plant taxon using Jacob's D index (Equation 3, Figure 4A). In general, the tendency of herbivores to avoid more common species was stronger than the tendency to select particular species, showing that the dominant species are not the most preferred ones. Overall, 10 plant taxa resulted in positive selection by cattle, 10 by deer and only 3 by horse. The most preferred plant taxa were not the same across herbivores. The most abundant plant taxon in the grassland, *Agrostis stolonifera*, was not preferred by any of the species. Among asterales species, *Cirsium sp.* (likely *C. arvense*) was very abundant and was only selected by cattle in year 1. Interestingly, the forb *Plantago lanceolata*, which is also very abundant, was not selected by any herbivore species despite being found in their diets across the years.

We calculated an alternative approach to Jacob's D index by combining the RRA and the relative abundance of each plant species as a proxy for habitat exploitation. We calculated this value separately for each individual and then we grouped them together (Figure 4B) to assess the relative utilisation of the area according to the diet composition. We observed horses were the group with the highest mean value and red deer showed the greatest variability across years, increasing habitat exploitation with increasing red deer population size.

We further tested the overall trend of each herbivore species in terms of selectivity, by modelling the selectivity values visualised above against the abundance of each plant taxon detected. We built linear regressions for each species and year using the RRA dataset (Suppl. Figure 4). We considered the model estimates as an indicator of the feeding strategy in terms of selectivity. Slopes above 1 implied an overall selective feeding strategy of the species whether regressions below 1 indicated a generalist feeding strategy and above a selective one.

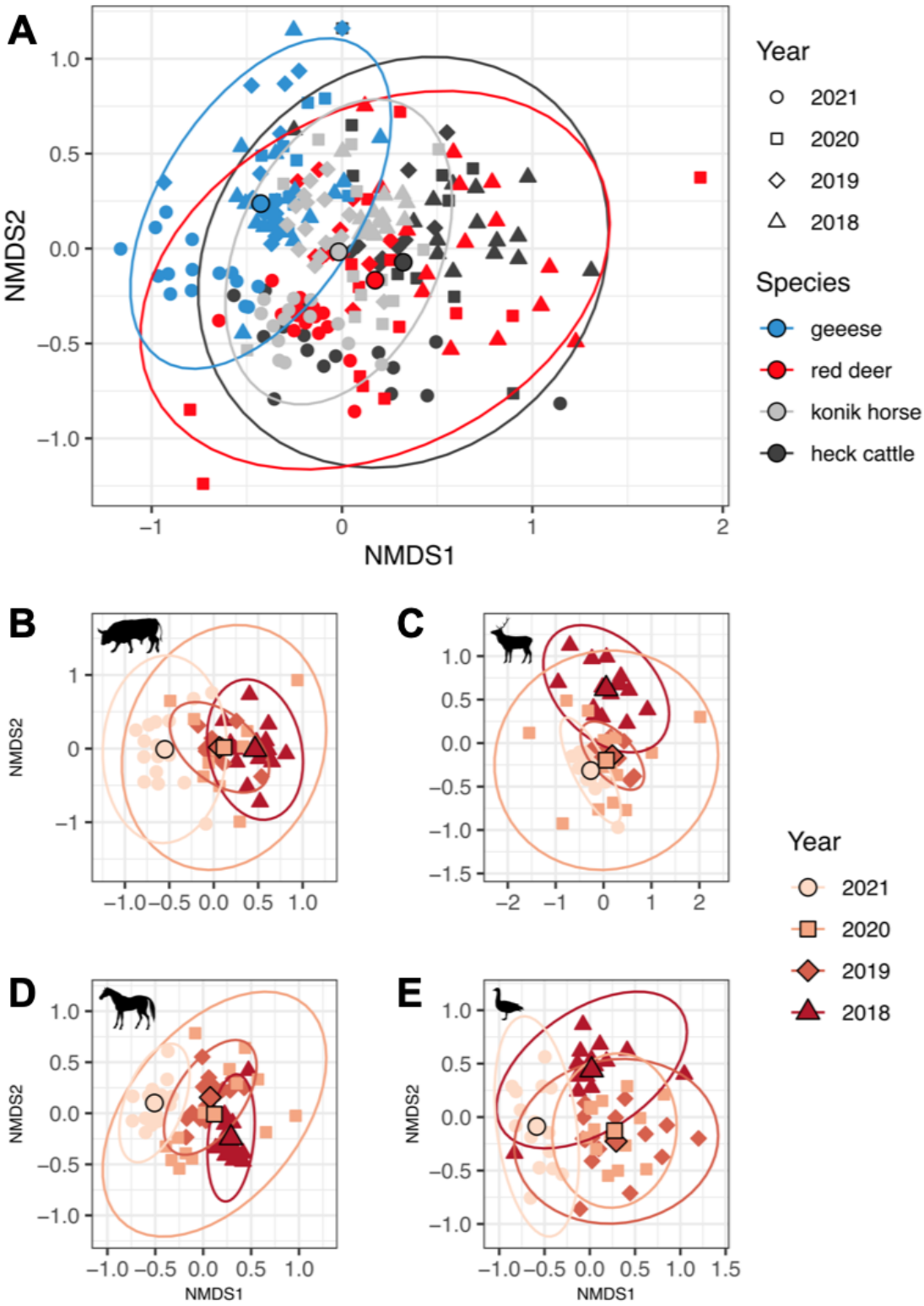

Figure 3. A. NMDS ordinations showing diet differences between species and years (A) and between years within a species (B-E). The NMDS calculation was done using the FOO data and including all the species together. B, C, D, E. NMDS visualisation for each species separately, to highlight the yearly differences within species. Points circled in black indicate the centroid for each group. Ellipses represent the 95% confidence interval assuming a normal distribution of the data.

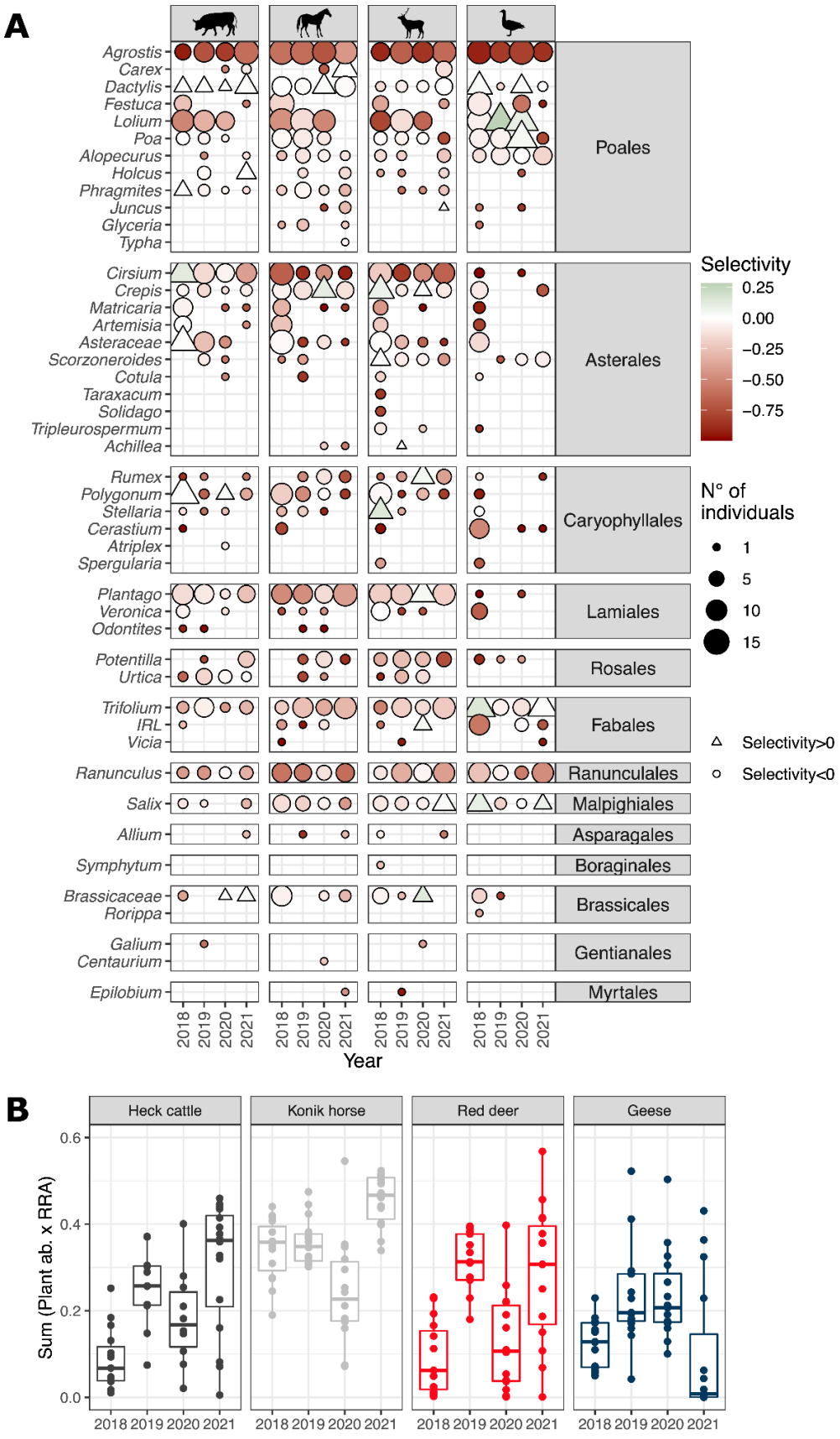

Figure 4. A) Selectivity for each herbivore species, year and plant taxon which are found in the reserve using the RRA data. Size of the dots correspond to the number of individuals (out

of a maximum of 15) where the plant taxon was detected in their diet. B) Alternative visualisation of the selectivity by each species and year. Represents the sum of the abundance of each plant species in the OVP times the RRA recorded per herbivore species and year. Geese were included but they have access outside the OVP. Dots indicate the value for each individual.

To put this information in perspective of the herbivore population shifts in the Oostvaardersplassen, we plotted the above-mentioned regression estimates (representing selectivity) against the total energy expenditure. However, we didn't find a clear correlation between the overall selectivity and red deer population (Figure 5). Only 4th year horses had an estimate above 1, associated with a selective grazing strategy, and also showed a significant correlation with the total energy expenditure. Nevertheless, we see a convergence of selectivity trends with increasing Total energy expenditure.

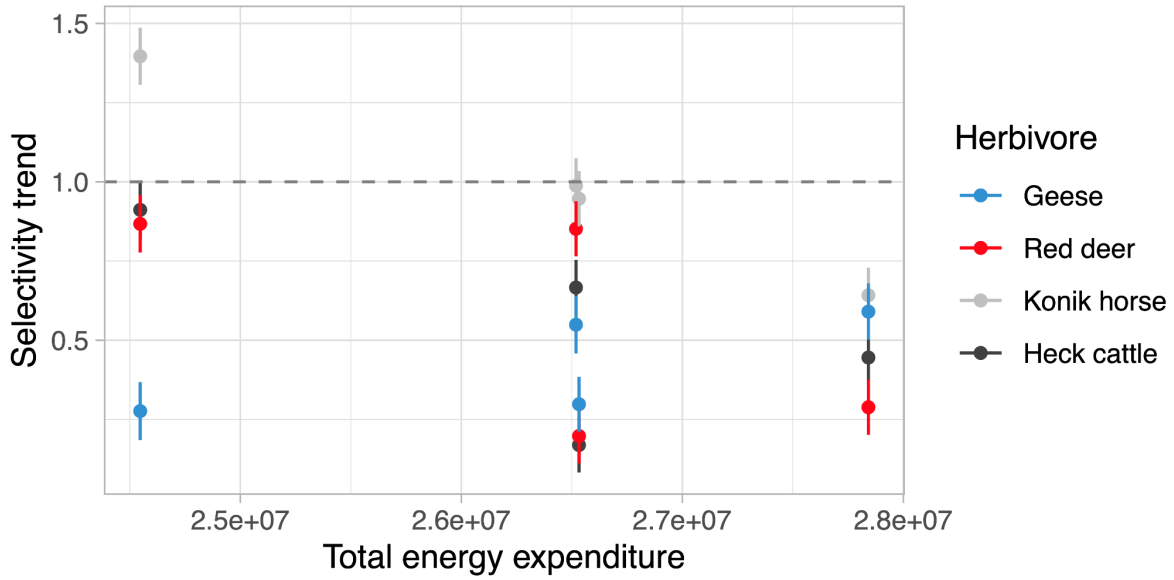

Figure 5. Selectivity trends against total energy expenditure (i.e. the slope of the regression line from Suppl. Figure 4) against the red deer population number using RRA. This figure is meant to show the overall lack of correlation in terms of Selectivity. Only horses showed a significant (negative) correlation.

Niche overlap

We then explored how the niche overlap between the four herbivores shifted between the herbivore species along the four years, and how this related to the herbivore population shifts and year-to-year fluctuations in the study area. We calculated the niche overlap between each individual sample in order to test for the dietary niche overlap between and within species (using FOO). Only comparisons between individuals sampled during the same year were kept, i.e. niche overlap comparisons across years were discarded. This model (Model 2) highlighted differences in predicted niche overlap between years for each combination of

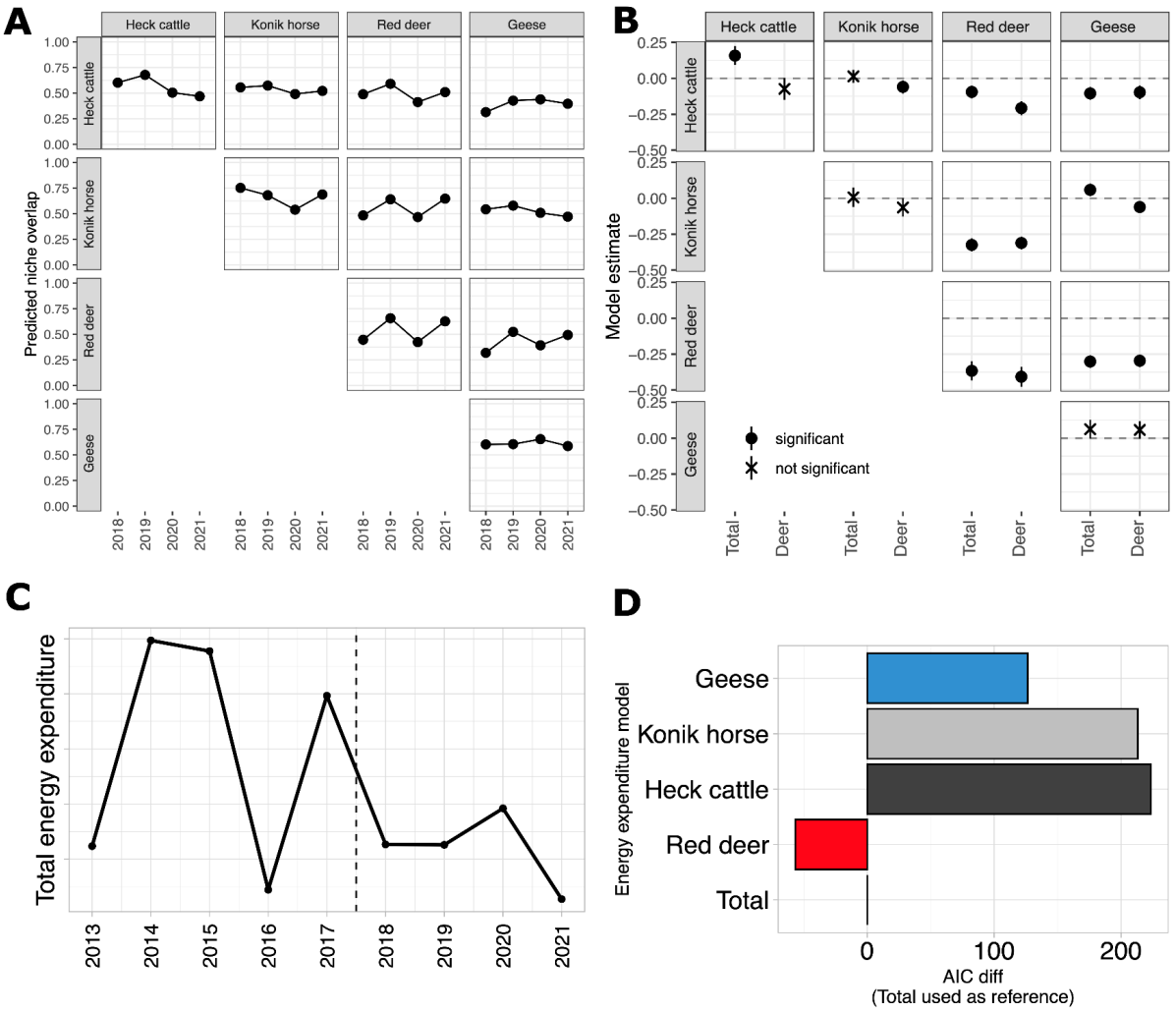

Figure 6. A) Predicted niche overlap for each year and species interaction, extracted from Model 2. B) Estimates of the regression line extracted from Model 3 for Total (all herbivore species together) and Red deer, as those were the best performing models. C) Detailed view of the total energy expenditure in the system during the last ten years (see Figure 1E for the total energy expenditure since 1983). D) AIC difference between models of niche overlap against energy expenditure. We use the model which accounts for the total herbivore energy expenditure as the zero reference. Notice that only the red deer model outperforms the "Total" model.

herbivore species (Figure 6A). We observe higher predicted niche overlaps in years 2 and 4, which aligns to the peaks of red deer population (Suppl. Figure 1B). Interestingly, this correlation becomes clear when red deer is one of the two species interacting. To assess it, we run linear correlations on each of the predicted niche overlaps from the aforementioned model predictors (Model 2) against the red deer numbers (Suppl. Figure 5A). Only red deer intraspecific niche overlap was significantly correlated to red deer population size. We also observed a marginal, non-significant correlation both between red deer and horse and between red deer and cattle. However, this approach is not capturing the fluctuations in the system as it only accounts for Red deer numbers. To overcome this, we modelled the observed niche overlap against the total energy expenditure of herbivores in the system (i.e. population level

energy expenditure, see Figure 6C for a detailed view of the energy expenditure in the system during the last ten years) but also against each herbivores' population energy expenditure and extracted the estimates from each. We visualised the estimates of each model in Figure 6B (see Suppl. Table 3 and Suppl. Figure 5B for all species comparisons). We extracted and visualised the AIC values of each *Model 3* in Figure 6D to compare their performance depending on the species' energy expenditure included in the response variable. This served to test the effect each species population shift had on the niche overlap calculated.
We observe a clear similarity between the estimates for the total herbivore energy expenditure and the red deer energy expenditure showing that the number of red deer influenced the diet selection of the other species. Both models ranked as the best in terms of AIC, far from the AIC value of the other models, which include only the energy expenditure of Heck cattle, Konik horse and Geese respectively. In particular, the estimates of Total and Red deer models (Figure 6B) are clearly negative for red deer related interactions, which aligns to the observed correlation of niche overlap being reduced with increasing red deer numbers (Suppl. Figure 5A). The results for geese-geese interactions are similar across models and non-significant in all cases.

Discussion

For the past two decades, the Oostvaardersplassen ecosystem has been a hotspot of strong debate on grazing management between scientists, policy makers and members of the public, including citizen action groups (Theunissen, 2019), especially around the question whether management should interfere in herbivore population numbers by proactive culling (before food limitation) to prevent density-dependent population regulation because of food availability or harsh winter conditions. In this discussion, the strength of competitive interactions through niche overlap between the different species always remained an open question. Nevertheless, in 2018 the management was changed from early reactive culling (mimicking food limitation regulation but without negative side effects of poor animal welfare) to proactive culling and supplementary feeding to prevent food limitation. We here explored the consequences of this for niche overlap. We found that the population numbers of the herbivore species did not substantially change from 2018-2021, despite the proactive culling, as red deer have a high reproductive rate and kept herbivore numbers at carrying capacity since 2008 (Figure 1D, 1E). The main shift from 2018-2021 was that cattle became relatively more important than horses (Figure 1C). But the whole herbivore community energy expenditure did not change much (Figure 1E), causing our study to shift focus towards resource partitioning of different-sized herbivores close to carrying capacity. Thus, this study focuses on investigating the functioning of an ecosystem at carrying capacity and how species coexist in relation to year-to-year fluctuations in abundance. We addressed this question through their diet comparing which plant taxon are consumed and in what frequency, i.e., their niche overlap, because diet is the evidence to understand long-term population dynamics.
Considering the herbivore population in terms of energy expenditure, the maximum was reached in 2011, at the same time when populations started to fluctuate from year to year in numbers (Figure 1A, 1E). Over time, the amplitude of the fluctuations seemed to increase. A

similar pattern occurs with the long-term feral population of sheep in St. Kilda island (UK), where the sheep population encounters no predators but limited resources. Their population oscillates around its carrying capacity (Crawley et al. 2021, Boyd et al. 1964). However, this system is composed of a single herbivore species. In the OVP we found that declines in one species were typically not compensated by an increase in another species suggesting strong niche overlap and shared constraints from winter conditions, with the exception of the relative increase in cattle since the start of active population regulation in 2018.
In ecosystems with larger predators, we expect smaller herbivores to be limited by predation and larger species by food quality and quantity. Without predation as in the Oostvaardersplassen, smaller herbivores are expected to outcompete larger ones due to higher population growth rates (faster recovery after strong winters) and their capacity to graze the grass too short for large species. The long-term population data of Oostvaardersplassen supports this expectation as the mean body size of the assembly consistently declines over time (Figure 1D).

Herbivore diet composition
The diet of the three ungulate species was overall similar as seen from the NMDS plots and the niche overlap calculations, suggesting overall strong resource overlap between these species of very different body mass. We used frequency of occurrence (FOO) instead of presence-absence or relative read abundance (RRA) because it informs on the plant taxa consumed but also on the spread of each taxon across the individuals of each species, providing a balanced ecological signal to study niche overlap from a dietary perspective (see Suppl. Fig 3 for a visual comparison of the three NMDS). It accounts for the different plant taxon consumed by herbivores and the digestion, extraction, amplification and sequencing drawbacks (Deagle et al., 2019). Given this data is calculated from FOO, we minimise the effect of the digestive differences between herbivores. When visualising the FOO of each plant taxon found in the OVP (Figure 2B) we found that the most abundant plant taxa (Figure 2A) are consumed by the four herbivore species in all the sampled years. However, they differ on the least abundant plant taxa, which align to the NMDS. Cattle and red deer (both ruminants) consume less abundant plants equally compared to horse and geese (both non-ruminants), which differ from the former two but do not group together. Our results suggest horses rely heavily on a few plant taxa, which are also eaten by red deer and cattle (full overlap in the NMDS, Figure 3A). The close distribution of individual horses in the NMDS (Figure 3A and Suppl. Figure 3) suggests horses have an homogeneous diet composition across individuals compared to red deer. This could indicate greater competition for horses to get resources but also lesser competition for cattle and especially red deer because of their greater choice of plant taxon. Also, it suggests that digestive strategy (Iason, G., & van Wieren, S. E., 1999) is more important in this system than body size to understand niche overlap. In the niche overlap part of the discussion we study in depth the effects of population dynamics on diet composition to also include between species interactions.

Plant selectivity
The classification of plant taxon as selected or avoided was based on the RRA data instead of the FOO data because the latter is not a good proxy to infer relative plant consumption.

Nevertheless, using the RRA implies that the results are sensitive to the herbivores' digestive biology, so only within-species comparisons are free of this bias. Differences in digestive tract morphology and functioning alters how plant material is broken down and impacts on the amount of plant DNA released during the DNA extraction.
Interestingly, the most abundant plant taxa in the grassland were not the most preferred ones by any herbivore (Figure 4A), despite being consumed by the majority of them. Herbivores tend to avoid most common taxa rather than selecting particular ones. This is a typical pattern for intensely grazed systems: the preferred plant species will decline in abundance due to consumption, making them rarer (Török et al., 2018). This tendency can lead to the point where they become more difficult to find, decreasing their preference by herbivores. This could explain why in the Oostvaardersplassen, grazing intensity has driven herbivores to accommodate their diet for the more common species, without really liking them. Examples are thistles (Asterales) and reed (*Phragmites* sp.), which are equally spread in the diet of all herbivores except geese, that fully avoids them because of their low palatability. Moreover, the selectivity trend observed for *Dactylis* sp. in cattle suggests the height of this graminoid makes it a preferred choice, despite its low abundance in the grassland. Overall, the consistency across years validates our methodology and provides a detailed view of the dietary choice of each herbivore species.

We also explored the potential of combining plant abundance and RRA as a proxy for the habitat use of each species, i.e. the relative area foraged (Figure 4B), to complement the above-mentioned selectivity approach. Red deer habitat use expanded when red deer numbers were greater (2018 and 2020), suggesting their feeding strategy changed to include a greater variety of plant species, which aligns to the niche overlap results (Figure 6A). We highlight *Alopecurus, Holcus and Juncus* sp. (Figure 4A), all from the Poales order as potential indicators for this shift on feeding strategy. Interestingly, we observed an increasing range of habitat use within red deer and cattle since culling began (Figure 4B), suggesting multiple feeding strategies coexist within species. Horses were the exception, which move in a single herd and thus have a similar diet composition across years between individuals. However, we are unable to disentangle if this feeding shift was due to a behavioural shift because of the culling disturbance or as a consequence of the red deer population reduction. This approach could be used to monitor habitat exploitation and is particularly suitable for within species comparisons or between herbivores species with similar digestive systems.

Niche overlap
When modelling niche overlap against energy expenditure (Figure 6B, *Model 3*), the energy expenditure by Red deer and by the Total assemblage were the best predictors compared to the other species (see Suppl. Figure 5B). Herbivore interactions involving geese were the least related to the population size conditions in the OVP because of their capacity to feed outside the reserve and serves as a control for our methodology. In fact, taking in account only the red deer energy expenditure results in a better model than using the total energy expenditure (Figure 6D). This result indicates the fluctuations in niche overlap are more linked to the shifts in red deer numbers than the overall herbivore population shifts, but the total energy expenditure is still a better predictor than cattle, horse or geese energy expenditure separately.

We detected negative estimates in all interactions involving red deer (Figure 6D, Suppl. Figure 5B), i.e. niche overlap decreases when total or red deer energy expenditure increases. We attribute this to the shifts within red deer niche overlap, which is probably more influenced by red deer population size shifts. The similarity between the two models is expected as Red deer account for the greatest biomass change in the OVP throughout the sampled years, but it reflects the relative impact of red deer population on the total energy expenditure and the ecosystem as a whole. These findings have an important ecological impact as it suggests that red deer responded to their population size increase in terms of diet, leading to more dissimilar diets and thus lesser niche overlap. They are the smallest of the three land herbivores and may then be superior competitors (e.g. by being able to graze preferred plants the shortest) forcing larger species to select other resources. This may indicate that smaller species are more limited by food quality and large species by food quantity. In that case higher total densities, especially of smaller species, are expected to reduce niche overlap, which aligns to what we have observed (Figure 6A, 6C).
We found niche overlap to increase together with the surface of the Oostvaardersplassen used by Red deer (Figure 4B). This indicates that despite the diet of Red deer becoming more similar, they used a greater feeding surface to reach their nutritional requirements. However, since the herbivore density remained high and stable along the sampled years (Figure 6C), we could be observing the result of facilitation, where red deer would have homogenised vegetation composition towards a higher quality one, benefiting other herbivores. In that case, increasing density is expected to promote resource overlap. Whether this is because of culling or because of herbivore density, could be used as a proxy for other ecosystems to assess herbivore population dynamics from a dietary perspective.

From an ecological perspective, these results suggest that the diet of horse and cattle remained mostly unaltered and it was the red deer diet the one that changed the most across the years. Red deer individuals being culled could disperse more and thus feed on a greater diversity of plant taxon, possibly beyond the dry grassland part and into the flooded part of the OVP. An alternative explanation could be that culling pressure led to a lower selectivity of plant taxon and thus their diet became more diverse as they put less effort in the selection. However, if that was the case, we should have observed a decrease in their selectivity index.

Conclusions
Our results indicate that culling of red deer led to more dissimilar diets in cattle and red deer. However, the total herbivore density did not change much (Figure 1C, 1E and Figure 6C). Thus, we may be observing a behavioural response, where red deer fled into the marsh after the culling began and complicated regulating their densities. After the harshness of 2016 and 2017 winters, the horse population was also reduced. This species was at the start of the public debate despite being reduced proportionally less than red deer (Figure 1A). However, our data suggest that their diet remained unaltered throughout the years and, at least in terms of diet variability, they remained the least unaffected by the red deer culling. In this line, culling of red deer had no clear effect on shifting the diet composition of the other species. Rather, it was the individual variation within red deer which responded the most to red deer numbers, with individuals having more dissimilar diets when red deer numbers were highest (Figure 6A). This

suggests the Oostvaardersplassen resources were not consumed mainly by red deer as the species exploited other resources when their population increased. They seem to be the most flexible ungulate in terms of diet but also the one which can feed on the greatest variety of plant taxa, which contributes to this plastic response observed. In the OVP, lack of predators and of migration has created a system highly beneficial for smaller herbivores, as they can graze the grass very short. They are the drivers of competition, if any, because their population has a high recovery rate compared to horses and cattle. Medium-sized herbivores, i.e. horses, are limited by food quality and could migrate to reduce the competition. Yet, deer-size species could also migrate as well, see e.g. historical mass migrations of springbok and kob (two antelope species) in Africa. In such a system, horse-like species, such as zebras, could not escape competition.

From a management perspective, the culling undergone in the Oostvaardersplassen was not motivated in order to restore a competitive balance between different-sized species or mimicking the effect of natural predation. Had that been the case, red deer culling would have been reinforced to further reduce their population size. That would have promoted horse and cattle growth and balanced the natural advantage of red deer in the absence of natural predators, which would have targeted this species and controlled their population growth. However, the intervention also affected horses, which were also translocated outside of the Oostvaardersplassen. Put together, the reasoning behind the overall intervention stems beyond ecological purposes. Rather, the decision was taken from an ethical perspective, in order to improve animal welfare in an incomplete ecosystem, shooting or translocating animals to prevent them from dying from malnutrition. Now, the Oostvaardersplassen has raised awareness that creating such an ecosystem comes with complex ecological challenges that will eventually have to be addressed, exposing the ethical contradictions of creating it in the first place, or what it has become.

Acknowledgements

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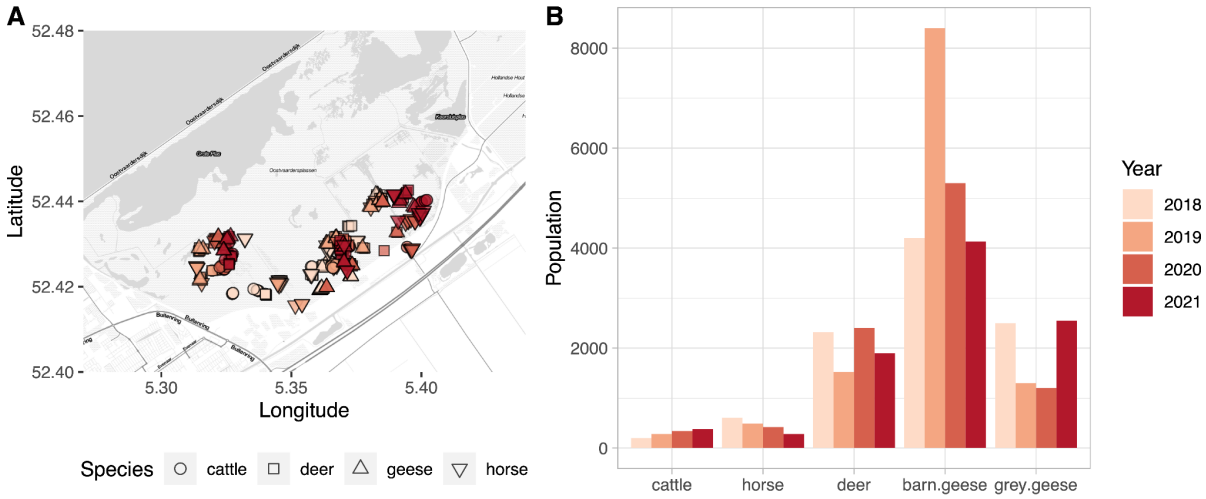
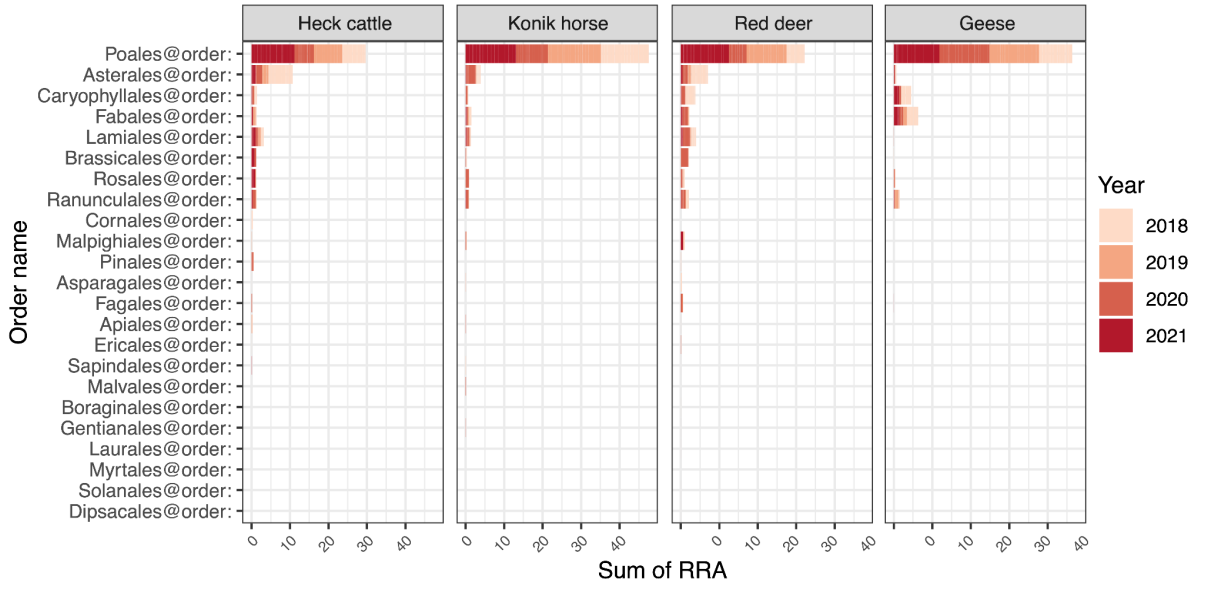
Conflicts of interest

The authors declare no conflicts of interest.

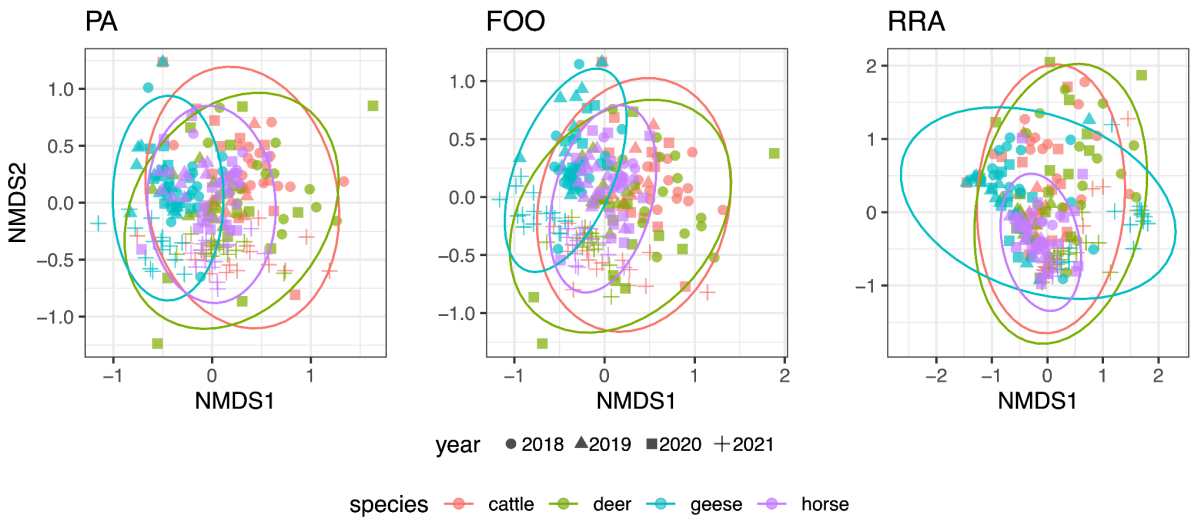
Supplementary material



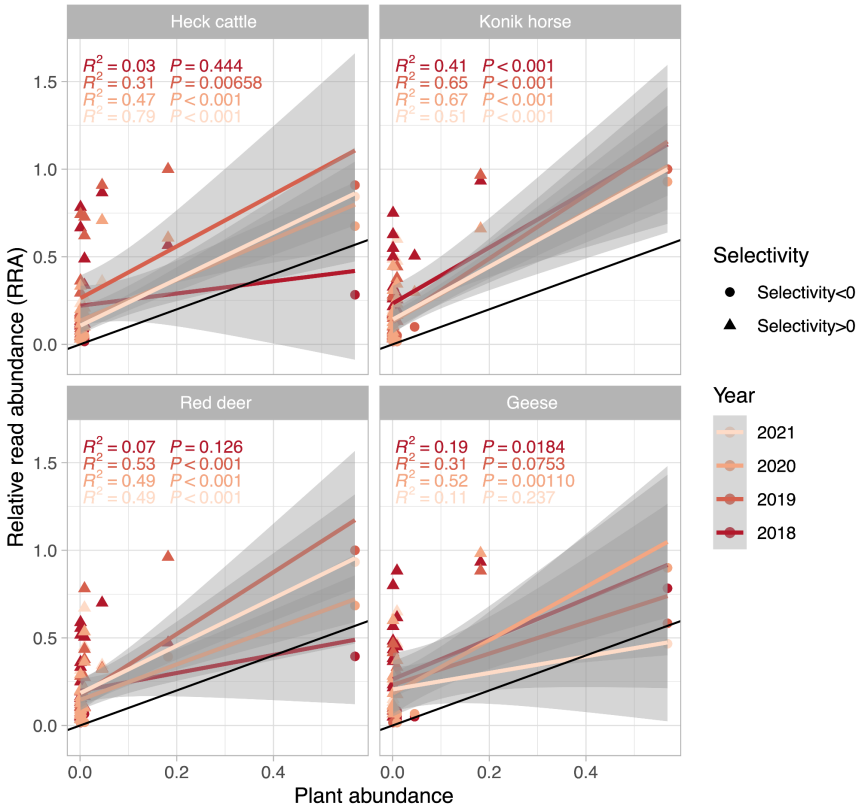
Supplementary Figure 1. A) Map of the study area. Points indicate the location where each scat sample was collected for each species and year of the grassland. B) Herbivore population numbers for each of the sampled years.



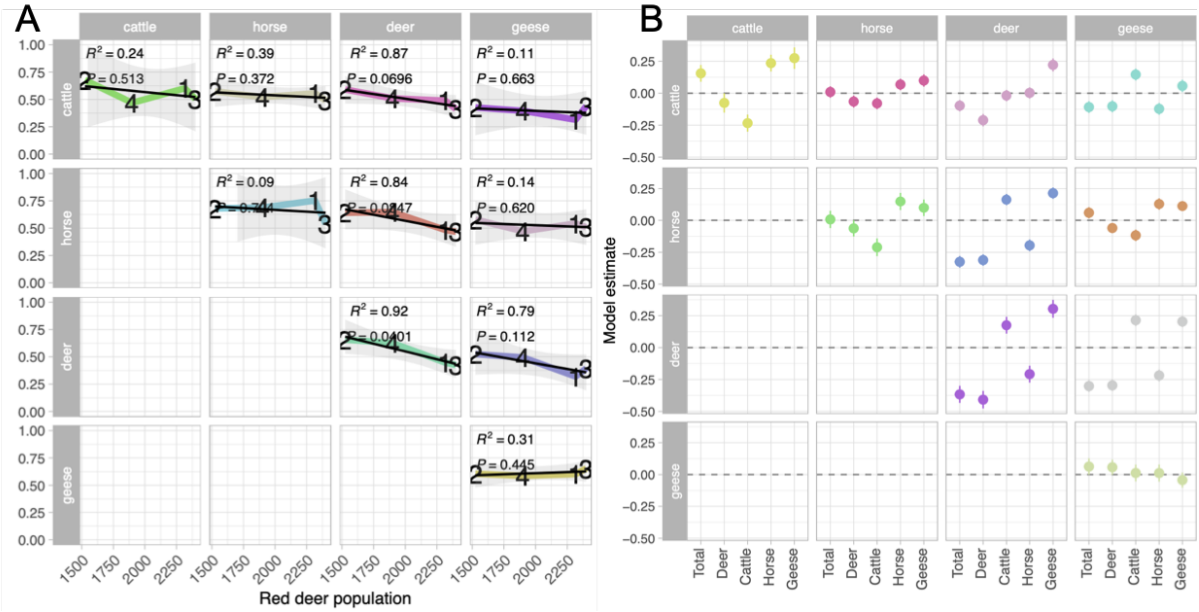
Supplementary Figure 2. Barplot of Sum of RRA for each plant order, herbivore species and year.



Supplementary Figure 3. NMDS comparing the three data transformation methods. PA stands for presence/absence. FOO for frequency of occurrence. RRA for relative read abundance.



Supplementary Figure 4. Selection regression lines for each herbivore species and year. Black line indicates the y=x line. Data points above this line indicate that the plant taxon was found in a higher proportion in the diet (in terms of sequences) than in the vegetation of the study area.



Supplementary Figure 5. A) Linear correlation between the predicted niche overlap from model 2 and red deer population. R2 and p-values are indicated for each species interaction. The bold numbers indicate the year of sampling, 1 being 2018 and 4, 2021. B) Estimates for all species models including total (Figure 6 includes only Total and Red deer). Dashed line indicates the 0.

Supplementary Table 1. Plant species abundance at the OVP. Genus name and Order name are also provided. Abundance is shown in percentage, extracted as mean from the Braun-Blanquet categories.

Plant species	Abundance	Genus	Order
Agrostis stolonifera	56.8698817	Agrostis	Poales
Lolium perenne	18.1983621	Lolium	Poales
Plantago lanceolata	4.54959054	Plantago	Lamiales
Cirsium arvense	4.54959054	Cirsium	Asterales
Potentilla anserina	0.90991811	Potentilla	Rosales
Ranunculus repens	0.90991811	Ranunculus	Ranunculales
Odontites vernus subsp. serotinus	0.90991811	Odontites	Lamiales
Plantago major subsp. major	0.90991811	Plantago	Lamiales
Trifolium dubium	0.90991811	Trifolium	Fabales
Trifolium repens	0.90991811	Trifolium	Fabales
Cerastium fontanum subsp. vulgare	0.90991811	Cerastium	Caryophyllales
Cirsium vulgare	0.90991811	Cirsium	Asterales
Jacobaea vulgaris	0.90991811	Jacobaea	Asterales
Matricaria chamomilla	0.90991811	Matricaria	Asterales
Convalvulus sepium	0.09099181	Convalvulus	Solanales
Urtica dioica	0.09099181	Urtica	Rosales
Ranunculus acris	0.09099181	Ranunculus	Ranunculales
Ranunculus sceleratus	0.09099181	Ranunculus	Ranunculales

Bromus hordeaceus	0.09099181	Bromus	Poales
Echinocloa crus-galli	0.09099181	Echinocloa	Poales
Alopecurus geniculatus	0.09099181	Alopecurus	Poales
Dactylis glomerata	0.09099181	Dactylis	Poales
Eleocharis palustris	0.09099181	Eleocharis	Poales
Elymus repens	0.09099181	Elymus	Poales
Festuca rubra	0.09099181	Festuca	Poales
Holcus lanatus	0.09099181	Holcus	Poales
Juncus articulatus	0.09099181	Juncus	Poales
Juncus bufonius	0.09099181	Juncus	Poales
Phleum pratense	0.09099181	Phleum	Poales
Phragmites australis	0.09099181	Phragmites	Poales
Poa annua	0.09099181	Poa	Poales
Poa pratensis subsp. pratensis	0.09099181	Poa	Poales
Scirpus sp.	0.09099181	Scirpus	Poales
Typha	0.09099181	Typha	Poales
Carex	0.09099181	Carex	Poales
Glyceria	0.09099181	Glyceria	Poales
Epilobium parviflorum	0.09099181	Epilobium	Myrtales
Epilobium tetragonum	0.09099181	Epilobium	Myrtales
Salix alba	0.09099181	Salix	Malpighiales
Ajuga reptans	0.09099181	Ajuga	Lamiales
Glechoma hederacea	0.09099181	Glechoma	Lamiales
Limosella aquatica	0.09099181	Limosella	Lamiales
Mentha aquatica	0.09099181	Mentha	Lamiales
Veronica arvensis	0.09099181	Veronica	Lamiales
Veronica catenata	0.09099181	Veronica	Lamiales
Veronica persica	0.09099181	Veronica	Lamiales
Lycopus europeus	0.09099181	Lycopus	Lamiales
Geranium dissectum	0.09099181	Geranium	Geraniales
Galium	0.09099181	Galium	Gentianales
Centaurium pulchellum	0.09099181	Centaurium	Gentianales
Vicia	0.09099181	Vicia	Fabales
IRL	0.09099181	IRL	Fabales
Trifolium fragiferum	0.09099181	Trifolium	Fabales
Trifolium pratense	0.09099181	Trifolium	Fabales
Equisetum arvense	0.09099181	Equisetum	Equisetales
Spergularia	0.09099181	Spergularia	Caryophyllales
Atriplex prostrata	0.09099181	Atriplex	Caryophyllales
Persicaria lapathifolia	0.09099181	Persicaria	Caryophyllales
Persicaria maculosa	0.09099181	Persicaria	Caryophyllales
Polygonum aviculare	0.09099181	Polygonum	Caryophyllales
Rumex conglomeratus	0.09099181	Rumex	Caryophyllales
Rumex crispus	0.09099181	Rumex	Caryophyllales
Rumex maritimus	0.09099181	Rumex	Caryophyllales
Rumex obtusifolius	0.09099181	Rumex	Caryophyllales

Stellaria aquatica	0.09099181	Stellaria	Caryophyllales
Stellaria media	0.09099181	Stellaria	Caryophyllales
Brassicaceae	0.09099181	Brassicaceae	Brassicales
Brassica nigra	0.09099181	Brassica	Brassicales
Capsella bursa-pastoris	0.09099181	Capsella	Brassicales
Rorippa palustris	0.09099181	Rorippa	Brassicales
Sisymbrium officinale	0.09099181	Sisymbrium	Brassicales
Symphytum	0.09099181	Symphytum	Boraginales
Solidago	0.09099181	Solidago	Asterales
Asteraceae	0.09099181	Asteraceae	Asterales
Achillea millefolium	0.09099181	Achillea	Asterales
Artemisia vulgaris	0.09099181	Artemisia	Asterales
Bellis perennis	0.09099181	Bellis	Asterales
Bidens cernua	0.09099181	Bidens	Asterales
Bidens tripartita	0.09099181	Bidens	Asterales
Carduus crispus	0.09099181	Carduus	Asterales
Cotula coronopifolia	0.09099181	Cotula	Asterales
Crepis capillaris	0.09099181	Crepis	Asterales
Erigeron canadensis	0.09099181	Erigeron	Asterales
Eupatorium cannabinum	0.09099181	Eupatorium	Asterales
Gnaphalium uliginosum	0.09099181	Gnaphalium	Asterales
Pulicaria dysenterica	0.09099181	Pulicaria	Asterales
Pulicaria vulgaris	0.09099181	Pulicaria	Asterales
Scorzoneroides autumnalis	0.09099181	Scorzoneroides	Asterales
Sonchus arvensis	0.09099181	Sonchus	Asterales
Sonchus asper	0.09099181	Sonchus	Asterales
Taraxacum officinale	0.09099181	Taraxacum	Asterales
Tephroseris palustris	0.09099181	Tephroseris	Asterales
Tripleurospermum maritimum	0.09099181	Tripleurospermum	Asterales
Tussilago farfara	0.09099181	Tussilago	Asterales
Allium	0.09099181	Allium	Asparagales
Daucus carota	0.09099181	Daucus	Apiales
Alisma lanceolatum	0.09099181	Alisma	Alismatales
Alisma plantago-aquatica	0.09099181	Alisma	Alismatales
Lemna minor	0.09099181	Lemna	Alismatales
Triglochin palustris	0.09099181	Triglochin	Alismatales

Supplementary Table 2. Herbivore counts at the OVP for each sampled year.

year	Heck cattle	Konik horse	Red deer	Barnacle geese	Greylag geese
1983	32				
1984	31	18			
1985	31	18			
1986	24	20			
1987	32	28			
1988	58	36			
1989	75	45			

1990	102	54			
1991	130	70			
1992	160	86	40		
1993	190	120	63		
1994	221	156	104	726	562
1995	269	198	133	471	455
1996	319	222	181	693	759
1997	385	281	242	357	1011
1998	434	329	307	546	1285
1999	442	377	378	380	1818
2000	500	448	457	1106	2049
2001	486	499	571	2337	5265
2002	539	572	750	2122	3057
2003	485	637	949	2035	2624
2004	573	712	1118	3253	2307
2005	442	768	1126	1647	2393
2006	379	763	1466	1745	2120
2007	379	763	1758	2450	2437
2008	404	947	1997	3495	3090
2009	311	921	2126	1854	3256
2010	258	852	2340	2778	1987
2011	283	867	2454	5003	2706
2012	216	808	2311	3453	1749
2013	163	818	2055	3449	1025
2014	183	899	2378	6086	2952
2015	177	1006	2229	6898	1735
2016	157	758	1778	3197	1692
2017	180	865	2650	3844	2096
2018	200	600	2000	4200	2500
2019	240	470	1800	8400	1300
2020	270	500	2450	5300	1200
2021	380	280	1900	4131	2548

Supplementary Table 3. Energy model estimates, std. error and p-values. Complementary information to Figure 6B.

	Species interaction	Estimate	Std. Error	P-value
TOTAL	cattle.cattle	0.155432	0.033367	3.19e-06 ***
	cattle.deer	-0.097167	0.022594	1.70e-05 ***
	cattle.horse	0.009801	0.022835	0.66776
	deer.deer	-0.366621	0.034057	< 2e-16 ***
	horse.deer	-0.324847	0.023407	< 2e-16 ***
	cattle.geese	-0.10817	0.023427	3.89e-06 ***
	deer.geese	-0.301708	0.023017	< 2e-16 ***
	geese.geese	0.062997	0.032814	0.05488 .
	horse.geese	0.058802	0.022442	0.00879 **
	horse.horse	0.006848	0.034447	0.84242
DEER	cattle.cattle	-0.07586	0.03857	0.04919 *
	cattle.deer	-0.21077	0.02481	< 2e-16 ***

	cattle.horse	-0.06495	0.02368	0.00609 **
	deer.deer	-0.40802	-0.03505	< 2e-16 ***
	horse.deer	-0.3112	-0.02278	< 2e-16 ***
	cattle.geese	-0.10197	0.02411	2.35e-05 ***
	deer.geese	-0.29609	-0.02237	< 2e-16 ***
	geese.geese	0.05749	0.03038	0.05846 .
	horse.geese	-0.06047	0.02057	0.00329 **
	horse.horse	-0.06302	0.03254	0.05277 .
CATTLE	cattle.cattle	-0.23395	0.03415	0 7.40e-12 ***
	cattle.deer	-0.01822	0.02281	8 0.424595
	cattle.horse	-0.07925	0.02317	0 0.000626 ***
	deer.deer	0.17512	0.03356	9 1.80e-07 ***
	horse.deer	0.16318	0.02319	6 1.98e-12 ***
	cattle.geesee	0.14638	0.02381	8 7.86e-10 ***
	deer.geese	0.21437	0.02336	7 < 2e-16 ***
	geese.geese	0.01253	0.03366	2 0.709672
	horse.geese	-0.1178	0.02299	4 2.98e-07 ***
	horse.horse	-0.21098	0.03527	2 2.21e-09 ***
HORSE	cattle.cattle	0.2355	0.033316	9 1.56e-12 ***
	cattle.deer	0.002672	0.022531	9 0.90558
	cattle.horse	0.068476	0.022968	1 0.00287 **
	deer.deer	-0.208456	0.033668	2 5.96e-10 ***
	horse.deer	-0.196143	0.023388	6 < 2e-16 ***
	cattle.geese	-0.121995	0.023576	4 2.29e-07 ***
	deer.geese	-0.218824	0.023313	6 < 2e-16 ***
	geese.geese	0.012	0.034058	2 0.72459
	horse.geese	0.128369	0.023225	7 3.25e-08 ***
	horse.horse	0.148323	0.034835	8 2.06e-05 ***
GEESE	cattle.cattle	0.27447	0.04287	1.53e-10 ***
	cattle.deer	0.22154	0.02605	< 2e-16 ***
	cattle.horse	0.09947	0.02438	4.51e-05 ***
	deer.deer	0.30318	0.03568	< 2e-16 ***
	horse.deer	0.21436	0.02279	< 2e-16 ***
	cattle.geese	0.05753	0.02469	0.01982 *
	deer.geese	0.20399	0.02223	< 2e-16 ***
	geese.geese	-0.04279	0.03028	0.15763
	horse.geese	0.11224	0.02065	5.49e-08 ***
	horse.horse	0.09872	0.03341	0.00313 **

Chapter 2 - Niche overlap between two large herbivores across landscape variability using dietary eDNA metabarcoding

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Chapter status: Submitted, under revision

Author contribution
EM-C, MC, DK and LF designed the study and supervised all analyses. EM-C and DK conducted the fieldwork. EM-C carried out the laboratory work, bioinformatics, data analyses and prepared the figures. EM-C wrote the paper, with input from all other authors.

Abstract

Understanding the relationship between a species feeding strategy and its environment (trophic ecology) is critical to assess environmental requirements and improve management policies. However, measuring trophic interactions remains challenging. Among the available methods, quantifying the plant composition of a species' diet indicates how species use their environment and their associated niche overlap. Nevertheless, most studies focusing on herbivore trophic ecology ignore the influence that landscape variability may have. Here, we explored how landscape variability influences diet composition through niche overlap. We used eDNA metabarcoding to quantify the diet composition of two large herbivores of the Bialowieza Forest, Red deer (*Cervus elaphus*) and European bison (*Bison bonasus*) to investigate how increasing habitat quality (i.e. higher abundance of deciduous forage species) and predation risk (i.e. density of wolf in the area) influence their diet composition and niche partitioning. Our findings indicate diet composition is non-homogeneous across the landscape, both within and between species. Red deer showed greater diet variability and lower niche overlap within species compared to bison. We detected a reduction of niche overlap for red deer with increasing predation risk, leading to more dissimilar diets, suggesting their feeding behaviour is affected by wolf presence. This correlation was not found for bison, which are rarely predated by wolves. Higher habitat quality was associated with higher niche overlap only within bison, probably due to their suboptimal feeding strategy as browsers. These results show the importance of integrating environment-induced diet variation in studies aimed at determining the landscape usage or niche overlap of a species. Niche overlap can therefore be a powerful tool for inferring predation risk for red deer, and estimating predator abundance or its perception. This approach has enormous potential for wildlife management in areas where predator estimates are unknown or difficult to quantify.

Introduction

Mammalian herbivore species, and more precisely ungulates, are important regulators of the structure and functioning of forest ecosystems (Bagchi et al. 2018, Bairey et al. 2016, Becker et al. 2021, Zhang et al. 2018). Environmental factors such as plant composition, herbivore and predator densities, human activity or protection regime influence each species differently (Speed et al. 2019, Kartzinel & Pringle 2020, Miles et al. 2019). They create a multivariate landscape of environmental influences that affect the species interactions, feeding strategy and modify their trophic ecology, i.e., feeding relationship between a species and its environment (Abdala-Roberts et al. 2019, Yoneya et al. 2015, Liu et al. 2015). Thus, understanding how environmental factors influence the trophic ecology of herbivore species is key to correctly assess their ecological needs and adjust management policies accordingly (Suraci et al. 2022). These factors can be used as an indicator to assess the status of a particular species in their environment and their potential impact (Sévêque et al. 2020, Jesmer et al. 2020).

Quantifying diet composition provides a proxy for measuring species trophic ecology. It reveals the plant species consumed, and has been used to study diet selection, diet overlap and niche partitioning between species (Lear et al. 2021, Jesmer et al. 2020, Sévêque et al. 2020). Traditional methods for diet quantification include micro-histology of scats (Garnick et al. 2018) or rumen content analyses (Gebert & Verheyden-Tixier 2001), but both methodologies are very labour intensive and are increasingly replaced by DNA metabarcoding (Piñol et al. 2019, Ruppert et al. 2019). DNA metabarcoding (i.e. PCR amplification of short but informative barcodes with universal primers and next generation sequencing (NGS) of DNA mixtures (Taberlet et al. 2018) allows the simultaneous identification of different species within communities. This technique can be applied using the plant DNA present in the scats of herbivores, and provides a quantitative approach of the relative presence of each plant in the diet composition of each individual herbivore. Compared to traditional diet quantification methods, DNA metabarcoding can handle many samples at once and provides detailed information on diet composition, unlocking a new path for community ecology to study ecosystem functioning. These dietary studies have the potential to unfold the relation between herbivore diet selection and the environmental variables of study, providing a non-invasive tool for wildlife management to assess the habitat use of a species in their ecosystem and the role each environmental variable has.

However, very little is known on the linkage between environmental factors, species interactions and diet composition among herbivore species (Pansu et al. 2018, O'Connor et al. 2019, Valls-fox et al. 2018, Manlick et al. 2020). The difficulties arise mainly from the many environmental variables acting simultaneously at landscape scale on each individual and how they influence diet composition. To tackle this problem, a new concept has been recently proposed to differentiate landscape-scale herbivory regimes, i.e., integrate multiple environmental variables and how they are perceived by herbivores, the so-called "herbiscapes" (Bubnicki et al. 2019). In the Bialowieza forest, these functional areas have been described and we aim to use the described spatial variability within environmental factors to study how they affect diet composition within and between herbivore species. Overall, this creates an

interesting experimental setup, where two sympatric species have to deal with distinct combinations of environmental factors within the whole ecosystem. The herbiscape approach could be advantageous for applied community ecology studies because they condense complex multivariate spaces into distinct functional areas.

We used scat DNA metabarcoding to characterise in the Bialowieza Primaeval Forest the diet composition of two common ungulate species, red deer and bison. We tested the role of predation risk (imposed by wolf, *Canis lupus*) and habitat quality (i.e. proportion of deciduous forest and mean altitude) as environmental factors on the diet overlap for each species and highlighted the key discriminant plant species. We further assessed the role of the environmental variables on the niche overlap within and between the two species. We hypothesised: (i) Low predation risk and high habitat quality are associated with higher interspecies niche overlap, as both species will select high nutrient plant species in low risk areas (for example, McArthur et al. 2014); (ii) Within species niche overlap is more sensitive to the environmental factors, but overall higher in bison due to their suboptimal feeding as browsers (Kowalczyk et al. 2011); (iii) Red deer behaviour changes due to wolf predation and its niche overlap is reduced with increasing predation risk compared to bison, which remains unaltered as the species is rarely predated (Bubnicki et al. 2019).

Materials and Methods

Study area

Scat sampling was conducted in the Polish part of the Bialowieza Primaeval Forest (Figure 1A and 1B), in the north-western part of Poland, on the border with Belarus. It has a total extension of 600 km^2 and covers a well-preserved fragment of the primaeval forest (Figure 1B), unique in Europe for its exclusion of forestry and ungulate management since 1921. The Polish part of the forest has two distinct management zones: the Bialowieza National Park (BNP, 105 km^2), without forestry and no ungulate management, and the state forest, with forestry and ungulate management. It is composed of different ecosystems, such as open grasslands, wet marshes and deadwood forests, but is mainly dominated by the primaeval forest. Deciduous forests are the dominant type and include hornbeam (*Carpinus betulus*), oak (*Quercus robur*) and lime (*Tilia cordata*). Tree stands in the state forest are dominated by spruce (*Picea sp.*) (Jedrzejewska et al. 1994). Herbivore species present include moose (*Alces alces*), bison (*bison bonasus*), roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*). Carnivore species present are wolves (*Canis lupus*) and lynx (*Lynx lynx*). The area is mainly known for hosting a free-ranging population of approximately 500 bison. In terms of wildlife management, the forestry department applies supplementary feeding in winter and culling of bison outside the park.

Sample collection

Sampling of red deer and bison scats was done in June 2019. 10 fresh scat samples were collected per area and species, adding up to a total of 100 samples, 50 for each species. Samples were taken distant from each other and the GPS coordinates were recorded. The scat samples

were collected fresh and stored in silica beads, in order to dry and preserve them without freezing until DNA extraction.

Herbiscapes

Five distinct areas, i.e. herbiscapes, were selected within the Polish part of the Bialowieza forest, as described in Bubnicki et al. 2019 (Figure 1C). Herbiscapes were defined using camera traps, remote-sensing technologies, human activity patterns and on-the-ground surveys. They were selected because of the high presence of red deer and bison in each of them and included two areas inside the National park and three in the managed part. These areas vary in terms of predation risk (predator encounter rate by ungulates) and habitat quality (proportion of deciduousness in the forest and altitude). Predation risk was inferred based on the landscape use of wolf and lynx (using camera traps) and habitat quality was defined based on forest composition, resource availability and its palatability. For both, the GPS locations of the scat samples were considered to associate the predation risk and habitat quality categories to each sampled area (Table 1; for a full description, see Bubnicki et al. 2019).

DNA extraction

We used between 0.5 and 1 g of dry scat material as the starting point for the extraction. Extractions were performed using the NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany) following the manufacturer protocol. A subset of the extractions was tested for inhibitors with quantitative real-time PCR (qPCR) applying different dilutions (2x, 10x and 50x) in triplicates. Following these analyses, all samples were diluted 5-fold before PCR amplification. All extractions were performed in a laboratory restricted to low DNA-content analyses.

DNA metabarcoding

DNA extracts were amplified using a generalist plant primer pair (Sper01, Taberlet et al. 2018), targeting all vascular plant species (see Supplementary Material for details). Sper01 targets the P6 loop of the *trn*L intron (UAA) of chloroplast DNA (10-220 bp). To reduce tag jumps (Schnell et al. 2015), we followed the library preparation as in Carøe & Bohmann (2020). Final libraries were quantified, normalised and pooled before 150 paired-end sequencing on an Illumina MiniSeq sequencing system with a Mid Output Kit (Illumina, San Diego, CA, USA).

Bioinformatic data analyses

The bioinformatic processing of the raw sequence output and first filtering was done using the *OBITools* package (Boyer et al. 2016). Remaining sequences were taxonomically assigned to taxa with a database for Sper01 (Supplementary material) generated using the EMBL database (European Molecular Biology Laboratory). Further data cleaning and filtering was done in R (version 4.0.2) using the *metabaR* package (Zinger et al. 2021, see Supplementary material for details). Remaining PCR replicates were merged by individual, keeping the mean relative read abundance (RRA) and the PCR ratio of each OTU. We also grouped the OTUs into functional plant types (broadleaf, shrub, conifer, graminoid and forb).

Statistics and modelling

All downstream analyses were carried out using R software (Version 4.0.2). Firstly, we calculated the dissimilarity matrix (Bray-Curtis distance) for each individual based on the final OTU table (transformed to relative read abundances, RRA) and visualised the dissimilarity between individuals using a non-metrical dissimilarity scaling (NMDS). Secondly, we quantified among-individual diet variation (V), i.e. the diet overlap between an individual and its population within the *RInSp* package (V = 1 – *Psi*, Zaccarelli et al. 2013). It estimates the diet similarity in terms of plant OTUs between an individual and the average diet of its species. Values close to 0 indicate similar utilisation of resources whether values close to 1 indicate greater difference in diet composition. Thirdly, we calculated Pianka's niche overlap index (Equation 1, Pianka 1973) using the *spaa* package to investigate niche overlap between individuals and across herbiscapes, which differ in terms of environmental variables, as follows:

$$Pianka_{□□} = \frac{\Sigma^{n}_{□} p_{ij} p_{ik}}{\sqrt{\Sigma^{n}_{i} p^{2}_{ij} \Sigma^{n}_{i} p^{2}_{ik}}} \qquad \textit{Equation 1}$$

where p_{ij} and p_{ik} are the proportion of plant OTU i by individual j and k, respectively, and n is the total number of plant OTU categories. Values close to 0 indicate no overlap, close to 1 indicates full overlap, i.e. same diets. Finally, we used generalised linear mixed models (GLMM), with the *glmmTMB* package, to investigate the significance of niche partitioning in terms of species interactions with herbiscapes and with the categorical variables associated with each herbiscape, i.e. predation risk and habitat quality. We used the *performance* package (Lüdecke et al. 2021) to assess which was the best distribution type for our data and compared multiple combinations of models. We retained the models with the highest marginal R^2.

Herbiscape	Elevation	Forest type	Reserve status	Predation risk	Habitat quality
1	Low	Mix (Coniferous)	National Park	High	Low
2	High	Coniferous	Managed forest	Medium	Low
3	Moderate	Deciduous	National park	Medium	High
4	High	Mix (Deciduous)	Managed forest	Low	High
5	Moderate	Deciduous	Managed forest	Low	High

Table 1. Herbiscape data used for the statistical analyses, adapted from Bubnicki et al. 2019.

Results

DNA metabarcoding

After all quality filtering steps, we retained 4,718,306 reads of 109 different OTUs (Operational Taxonomic Units) for the Sper01 assay that were assigned to 105 different taxa. Most relevant taxa were *Carpinus/Corylus sp.*, *Rubus idaeus* and *Quercus sp.*, which represent 80.8% of the sequences retained. OTUs grouped by plant type and herbivore species are shown in Suppl. Figure 1, where the sum of RRA of all individuals is shown by plant group.

Red deer and bison diet composition

Bison clustered more than red deer for both axes of the NMDS, with both sharing a partial overlap in the multivariate space (Figure 2A). We visualised diet composition also by plant group. Both species show a clear dominance of broadleaves in their diet (Figure 2B). Red deer register an overall higher contribution of the other plant groups compared to bison, except for conifers that were higher in bison.

Among-individual diet variation

We found the two species to show a non-overlapping mean value of among-individual diet variation (V), with red deer having higher mean variation among individuals than bison (Figure 3). However, few bison individuals show a higher value of V, which indicates more diet variability compared to the majority of bison and will be investigated later on. In fact, diet clustering revealed that the majority of individuals grouped by species, except for 20 out of the total 100 individuals of both species, which clustered within the other species (Suppl. Figure 2, also for details). We modelled V against the herbiscape categorical variables (*Predation risk* and *Habitat quality*):

V ~ Species/Food quality	*Model 1*
V ~ Species/Predation risk	*Model 2*

We found *Predation risk* to be significantly correlated to V, meaning that with increasing predation risk the variation in diet composition between individuals increased (p-value=0.003) but not habitat quality (p = 0.11). These results did not change when including herbiscape as a nesting variable (Suppl. Figure 3).

Niche overlap

We calculated the niche overlap (pianka's index, Pianka 1973) between each individual sample in order to test for the dietary niche overlap between and within species. We kept only the comparisons within the same herbiscape, to exclude the potential differences in plant composition between areas.

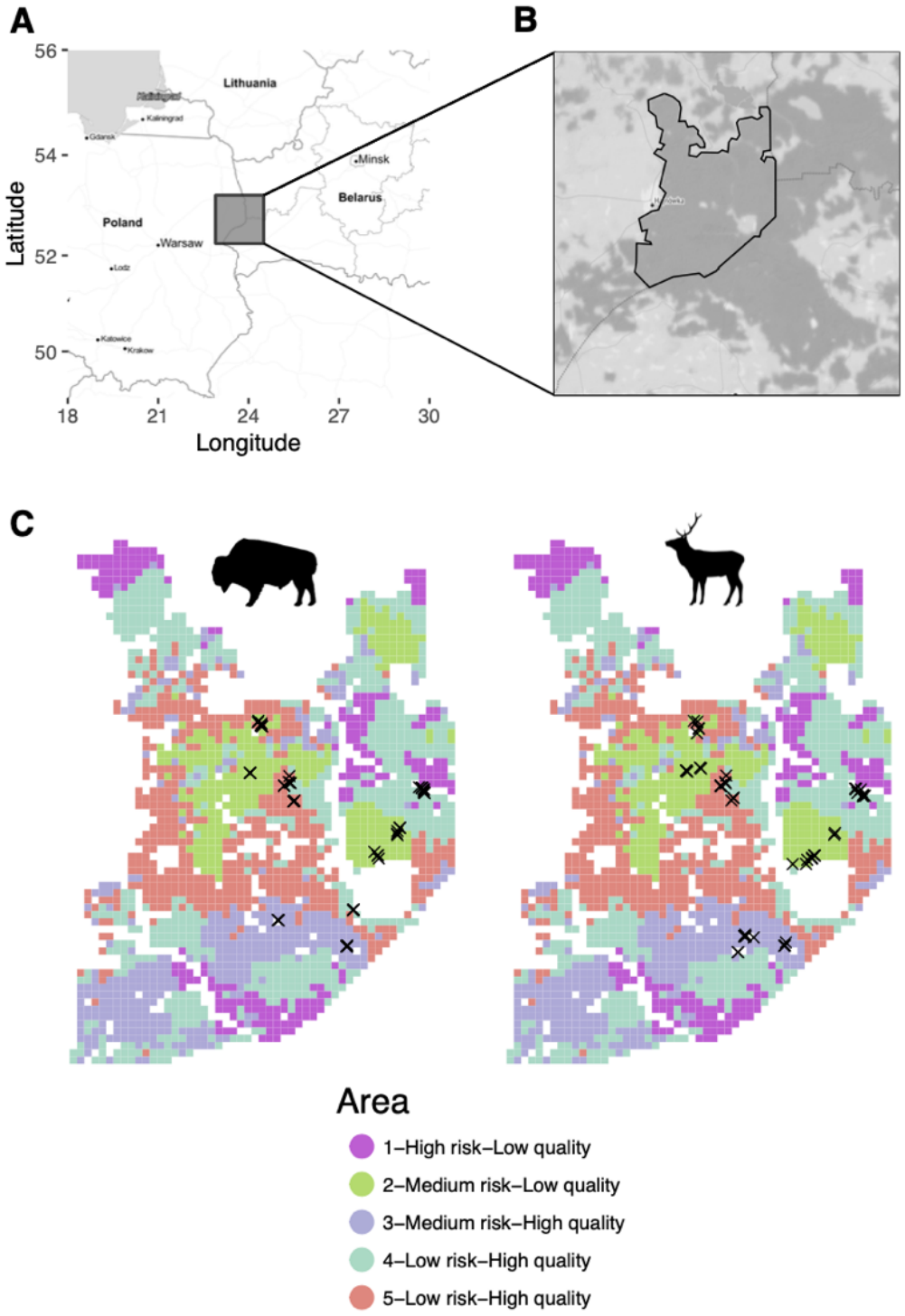

Figure 1. Maps of the study area. A) Large scale map of the area. Grey square marks the area where the Bialowieza forest is located. B) Dark grey areas indicate forested areas. Full black lines mark the area of study within the Bialowieza forest. Dashed black line indicates the limits of the Bialowieza National Park. Notice the eastern edge of the study area is limited by the border with Belarus. C) Area of study divided by the different herbiscape described in Bubnicki et al. 2019. We used the same herbiscape numbering. Each herbiscape has a unique and arbitrary colour (herbiscape numbering is maintained as in Bubnicki et al. 2019). Crosses indicate the location where each scat sample was collected for each species.

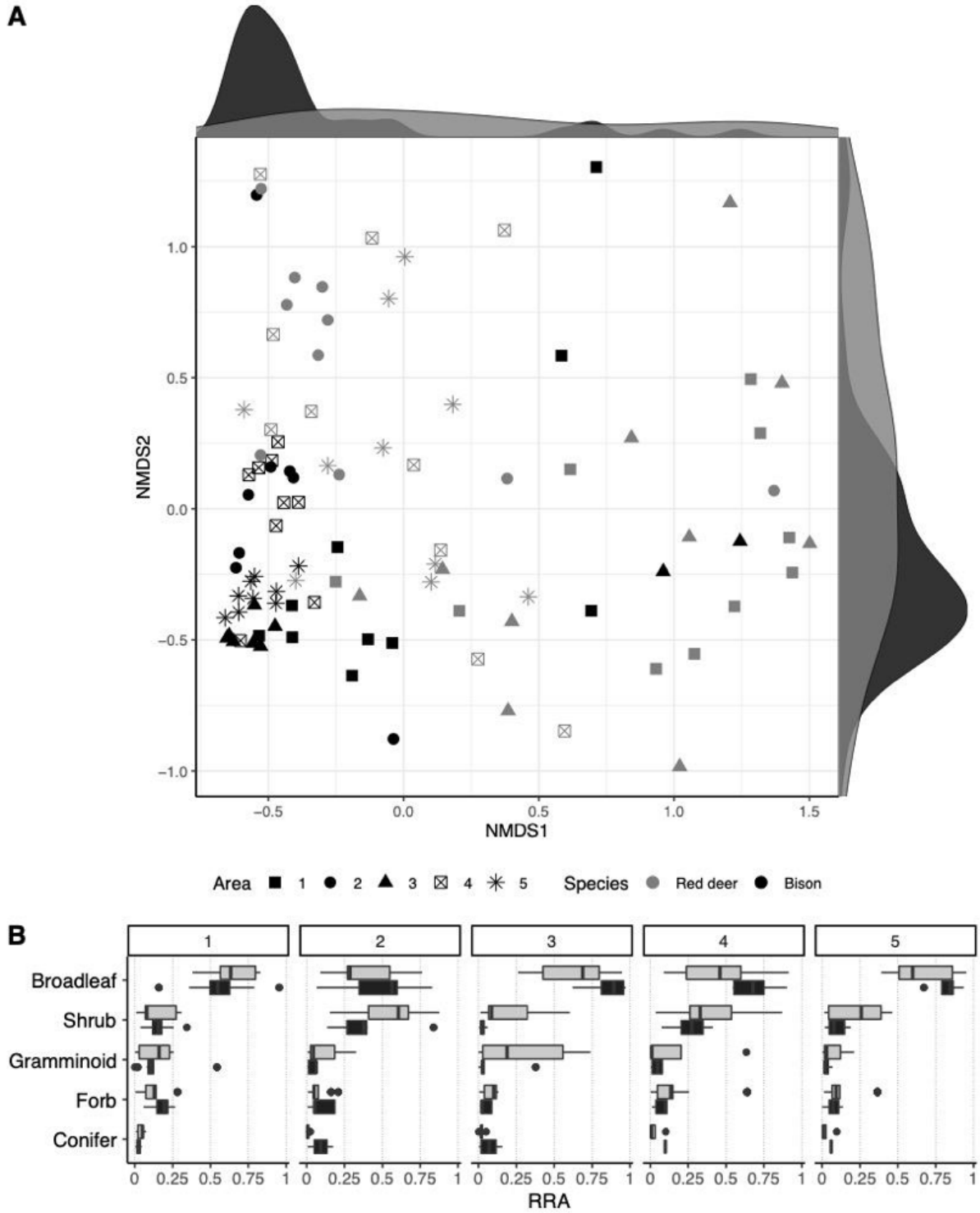

Figure 2. A) NMDS visualisation of the diet composition of all individuals. Density lines on the NMDS1 and NMDS2 axis indicate the density of points on each axis and each species. Dark density lines stand for bison and light grey ones for Red deer. B) Relative read abundance (RRA) for each plant type is shown separately for each species in each of the sampled areas.

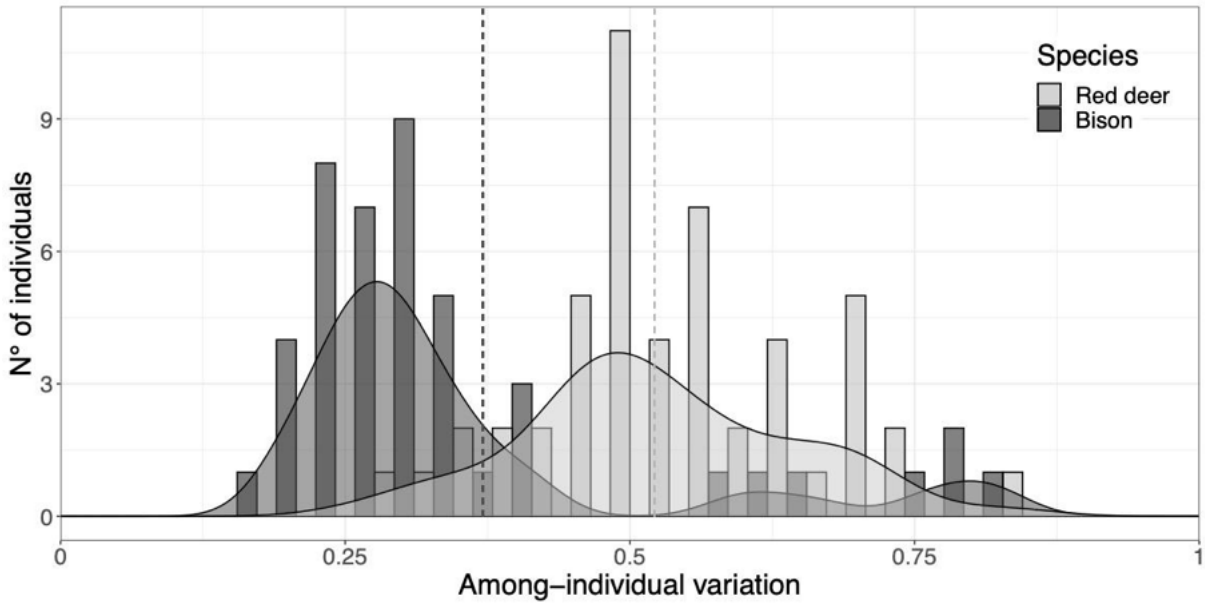

Figure 3. Among-individual diet variation (V). Dashed line indicates the mean value for each species.

We first modelled the observed niche overlap against the three types of species interactions and the sampled areas (herbiscapes) using a beta distribution and with the *glmmTMB* R package, as follows:

Observed Niche overlap ~ Species interaction + Area *Model 3*

This model highlighted differences in predicted niche overlap between species across the sampled areas, which reflects the variability within the forest ecosystem and suggests an interesting interplay of factors (Figure 4A). In line with Figure 3, niche overlap within bison was much higher than within red deer.

We further tested niche overlap against predation risk and habitat quality. Both variables were significant in a linear mixed effect model, together with species interaction, which was included as an interaction variable. The models used was as follows (using a Beta family data distribution and with the *glmmTMB* R package) and held the best fit for the data with a meaningful combination of environmental variables:

*Observed Niche overlap ~ Species interaction + Food quality + Predation risk + Species interaction*Food quality + Species interaction*Predation risk + (1|Area)* *Model 4*

We visualised both the model estimates of each environmental variable on the observed niche overlap (Figure 4B and 4D) as well as the predicted niche overlap range calculated (Figure 4C and 4E) in Model 4. Habitat quality had the same effect within red deer and between red deer and bison, reducing niche overlap in high quality areas, contrary to within bison (Figure 4B and 4C). We detected an increase in predicted niche overlap when predation risk is reduced, but this pattern is only clear within red deer (Figure 4D and 4E). Between species, high risk

reduced niche overlap contrarily to low risk, but the overall niche overlap was similar across predation risk levels.

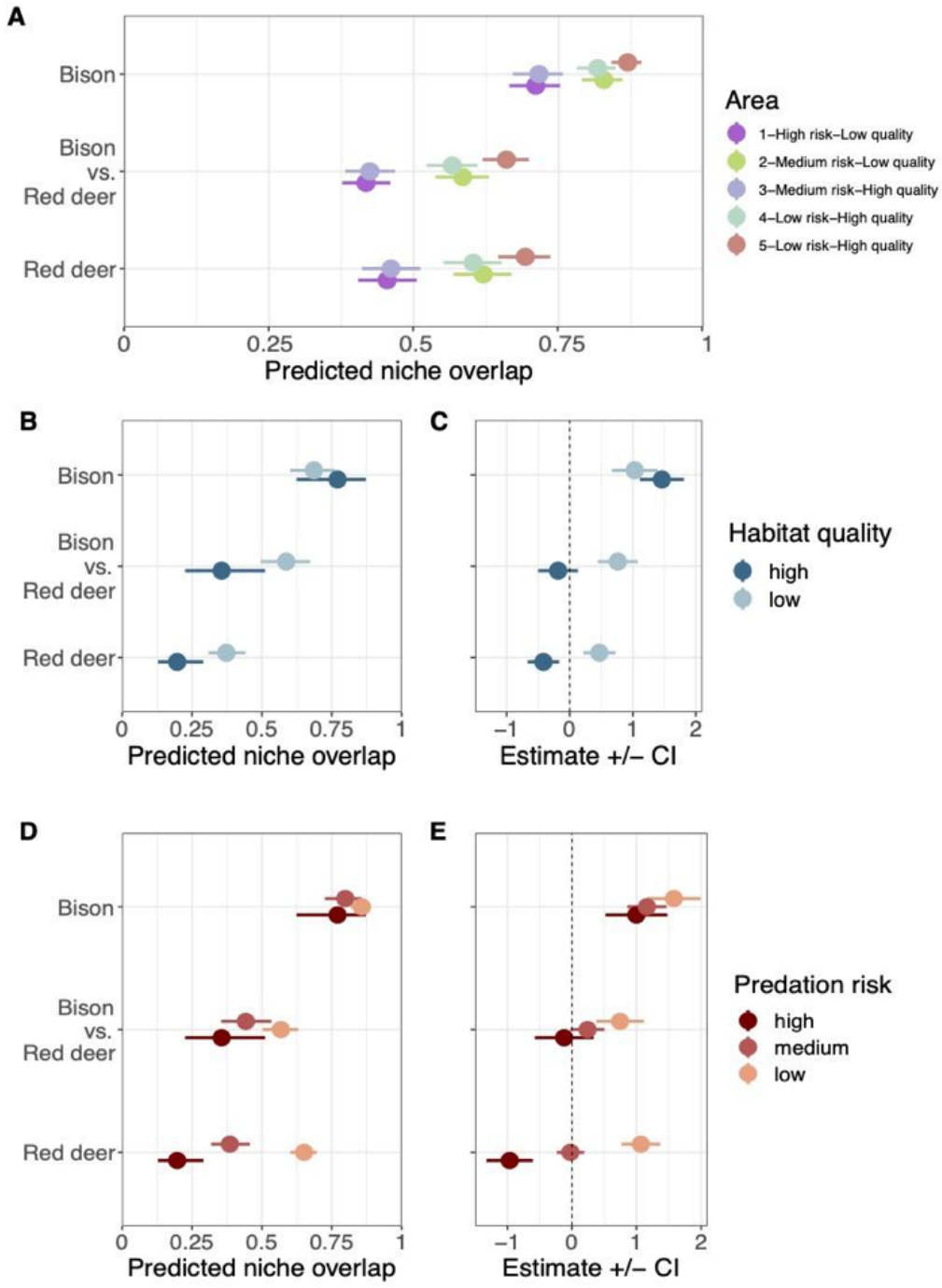

Figure 4. Niche overlap modelling for each type of species interaction. A) Predicted niche overlap by area (herbiscape). B) Predicted niche overlap for each habitat quality level. C) Estimates of the model for each species interaction and habitat quality. Dashed line indicates estimate value of 0. D) Predicted niche overlap for each predation risk level. E) Estimates of the model for each species interaction and predation risk.ashed line indicates estimate value of 0.

Discussion

In this project, we studied the role of landscape variability on the diet of two large herbivores, in terms of plant species consumed and in terms of niche overlap between and within species. Areas were classified in five different categories, which reflect different herbivory regimes across the landscape depending on the composition of the mammal community and other environmental variables. We used this framework to study the diet differences across the landscape between the two main ungulates of the forest, red deer and bison. We retrieved the plant composition of their diets and calculated their niche overlap. We tested these results against the different combinations of habitat quality and predation risk in order to investigate the relationship between herbivores' diet and the variability of the environment.

Comparison of diet composition

The diet of both species was dominated by broadleaf plants (mainly *Corylus/Carpinus sp.*) during the sampled period in summer (Fig 2B). These tree species, that abundantly occur in the area, produce new leaves during June and become the primary choice for both species, as previous studies have shown (Gębczyńska 1980, Kuijper et al. 2010a, Kowalczyk et al. 2011). However, we found a higher proportion of all the other plant groups in red deer compared to bison (Figure 2B), suggesting a more diverse diet. This is consistent when observing the NMDS visualisation (Fig 2A), as red deer individuals are distributed sparsely in the multivariate space compared to bison, which are more clustered (except few outlier individuals). In general, red deer are associated with eating a higher proportion of woody plant species (browsing), compared to bison preferring to forage on herbaceous plant species (grazing) (Kowalczyk et al. 2011, Churski et al. 2021). As a result, bison prefers natural and human-made openings in the forest to profit from the grassy vegetation (Kerley et al. 2012, Kuijper et al. 2010b). They have clear seasonal patterns with more woody vegetation in their diet during autumn-winter (Kowalczyk et al. 2011).

Among-individual diet variation

Overlap in the diet composition between individuals and the average within species provides a straightforward method to assess the intraspecific variation in diet and thus can inform on the dietary plasticity of a species in their environment. Mean diet diversity was higher for red deer than for bison (Figure 3), i.e. red deer have higher diet variation within species than bison, in line with the NMDS (non-metric dimensional scaling, Figure 2A), where greater dispersion of individuals means greater dissimilarity across their diets. In terms of their feeding strategy, we consider that the lower mean diet diversity of bison aligns to grazers because the species is less selective than typical browsers (Hofmann 1989, Bocherens et al. 2015). However, we did not detect a clear signal of meadow plant species when comparing the two species (we did not observe any clear differences in graminoid proportion between the diet of both species, as seen in Figure 2B). Red deer browse forest gaps with high regeneration growth (Churski et al. 2017, Kuijper et al. 2009), which are heterogeneously spread in an old forest like the Bialowieza forest. This implies covering more distance than grazers giving them access to a greater variety of resources (Hofmann 1989). Taking in account the differences between sampled areas, this

result points to a greater pool of preferred plant species for red deer compared to bison, which could explain the mean diet diversity results.

Niche overlap, intraspecific and interspecific interactions

We observed bison to have the highest niche overlap within species, regardless of the sampled areas. Red deer niche overlap within species was clearly lower than bison and aligned to the interspecific niche overlap. The three areas outside the National park (2,4,5) show the highest niche overlap between species, i.e. most overlapping diet composition between bison and red deer. This could be related to behavioural differences between the two herbivores. However, we cannot rule out differences in habitat composition outside the reserve, exploited for timber, which might offer a lower variety of plant species for the two species.

We associate the overall high niche overlaps within both species, particularly for bison, to the availability of food plants in the area. In fact, the abundance and diversity of resources available in spring probably exceeds the food requirements of the bison and red deer community in all the sampled areas and complicates the distinction between selectivity and competition. Niche overlap is likely to align closer to competition during periods of limiting resources (winter). Thus, extending the experimental setup presented in this study to a year-round survey could reveal the seasonal dynamics between niche overlap and competition.

Niche overlap and habitat quality

In terms of habitat quality, high quality areas show greater niche overlap within bison but not within red deer and between species (Figure 4B). Both estimates of the model were positive, which suggests the differences in habitat quality does not affect the niche overlap within bison. This could be explained by the higher availability of their preferred plant species for bison in high quality areas, and contradicts our first hypothesis, which stated higher interspecies niche overlap would occur at low predation risk and high habitat quality, with both species selecting high nutrient plant species. Interestingly, red deer estimates diverged compared to bison, as the model indicates low habitat quality increases the predicted niche overlap within species, contrary to high habitat quality (Figure 4C). The higher diversity in diet composition of red deer comes into play to explain this pattern (Figure 3). More likely, the browsing behaviour of red deer provides a fine-scale choice of plant species. Our data indicate red deer have more similar diet choices in lower quality areas. We detected a significant effect of low habitat quality to increase niche overlap, i.e., red deer has its niche overlap increased in low quality areas (Figure 4B and 4C). This could be due to red deer selecting for the same (and more palatable) plants, thus increasing their niche overlap. However, the plant composition of the environment or the competition between individuals could be also playing a role. Between species, the model shows the same pattern as within red deer (Figure 4B and 4C). This suggests more diet convergence in low quality areas compared to high quality areas, where the greater plant diversity allows for both species to be more selective. We suggest that in high quality areas the greater diversity of plant species translates into lower predicted niche overlap between both species, as they feed on their preferred plant species. Moreover, the similarities between bison-red deer and red deer-red deer niche overlap confirms the facultative browsing nature of bison. Despite the many meadows surrounding the forest, our data suggest that bison spend most of their feeding time browsing within the forest, rather than grazing on the open meadows

(Kowalczyk et al. 2011). Grazing bison would reveal as a low niche overlap with red deer, which is not the case and aligns to our second hypothesis, i.e., bison have higher within species niche overlap due to their feeding behaviour. This could be confirmed by crossing our results with GPS data monitoring bison habitat use and testing if niche overlap within bison correlates with the proportion of time spent on meadows. Our results show higher niche overlap variability within than between species, but they are not conclusive enough to answer if interspecific interaction can be used by management authorities as a fine-scale tool to measure the role of the environmental conditions.

Niche overlap and predation risk

We observed a complex relation with predation risk imposed by wolf and lynx on the two herbivores in terms of niche overlap, as firstly investigated in Churski et al. 2021. Our study covers more areas and more variables interplaying along the landscape, but measuring accurately these indirect effects using dietary data remains a challenge (see Suraci et al. 2022 for an overview on the advances in measuring predator-prey interactions). Between species, we detected a negative effect of predation risk on the diet overlap in high risk areas and positive in low risk areas (Figure 4E). This trend was more pronounced when comparing diet overlap between species than within species. However, the three types of species interaction converged in similar predicted niche overlap (Figure 4D), contrary to our second hypothesis, and illustrates the complex interplay of factors in the studied system. If both species were influenced by predation risk, we would have observed niche overlap increasing together with predation risk, which is not the case (Figure 4D). Such confounding results could be explained by the disparity in predation risk perception between red deer and bison, which modify the diet choice of red deer but does not affect the diet choice of bison, resulting in opposite estimates in the model but not in the predicted niche overlap.

Within bison, the higher niche overlap was kept across predation risk levels. The positive estimates detected for all predation risk levels (Figure 4E) is likely to be an artefact of the dietary choices, i.e. match between diet preference and plant availability, rather than a consequence of predation risk since bison is rarely predated. Their diet choice is only driven by selectivity and availability, and we can use them as a reference diet to assess the role of predation risk on red deer.

Red deer are highly predated by wolves and we hypothesised higher predation risk would result in lower within species niche overlap. In line with our third hypothesis, we found red deer had lower niche overlap within species with increasing predation risk, in contrast to bison, which is rarely predated, and supports our model results. The negative estimate for high risk and positive for low risk (Figure 4E) indicates predation risk is an active factor driving red deer feeding strategy. We suggest these results respond to red deer reducing their selectivity and thus have a broader diet composition, leading to more dissimilar diets. The niche overlap in this species can be thus used as a tool to infer predation risk in the sampled area but also to estimate the abundance of predators, or at least the perception of predator abundance by red deer. We advocate this approach has great potential to be used in wildlife management for areas where predator estimates are not known or hard to quantify.

Conclusion

This study shows eDNA dietary metabarcoding can be used to study herbivores' diet variation over the landscape and how diet is affected by environmental factors and interaction with other herbivore species. The integration of this technique in ecology studies will provide a new pathway to answer complex ecological questions. Moreover, this approach yields great potential to serve as a complementary tool for wildlife monitoring and species assessment in natural environments. Through diet composition, it can bring useful information for conservation purposes on herbivores' habitat use and feeding interactions. However, it requires an exhaustive characterization of the landscape so the diet composition of the study species can be used as a proxy to monitor the habitat use of species in their environment. We advocate future studies to explore this direction, but more comparative studies should be designed to assess the pros and cons of combining landscape ecology and dietary metabarcoding before drawing conservation and management policies using this methodology.

Acknowledgments

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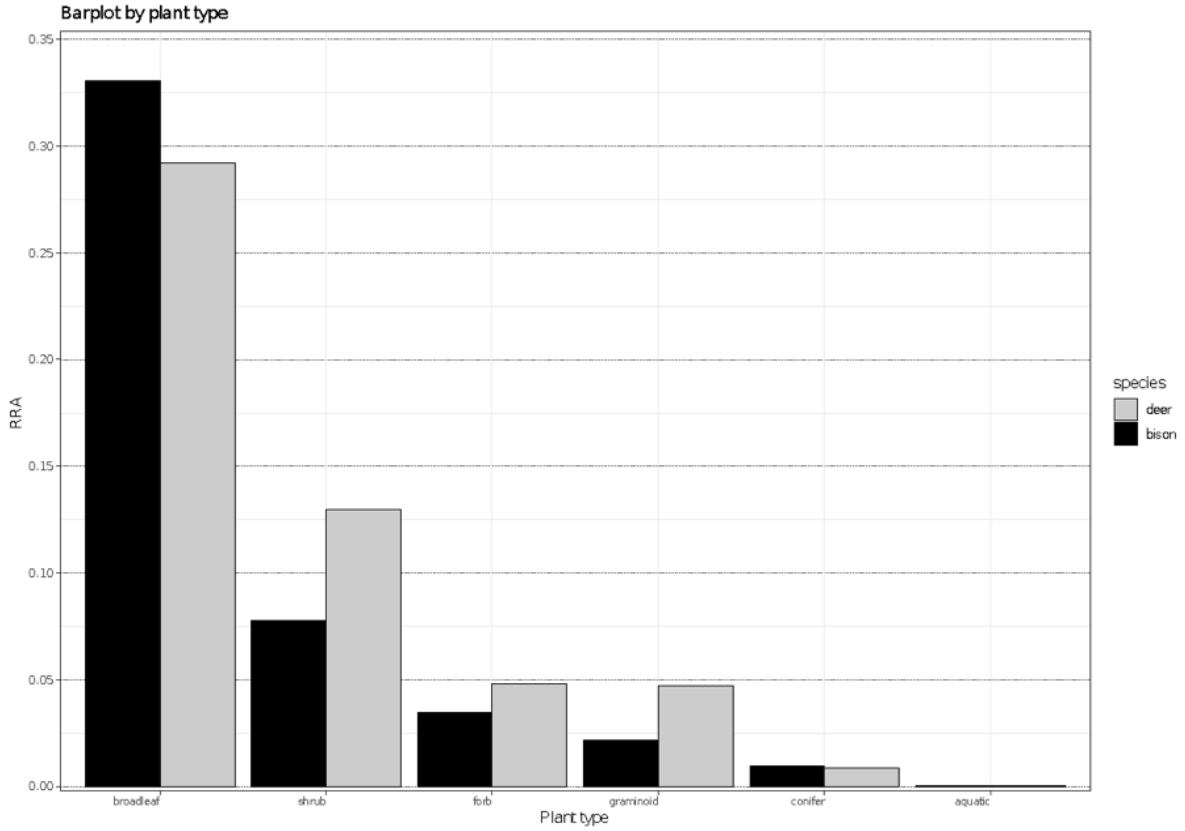
Conflicts of interest

The authors declare no conflicts of interest.

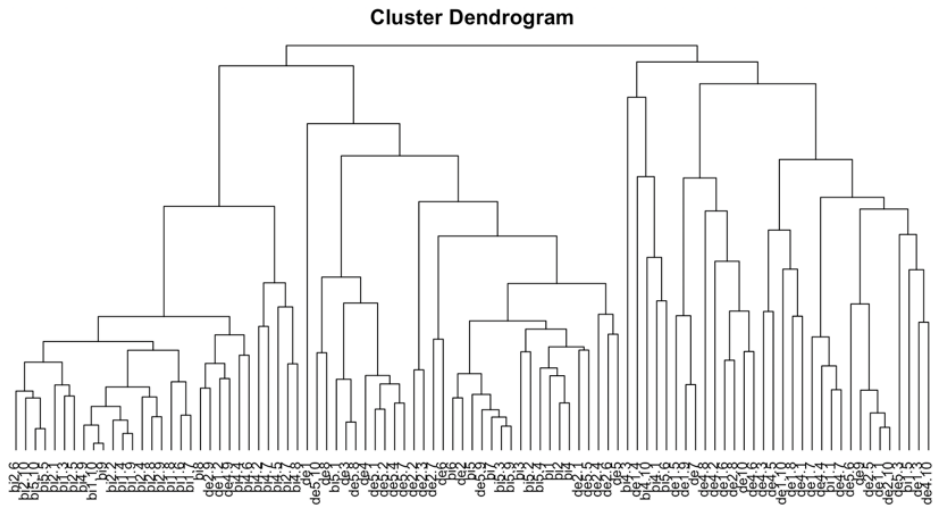
Data availability statement

All data used for this study are available in the Dryad repository at the following link: https://doi.org/10.5061/dryad.k0p2ngfbn

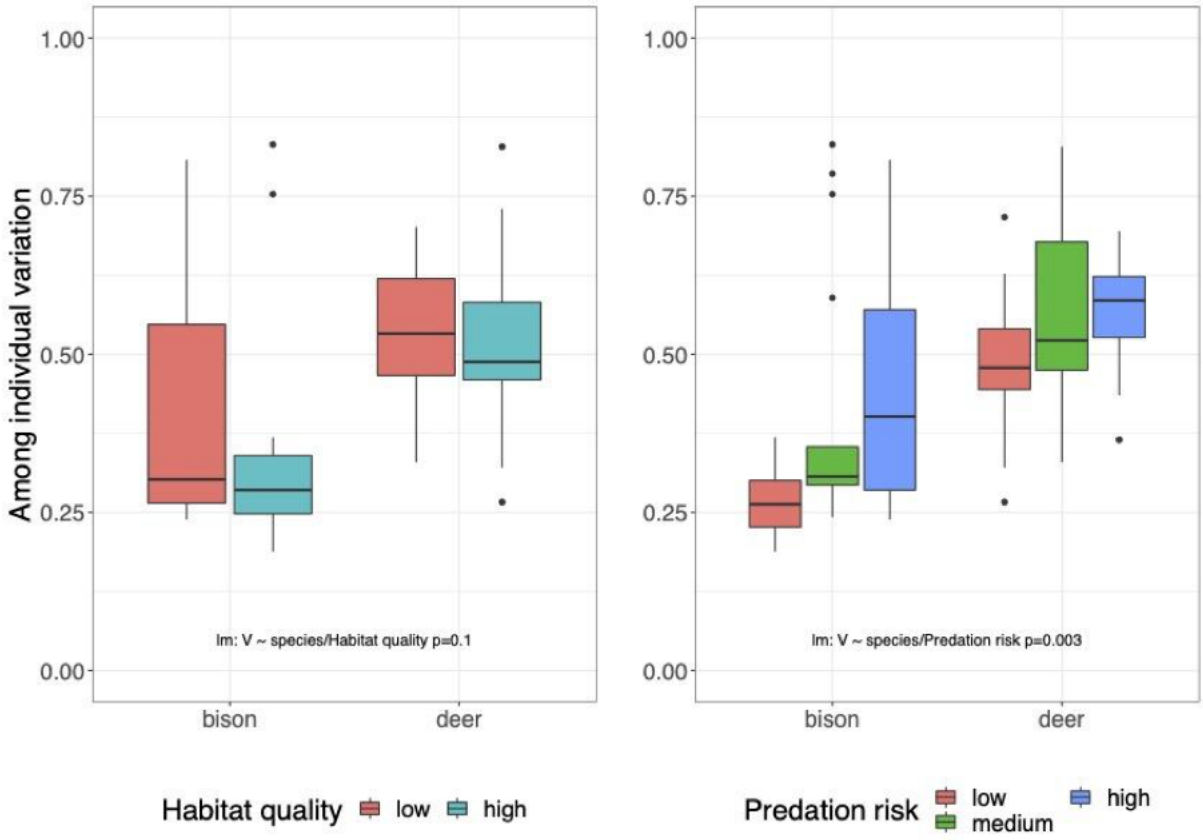
Supplementary material



Supplementary Figure 1. Barplot by plant type and species, all individuals summed.



Supplementary Figure 2. Cluster dendrogram of diets (de = deer, bi = bison). Clustering analysis group species by diet composition similarity in a tree-like visualisation. If the first split of branches is grouped by species, all diets within species are more similar between each other than to any of the other species' individual diets.



Supplementary Figure 3. Among individual diet variation modelling against the two studied environmental factors, i.e., habitat quality (left) and predation risk (right).

Supporting information

DNA amplification and library preparation

qPCR reagents and conditions were the same as in DNA metabarcoding PCR reactions (see below), with the addition of 10,000-fold diluted SybrGreen (Thermo Fisher Scientific, USA). To assign the DNA sequences to each sample, primers were tagged with eight variable nucleotides added to their 5'-end with at least five differences between tags. Tags also included 2 to 4 random nucleotides on the tag 5'-end to increase variability and improve sequencing performance. The PCR reactions were performed in a final volume of 20 µL. The mixture contained 1 U AmpliTaq® Gold 360 mix (Thermo Fisher Scientific, USA), 0.04 µg of bovine serum albumin (Roche Diagnostics, Basel, Switzerland), 0.2 µM of tagged forward and reverse primers and 2 µL of 5-fold diluted template DNA. PCR cycling conditions were denaturation for 10 minutes at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 52 °C and 1 min at 72 °C, with a final elongation step of 7 min at 72 °C. Amplifications were performed separately for each species and in replicates (3 per sample divided in 6 plates, 3 for each species) in PCR plates with controls. Amplicons were purified using a MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Purified pools were quantified using a Qubit® 2.0 Fluorometer (Life Technology Corporation, USA).

Plates contained 50 DNA extracts, 11 blanks as well as 8 extraction, 8 negative and 8 positive PCR controls (DNA assembly of 10 species with increasing relative concentrations). The use of blanks allows estimating the proportion of tag switches (i.e., false combination of tags, generating chimeric sequences) during library preparation (Schnell et al. 2015). Amplification success and fragment sizes were confirmed on a 1.5 % agarose gel. PCR products were subsequently pooled per PCR plate.
Library preparation was done following the recently published TagSteady Protocol (Carøe & Bohmann 2020). After adapter ligation, libraries were validated on a fragment analyzer (Advanced Analytical Technologies, USA).

Sequence filtering using obitools
Forward and reverse reads were assembled with a minimum quality score of 40. The joined sequences were assigned to samples based on unique tags combinations. Assigned sequences were then de-replicated, retaining only unique sequences. All sequences with less than 100 reads per library were discarded as well as those not fitting the range of metabarcode lengths. This was followed by two different clustering methods. First, pairwise dissimilarities between reads were computed and lesser abundant sequences with single nucleotide dissimilarity were clustered into the most abundant ones. Second, we used the *Sumaclust* algorithm (Mercier et al. 2013) to further refine the resulting clusters based on a sequence similarity of 97 %. It uses the same clustering algorithm as UCLUST (Prasad et al. 2015) and it is mainly used to identify erroneous sequences produced during amplification and sequencing, derived from its main (centroid) sequence.

Sequence filtering using metabaR
Sequences that were more abundant in extraction and PCR controls than in samples were considered as contamination and removed. Operational taxonomic units (OTUs) with similarity to the reference sequence lower than 97 % were also eliminated from the dataset. Removal of tag-leaked sequences was done independently for each library. This approach allowed us to discard single OTUs instead of whole PCR replicates. However, PCR replicates with too small reads count were also discarded.

Chapter 3 - The role of pesticides and vineyards management on soil arthropod community using eDNA

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Author contribution
PD and MVdH designed the study and supervised all the analyses. PD conducted the farmer pesticide survey. EM-C conducted the soil sampling fieldwork, laboratory work, bioinformatics, data analyses and prepared the figures. EM-C wrote the first draft of the manuscript, with input from all other authors.

Abstract

The use of pesticides in vineyards has multiple implications for the soil biological community and the ecosystem. They are designed to target certain species in order to ensure the production of grapes, but their combined effects on the soil ecosystem are largely unknown. Conventional viticulture relies on multiple pesticides to control pests and diseases, but these chemicals can infiltrate in the soil after being sprayed and disrupt the soil community. Contrarily, organic viticulture is becoming more popular nowadays because it reduces the use of pesticides. However, further studies are needed to better understand the effects of pesticides in the soil ecosystem and the benefits organic management can bring to improve the soil ecosystem. Arthropods are responsible for many ecological services and are good indicators for the health of the soil. Studying this group to understand the effects of pesticides can serve as a reference to quantify the ecological impact of pesticides and provide effective and sustainable solutions to reduce dangerous pesticides. However, the variety of pesticides used and types of soil implies that a whole-ecosystem approach is needed. Recently, environmental DNA (eDNA) has become a robust approach to describe soil communities as it can capture the DNA signal of the species present in the soil. Here, we used eDNA to study pesticide use on the soil arthropods. We sampled soil from forty vineyards within two regions in Switzerland, retrieved their arthropod soil community and studied how it is influenced by the pesticides used. We profited from a detailed list of pesticides used by farmers from both conventional and organic management to investigate their influence on the soil arthropods. The two types of management used distinct pesticide combinations along the retrieved years, but both heavily relied on fungicides. We found the soil arthropod community of organic vineyards to have greater diversity compared to conventional ones. However, the community differences between regions indicated that the nature of the soil was a stronger driver of the sampled community than the management applied. Pesticide-wise, we were able to identify several active compounds significantly correlated to the shifts in the arthropod community that could be having a negative impact on the soil ecosystem, highlighting the need to focus on these pesticides when designing new sustainable management practices. Our study provides an ambitious but scalable approach to monitor pesticide impact on the soil biological community to improve vineyard ecosystems but further research is needed in order to better understand the combined effects of pesticides on the soil ecosystem.

Introduction

Vineyards are a well-known and cherished type of agricultural practice with deep connection to communities and traditions, especially in southern Europe (Biasi et al., 2012; Brevik & Hartemink, 2010; Purcell, 1985). In vineyards, the grape vine is the cultivated species, yet many more species co-occur, in the soil, on the leaves and in the vines themself, which altogether influence the quality of the final product, the wine (Huggett, 2006; Jackson & Lombard, 1993). Especially belowground, the soil community, which includes microorganisms, fungi, bacteria, and other organisms, plays a crucial role in vineyard health and productivity, but has been underestimated until recent years (Zarraonaindia et al., 2015). It is a complex network of species which perform key regulatory processes within the soil, comprising soil bioturbation, fertilisation, organic matter degradation, etc (Crowther et al., 2019). As such, this soil community is a key and direct driver of soil fertility and thus, of wine quantity and quality (Liu et al., 2019). We are now starting to comprehend the extent of the complexity of the soil ecosystem, how it interacts with the physicochemical properties of the soil and how they affect agriculture and aboveground ecosystems (Ghiglieno et al., 2019; Lukac et al., 2017).

In this line, vineyard management can be divided mainly into two categories: conventional and biological management (Shennan et al., 2017). Conventional viticulture is oriented to maximise the grape yield from vines. To do so it uses synthetic (but also organic) fertilisers and pesticides, i.e. insecticides, herbicides or fungicides, which overall have a complex impact on the soil biological community difficult to disentangle and relate to each single pesticide (Lukac et al., 2017). On the contrary, organic viticulture prohibits the use of synthetic chemicals and relies mostly on organic products and traditional practices to face the potential weeds, diseases and pests that could damage their crops. These practices include using natural pest control methods, such as natural predation, hand picking or companion planting. To fertilise, they rely on organic fertilisers, such as compost or cover crops, to enrich the soil while limiting damage to its biological communities and natural functioning. Overall, organic viticulture places a greater emphasis on sustainability, soil health, and natural practices, while conventional viticulture relies more heavily on synthetic inputs and technologies. Nevertheless, the classification of practices is not binary. Rather, in practice, a range of methods are implemented between the so-called conventional and organic practices.

Current research is aiming to disentangle the complex relationship between wine quality, soil properties and its biological community, but the multivariate nature of these interactions complicates the attainment of transversal conclusions (Lukac et al., 2017). The chemical properties of the soil strongly influence the wine produced. In fact, wine varieties are distributed by regional areas, which align to soil types, which vary in terms of pH, consistency, water retention and nutrient concentration (White, 2020). In this line, much research has been developed on the influence of the physicochemical properties of the soil on the wine produced. The soil properties and climate will not only influence the yield and quality of the grape, but will also influence how the vineyards are managed and the soil communities that can inhabit

it. However, less is known about the influence agricultural management has on the soil community of the vineyards.

Conventional and often organic vineyard management usually rely on some type of pesticides to control pests and diseases that can damage the grapes (Ghiglieno et al., 2019). However, they can disrupt the existing soil community, depriving it of beneficial microorganisms and leading to decreased soil health and productivity. Pesticides can also harm beneficial arthropods, depriving the soil from key bioengineers, such as earthworms, springtails or bees (Gunstone et al., 2021). Fertilisers are also commonly used in vineyards to provide essential nutrients to the vines. However, excessive use of fertilisers can lead to soil degradation, nutrient runoff and contamination of nearby ecosystems (Karlen & Rice, 2015; Kopittke et al., 2019). To minimise the impact of pesticides and fertilisers on soil communities, sustainable viticulture practices are increasingly being adopted by vineyard growers.

Traditional methods for assessing soil communities, such as pitfall trapping (Hohbein & Conway, 2018) are time-consuming, labour-intensive and invasive sampling methods (i.e. they may require the sacrifice of the collected individuals). In contrast, recent advances in next generation sequencing has opened a new approach to sample soil communities through environmental DNA (eDNA), i.e., DNA left by organisms in their environment (Taberlet et al., 2018). eDNA can be collected in an easy, cheap and non-invasive way. This approach was first used to study the soil microbial community (Ogram et al., 1987), but has now expanded to all groups present in the soil, but also other ecosystems. Another advantage of using eDNA is that it can provide a more comprehensive assessment of soil biodiversity than traditional methods. eDNA can detect soil species that may be difficult to identify visually or through traditional sampling methods, such as small or cryptic species. This can provide a more complete understanding of the impacts of pesticide use on soil communities (Kestel et al., 2022).

Soil arthropods have been extensively studied using traditional methods and are good indicators of the soil community and functioning in agricultural soils (Parisi et al., 2005). In this project, we aimed at better understanding the role of agricultural management on the soil community with a focus on the soil arthropod community. We used eDNA techniques to assess the soil arthropod community in vineyard soils following distinct management regimes, divided in conventional and organic viticulture. In parallel, we conducted a survey of farmers to obtain data on the management practices and pesticides applied over the past five years. To better understand the impact of vineyard management strategy (i.e., conventional or organic) on soil arthropod communities, we have focused our research efforts within the same region to reduce the influence soil physicochemical properties have on the soil biological community. We hypothesised: (i) arthropod diversity will be higher in organic vineyards, (ii) community composition will be more similar between geographically close areas and (iii) between similar combinations of pesticides used. By doing so, we aim at comparing the effect agricultural practices have as the main difference between the sampled vineyard soil communities. Understanding the impact of these practices on soil health and productivity can help vineyard owners and growers make informed decisions about how to manage their vineyards while minimising negative environmental impacts.

Materials and methods

Sampling area

Vineyards selected for soil sampling were distributed between the Canton Vaud and Valais, Switzerland (Suppl. Figure 1). Soil type differs between the two cantons because of the geological history and characteristics of each area, making its physicochemical properties also distinct (Gubler et al., 2022). On the one hand, Valais vineyards are located within the Alps and their soil is dry, rocky and with little organic matter. The depth of the valley and the orientation favours a sub-mediterranean climate, permitting viticulture from 400 up to nearly 1150 m a.s.l. in some locations, depending on the orientation and exposure to sunlight. The plain within the valley is reserved for other types of crops, as the soil is less rocky and richer in organic matter thanks to river deposition of sediments. Thus, the suboptimal conditions on the rocky slopes have favoured the proliferation of terraced vineyards, i.e. laid out design to cultivate vines on steep slopes. On the other hand, Vineyards in Canton Vaud are located outside the Alps mountain range, on the Leman lake basin. The soil has a higher clay composition, with more organic matter and vineyards are not terraced as in Valais. Because of this, the vineyards in Vaud are located scattered among other types of agricultural exploitations, such as fruit trees, corn or wheat.

Vineyard management regime

For each location, we recorded the GPS coordinates, elevation and management strategy (Suppl. Table 1). Each farmer was provided with a survey to complete in order to retrieve data on the details of their agricultural management, beyond the categorization as conventional or organic, indicating the vine variety, the years since organic practices are followed, if so, amount and frequency of pesticides applied, together with the tillage and mowing frequency among other physical disturbances applied to the soil as part of their management.

Regarding the use of pesticides, for each year since 2016, farmers provided a detailed list of agricultural products applied in the soil, which were grouped in fertilisers, herbicides, insecticides and fungicides. The concentration of the active compound and the number of times and quantity of each pesticide applied was also calculated using the manufacturer's information for each pesticide so as to standardise their effect on the soil and used this metric for all downstream analyses. For each fungicide, herbicide and insecticides, we calculated the number of days pesticides were applied, the number of different pesticides applied and their interaction (i.e. number of pesticides times number of days applying pesticides). This created an estimate of the impact applied pesticides could have and standardised the pesticide application regime across parcels, as farmers often used unique combinations of pesticides.

Sampling methods

In each parcel, two soil samples of about 1 kg were collected. One sample was collected between vine lines (L) and the other was collected between vine stems (S). The soil was less compact between vines because it remains year-round free of tillage, machinery or farmers going over it. To sample soil, we used a soil corer (Wolf-Garten MTD Schweiz AG) and

collected only the top 10cm from each core. Soil was collected in a zip plastic bag and all the material required for sampling was sterilised with bleach and 96% alcohol in between samples. To reach 1Kg, between 20 and 40 soil cores were required. The variability is due to the different conditions of compaction and humidity of the soil. Each soil sample was weighted before starting DNA extraction.

DNA extraction

We used all the soil collected from each parcel as the starting point for the extraction. We first prepared a phosphate buffer solution (as in (Taberlet et al., 2018)) and mixed it within the zip bag used to collect the soil using a 1:1 ratio. After thoroughly mixing the soil and the buffer, we transferred the soil-buffer mix into a 50mL falcon tube. Falcon tubes were mixed using a spinner for 10 min. Then, 1.5ml of the supernatant was transferred to a 2mL Eppendorf tube. Tubes were then centrifuged at maximum speed for 10min to remove all the soil particles suspended in the buffer. After this step, DNA extraction was performed using the NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany) following the manufacturer protocol. A subset of the extractions was tested for inhibitors with quantitative real-time PCR (qPCR) applying different dilutions (2x, 10x and 50x) in triplicates. Following these analyses, all samples were diluted 5-fold before PCR amplification. All extractions were performed in a laboratory restricted to low DNA-content analyses.

DNA metabarcoding

DNA extracts were amplified using a generalist arthropod primer pair *Arth02*, targeting all arthropod species (see Taberlet et al., 2018 for details). *Arth02* targets the 16S mitochondrial rDNA (76-168 bp). The PCR reactions were performed in a final volume of 20 µL. The mixture contained 1 U AmpliTaq® Gold 360 mix (Thermo Fisher Scientific, USA), 0.04 µg of bovine serum albumin (Roche Diagnostics, Basel, Switzerland), 0.2 µM of tagged forward and reverse primers and 2 µL of 5-fold diluted template DNA. PCR cycling conditions were denaturation for 10 minutes at 95 °C, followed by 45 cycles of 30 s at 95 °C, 30 s at 49 °C and 1 min at 72 °C, with a final elongation step of 7 min at 72 °C. Amplifications were performed in separate PCR plates for each Canton and in replicates (4 per sample) with controls. Amplicons were purified using a MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Purified pools were quantified using a Qubit® 2.0 Fluorometer (Life Technology Corporation, USA). To reduce tag jumps (Schnell et al., 2015), we followed the library preparation as in Carøe & Bohmann (2020). Final libraries were quantified, normalised and pooled before 150 paired-end sequencing on an Illumina MiniSeq sequencing system with a Mid Output Kit (Illumina, San Diego, CA, USA).

Bioinformatic data analyses

The bioinformatic processing of the raw sequence output and first filtering was done using the OBITools package (Boyer et al., 2016). Remaining sequences were taxonomically assigned to taxa with a database for *Arth02* generated using the EMBL database (august 2022 release). We double checked taxonomic assignment using BLAST within NCBI, to assess the automatic assignment done with our reference database and correct any potential errors or refine the results for each sequence. When more than one species was equally possible and co-occuring

in the studied geographical area, we assigned that sequence to a higher taxonomic level. Further data cleaning and filtering was done in R (version 4.0.2) using the *metabaR* package (Zinger et al., 2021). Remaining PCR replicates were merged by sample type (i.e., S and L samples for each sampled vineyard), keeping the mean relative read abundance (RRA), the frequency of occurrence (FOO) and presence/absence data resulting in two communities per sampled parcel. Finally, we grouped the OTUs into arthropod orders and families.

Statistics

All downstream analyses were carried out using R software (Version 4.0.2). First, we calculated the dissimilarity matrix for each sample based on the final OTUs table using the three data transformations, but for the follow up analyses we used the RRA dataset. We visualised the variation in OTUs composition between samples using non-metrical dissimilarity scaling (NMDS) and using PERMANOVA (Anderson, 2017). We also grouped OTUs by region and by order. The results of the NMDS were compared to meaningful metadata variables of the parcels, i.e. altitude, location and management applied and tested using linear regression.

Second, we calculated alpha diversity for each sampled community. We calculated three alpha diversity measures, including species richness, Shannon and Simpson indexes (Thukral, 2017) and tested if there were significant differences between treatments and sample types (using generalised linear mixed models (Beta family) (*glmmTMB* package):

$$Alpha\ diversity \sim Sample\ type + Treatment + (1 \mid Canton) \qquad Model\ 1$$

We also modelled Shannon and Simpson alpha diversity metrics using the management variables in terms of Nitrogen fertiliser used and number of pesticides applied, accounting separately for herbicides, insecticides and fungicides. We excluded Richness to focus on the relative abundance of arthropods. For this model (Model 2), we only included conventional parcels in the modelling because organic parcels do not use herbicides and barely any insecticide (Figure 5). We also included the amount of nitrogen added (excluding natural sources such as manure, because they were not quantified by the farmers) as an explanatory variable to model alpha diversity. We used these explanatory variables because they are standardised across parcels and did not lead to an overparameterization compared to using each single pesticide active compound as an explanatory variable. :

$$Alpha\ diversity \sim N\ Fert. + Numb.\ Of\ Herbicides + Number\ of\ Insecticides + Number\ of\ Fungicides + (1 \mid Canton) \qquad Model\ 2$$

In order to compare the management regimes prior to including soil arthropods, we performed a principal component analysis (PCA) of the management data to have an overview of which factors determine the labelling of a parcel as organic or conventional by each farmer. For this, we included the number of mowing events, total amount of Nitrogen added (in any form), the number of pesticides applied and the number of times pesticides were applied.

Active compounds of pesticides applied during all the previous years before soil sampling were used to compute an NMDS ordination to visualise the differences between management

regimes along the years recorded, i.e. 2016 until 2020. To further complement the two previous calculations, we compared the differences in dosage between the pesticides applied, grouping them by management type, i.e. conventional or organic, and type of pesticide, i.e. fungicide, herbicide and insecticide. We also grouped the total amount of pesticides applied, corrected by the amount of active compound to be comparable across commercialised pesticides, and visualised this by management regime and Canton.

Finally, we linked the NMDS ordination, based on the arthropod eDNA data, to the pesticides applied to each parcel. We used the *envfit* function within the *vegan* package in R to fit environmental vectors, i.e. the amount of active compound applied on each parcel per surface unit, on the NMDS ordination. Mapping pesticides on an ordination allows for testing the arthropod community as a whole. However, the ordination is a representation of the multidimensional scaling fitted into a 2D space, which adds a bias to consider as we are representing pesticides as 2D vectors on the NMDS ordination. Nevertheless, we considered this to be an interesting approach to identify pesticides with potential effects on the soil community. Each active compound was treated separately as the dependent variables to explain the ordination score of the NMDS, which was treated as the explanatory variables. We only retained significant active compounds ($p<0.05$), which was tested using a permutation test. This was done for the NMDS calculated separately for each Canton and for both together.

Results

DNA metabarcoding

After quality filtering, we retained a total of 1,193,760 sequences, distributed across 94 unique arthropod sequences. The three dominant orders were Hemiptera, Coleoptera and Diptera. Orthoptera, Lepidoptera and Thysanoptera were more abundant in Vaud than Valais (Figure 1). We visualised the sequencing results between treatments in order to have an overview of the main differences between arthropod groups (Figure 1). We also visualised the abundance of each species by arthropod order and family across the two sampled Cantons and sample types (between lines (L) and between stems (S)) (Suppl. Figure 2).

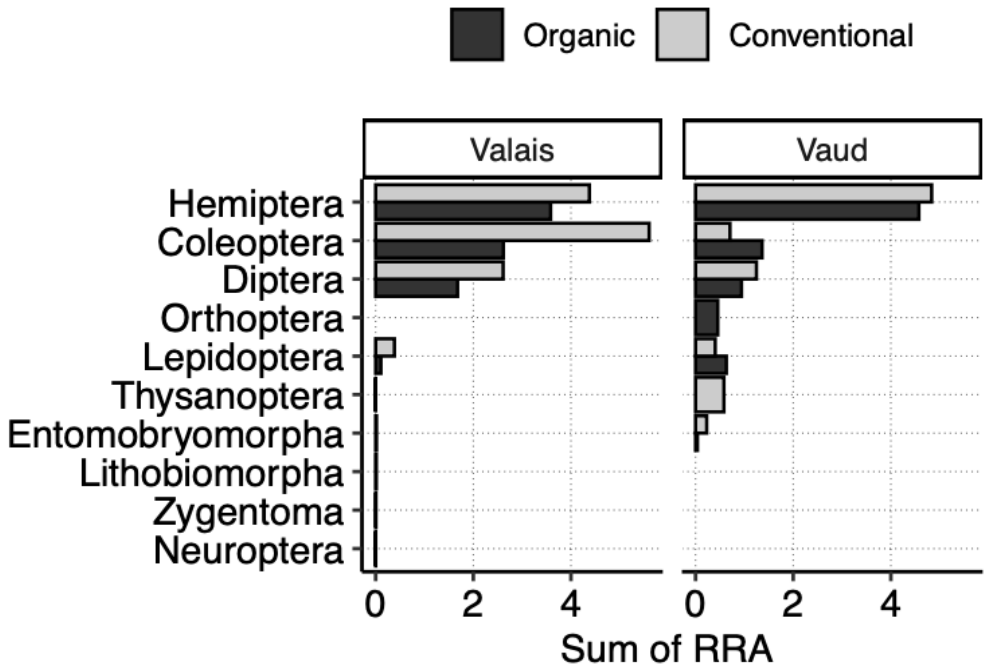


Figure 1. Barplot for the Sum of relative read abundance by arthropod order, Canton and management regime.

Soil community composition

We computed a non-metric multidimensional scaling (NMDS) to visualise differences in soil arthropod composition between the two types of treatment and sampled areas using a Bray-Curtis distance matrix using the relative read abundance (RRA) dataset (Figure 2A). We observe a clear distinction between the arthropod communities between the two Cantons (PERMANOVA, $R^2 = 0.11$, $p<0.001$, $F = 7.45$), this was also true for the P/A and FOO datasets (Suppl. Figure 3). However, the sampling position (i.e., samples taken between lines (L) or between stems (S)) does not seem to explain the differences in arthropod species compositions (PERMANOVA, not significant). Therefore, we treated L and S samples as biological replicates for each parcel in further analyses. We then computed separate NMDS for each Canton (Figure 2B, 2C) to minimise the soil type differences between Cantons.

Regarding the dissimilarity of arthropod species communities between management strategies (i.e. conventional vs. organic), results from the PERMANOVA by Canton, it does not seem to reveal a clear impact (PERMANOVA, not significant), although organic parcels seems to be more similar to each other than the conventional ones (Figure 2A, B, C).

Management regime

We observe a clear separation between management regimes but not as clear between regions in the PCA (Figure 3, extended in Suppl. Figure 4). Organic parcels are mowed more often and fungicides are applied during more days compared to conventional parcels, which have more herbicides applied (organic parcels don't apply herbicides). Moreover, organic parcels are less dispersed in the multivariate space compared to conventional ones, suggesting that the management practices are more similar between organic parcels than between conventional

ones. However, part of the variability observed could not be explained by any of the management variables provided.

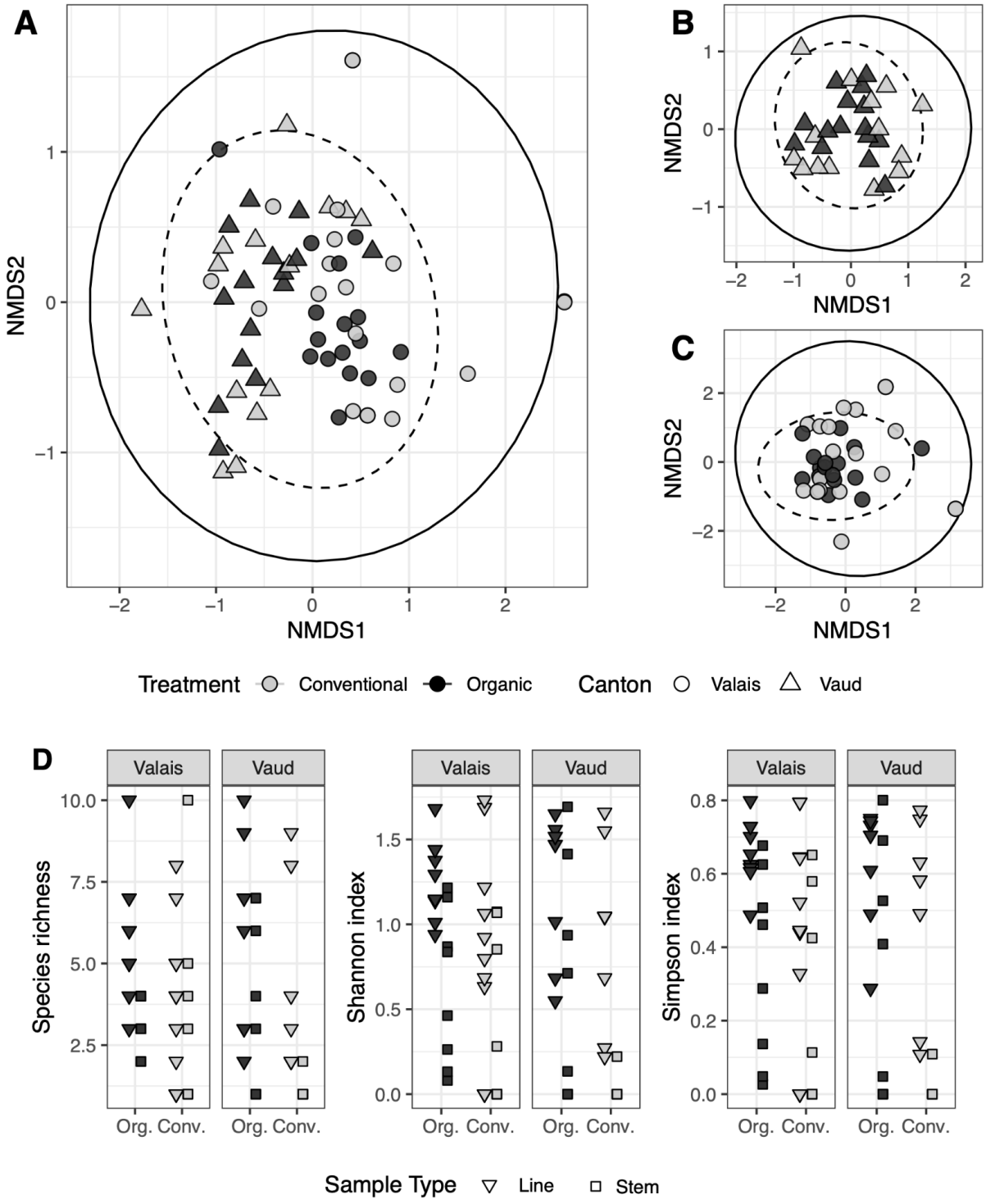


Figure 2. A) NMDS on the eDNA arthropod data using Bray-Curtis dissimilarity matrix (RRA) having two dots per parcel, one for the Stem sample and one for the Line sample. We grouped parcels by Canton. B) NMDS calculated only using parcels in Canton Vaud. C) NMDS calculated only using parcels in Canton Valais. The colour corresponds to the management

regime and the shape to the Canton. D) Alpha diversity measurements calculated for each soil sample collected (visualising the differences between Stem and Line samples).

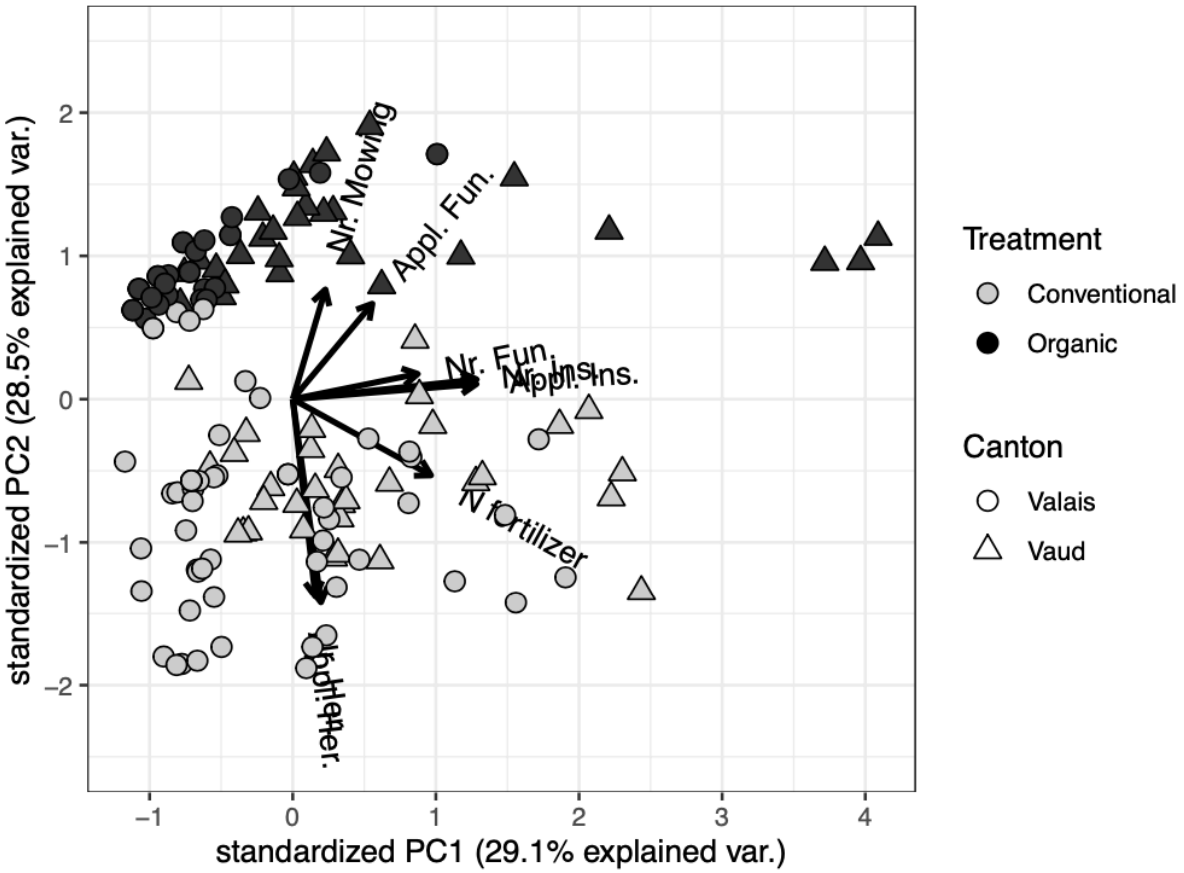


Figure 3. PCA of management practices for each parcel. Colour indicates the management regime and shape indicates the Canton. List of acronyms: *Nr. mowing*, number of mowing events during one year; *Appl. Her/Fun/Ins*, number of times pesticides were applied during one year; *Nr. Her/Fun/Ins*, number of different pesticides applied during one year; *fun*, fungicide; *ins*, insecticide; *her*, herbicide; *N fertiliser*, total amount of Nitrogen applied to a parcel as fertiliser, regardless of the type of fertiliser used.

We further investigated the differences between management regimes before studying its link to the arthropod data obtained. The NMDS ordination of the pesticides applied and the quantity used revealed a clear separation between the two types of managements along all the recorded years (Bray-Curtis distance, Figure 4A). Compared to the PCA, here we used as input the active compound of each pesticide applied and the amount used per surface unit. We observe clear distinction between management regimes, as in the PCA (Figure 3), but not between regions. Overall, we observe lesser dispersion among organic parcels than conventional ones consistently across the years. The only overlap occurs in 2020.

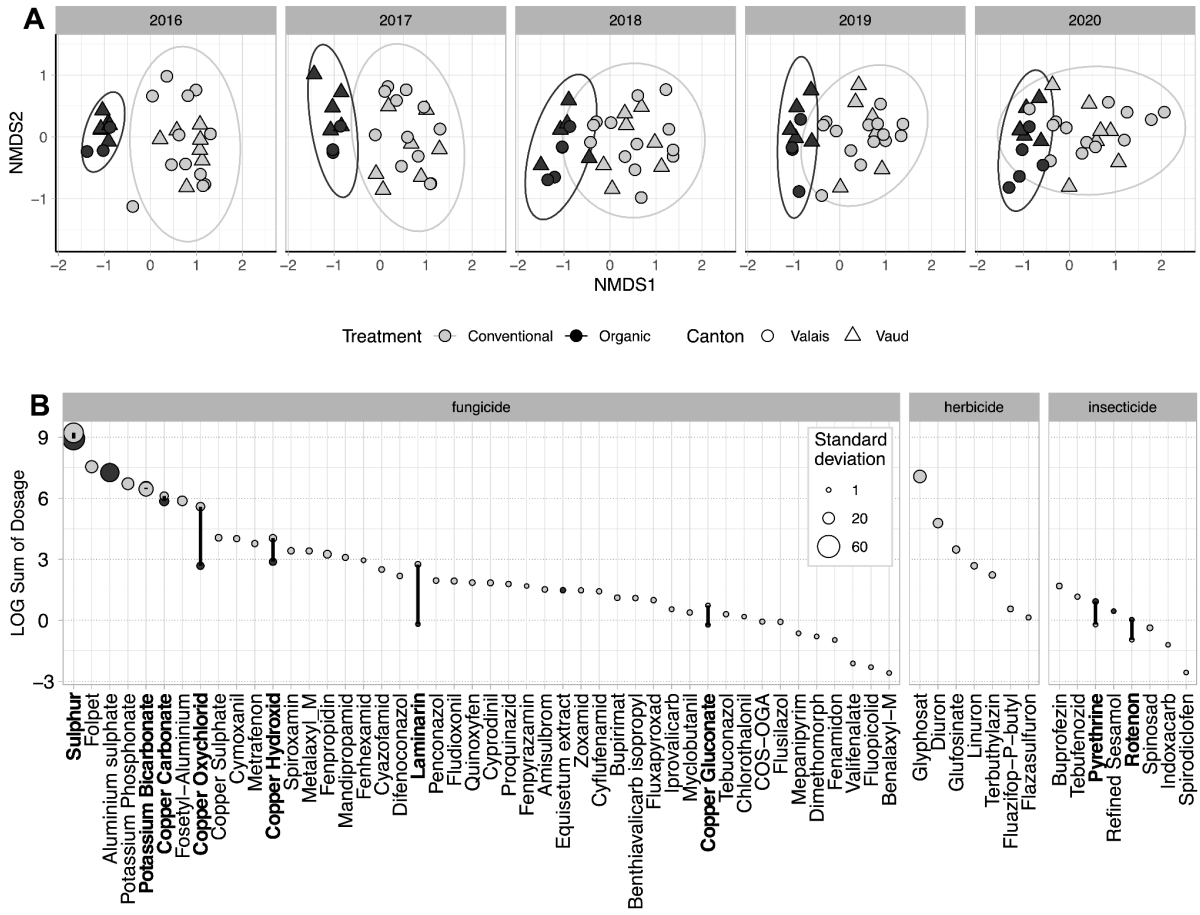


Figure 4. A) NMDS of pesticides used divided by year and management regime. Colours of the points indicate the canton and colour of the area indicate the management regime. B) Active compounds of pesticides applied by management regime and type of pesticide. Each active compound is shown in order from most to least applied components in terms of active compound per surface unit. The black lines indicate the difference in dosage between management regimes. The size of the dots refer to the variance between parcels. Active compounds in bold indicate that they are used by both types of management.

When comparing the dosage of added pesticides to the vineyards, only 9 substances were shared between management regimes (the ones with a black line in the figure), being sulphur the most used pesticide, for both (Figure 4B). Shared fungicides between managements were: Sulphur, Potassium bicarbonate, Copper Oxychloride, Copper hydroxide, Laminarin and Copper gluconate. Shared insecticides were Pyrethrin and Rotenon. The other pesticides are mainly used within conventional parcels, and the dosage used is similar between parcels compared to Sulphur. Another form of sulphur, aluminium sulphate, is only used in organic parcels. In terms of types of pesticides applied, i.e. herbicides, fungicides and insecticides, we observed clear differences between management regimes (Figure 5). Comparatively, the use of fungicides is similar between management regimes, and it is used abundantly in both types of management. Organic parcels completely avoid the use of herbicides and the use of insecticides is limited to few parcels, including two organic ones and six conventional ones.

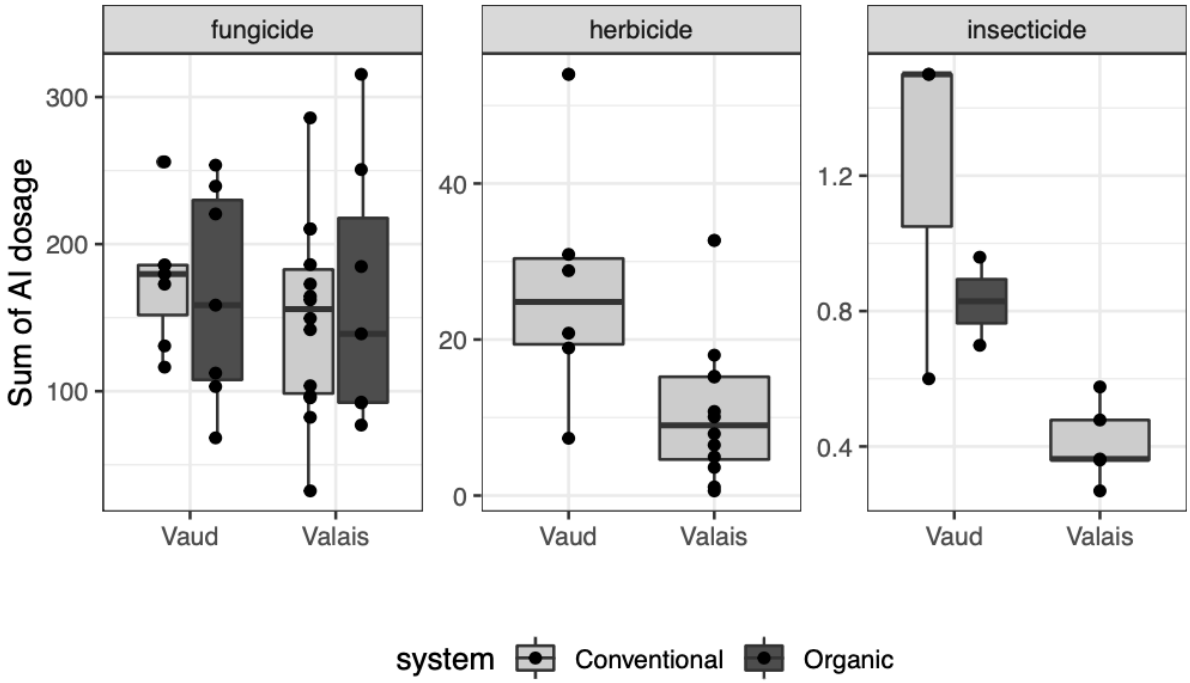


Figure 5. Comparison by regime on the amount of active compound (AI) added in total per parcel. Results are shown for each Canton and are grouped by type of pesticide. Herbicides are not used in organic parcels and no organic parcels within Valais used insecticides.

Alpha diversity

We were interested in comparing the effect of conventional and organic agricultural management strategies on the alpha diversity, estimated by three indexes, i.e, species richness, Shannon and Simpson indexes. Our results showed that organic parcels have an overall higher alpha diversity compared to conventional parcels for all the calculated metrics, which were more sparsely distributed (Figure 2D). However, some conventional parcels aligned to organic parcels and also showed high alpha diversity metrics. When comparing the alpha diversity between the two types of soil sampled in each parcel, soil samples taken between vine lines (L) showed a higher diversity than soil samples taken between vine stems (S) (Model 1, Figure 2D, Suppl. Table 2).

Next, we were interested in assessing the impact of management variables (i.e., the number of each pesticides and of the fertilisers) on the alpha diversity measured as Shannon and Simpson indexes (c.f. methods for details). When looking at the overall alpha-diversity, considering Line and Stems samples together, our model revealed a significant impact of herbicides on the two alpha diversity metrics, but only marginally when keeping sample types separately, both showing lower alpha diversity with greater use of herbicides (GLMM: Shannon index, p-value=0.07; Simpson index, p-value=0.09, Suppl. Table 3). In contrast, our results suggested that the number of insecticides, fungicides and Nitrogen fertilisation did not affect either of the two alpha diversity metrics (Supl. Table 3).

Soil arthropod community vs. management regime

The main objective of this study was to investigate the influence of the management regime and the applied pesticides on the arthropod soil community detected, using eDNA metabarcoding in each vineyard parcel. We first tested the correlation between management data (i.e. quantity of applied pesticides in terms of active compound) and RRA data using Mantel test but found no significant correlation between the two ($p=0.44$), suggesting that the management regime alone does not explain the arthropod species communities.

Next, we tested if there was a correlation between the management practices and the geographical location of the parcel in terms of the soil arthropod community, i.e. eDNA NMDS coordinates extracted from Figure 2. The two cantons, because they are located along the same river basin, have different altitude and longitude (Figure 6A). When comparing the two NMDS components, we only found significant differences on NMDS1 between Cantons (p-value < 0.001, Figure 6B) but not between management regimes. However, we found a significant correlation between the first NMDS component and the two geographical variables defining each canton, altitude and longitude (Figure 6C and Figure 6D). Overall, this seems to suggest that the differences of arthropod communities between parcels are mainly explained by geographical dissimilarities in environmental conditions.

In order to disentangle how the management regime affects the soil arthropod community, we used the *envfit* function to fit environmental vectors, in our case, herbicides, insecticides and fungicides, onto the arthropod NMDS ordination shown in Figure 2. We retained only the significant pesticides and visualised them globally (Figure 7A), including all three types of pesticides. The pesticides with the greatest R^2 value, among the significant pesticides found using the *envfit* function, were Linuron and Terbuthylazin (herbicides) and Tebufenozid (insecticide). We also detected many fungicides, with lower R^2 values, including Sulphur, Tebuconazol and Copper Sulphate among others. For Vaud Canton (Figure 7B), the significant pesticides were mainly fungicides, including Folpet and Laminarin, except for the insecticide Buprofezin. For Valais Canton (Figure 7C), we identified Terbuthylazin and Linuron (herbicides), Tebufenozid (insecticide) and five different fungicides, including Sulphur, copper sulphate and Fosetyl-Aluminium.

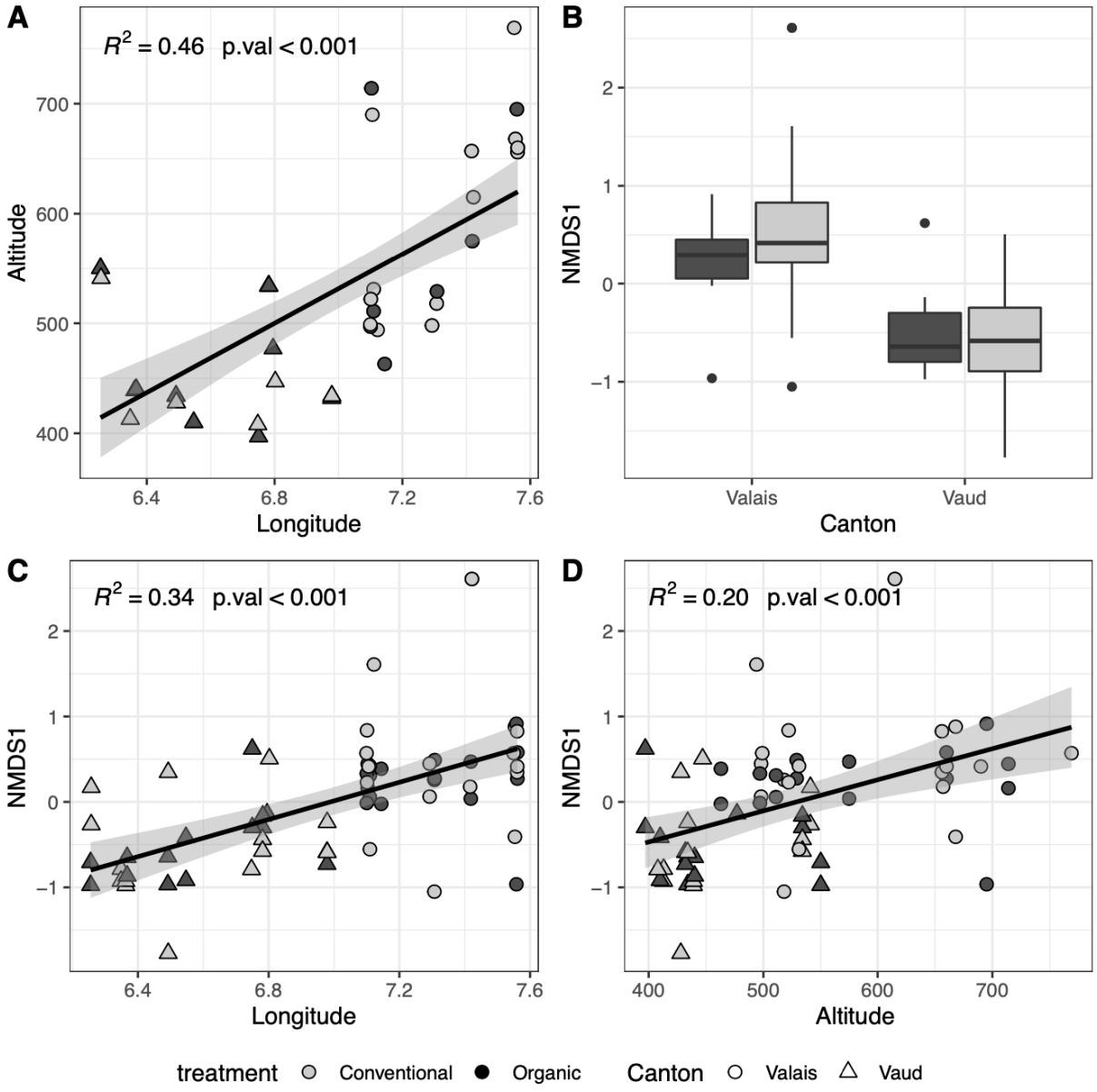


Figure 6. A) Altitude compared to longitude across the sampled parcels. Colour indicates the canton. B) NMDS1 by treatment and Canton. C) NMDS1 by longitude. D) NMDS1 by altitude. All correlations are significant. The R^2 value of each correlation is indicated within the figure, together with the p-value. All correlations are significant.

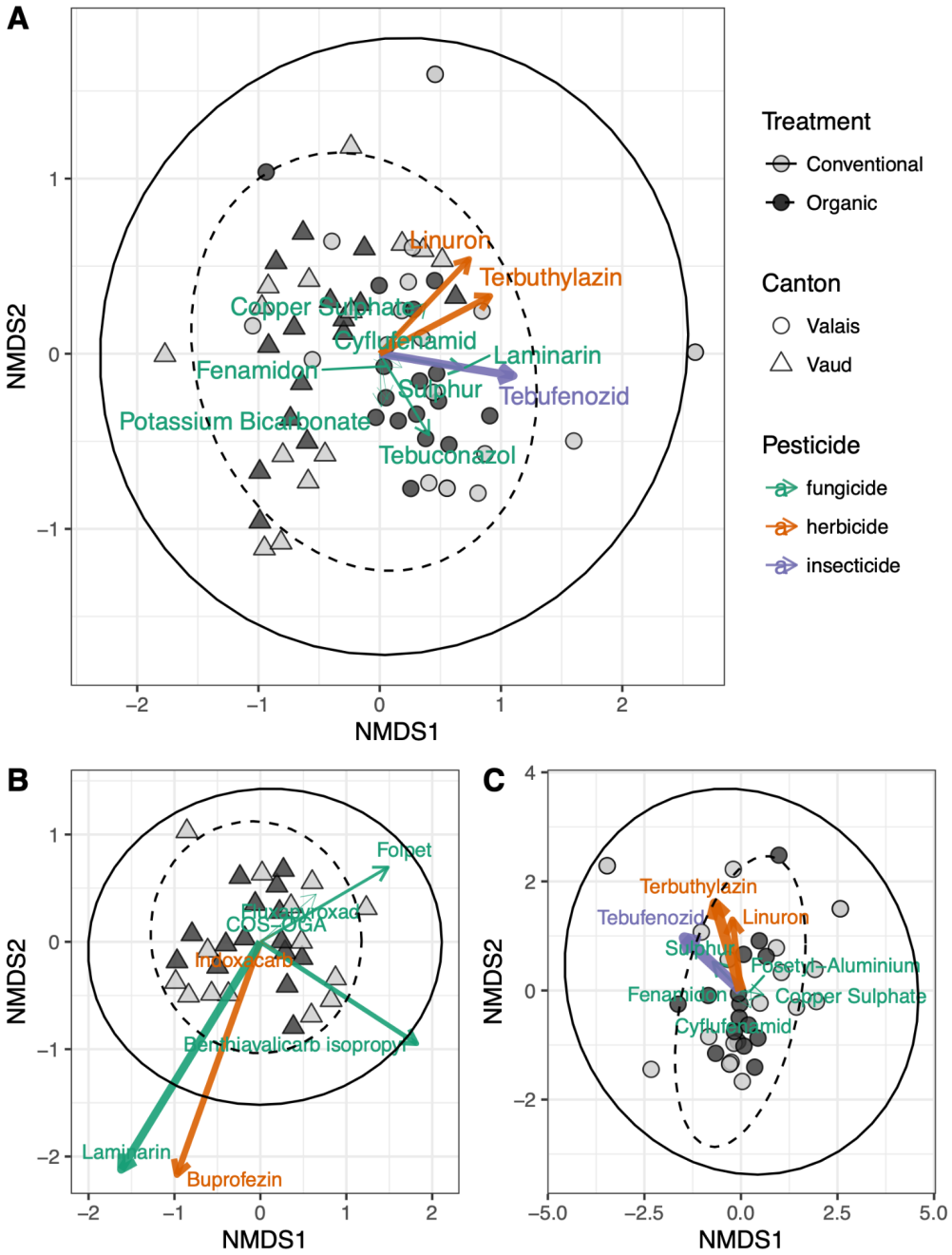


Figure 7. A) NMDS visualisation using the RRA distance matrix (Bray-Curtis) of the soil arthropod community as in Figure 2, with the results of *envfit* function represented. Each arrow corresponds to a pesticide which is significantly correlated to the dissimilarities in the soil community. The length and orientation of each arrow indicates the direction of correlation. The colour of the arrow indicates the type of pesticide and the thickness of the line increases with the R2 value of the correlation with the ordination. B) NMDS visualisation using the p/a distance matrix (Bray-Curtis) of the soil arthropod community for Canton Vaud with the results of *envfit* function represented. C) Same as B) but for Canton Valais. Only significant pesticides are displayed.

Discussion

Promoting the belowground biological community while maximising the aboveground productivity is a major challenge for agricultural management (Bender et al., 2016). Any potential modification on the management can have unknown consequences on the above- or belowground parts of the agricultural ecosystem, putting the success of any agricultural campaign at risk (van der Putten et al., 2009). Adapting management practices to improve the soil ecosystem can benefit the productivity of the crops, but this is a complex task because of the complexity and connectivity of the two parts of the agricultural ecosystem. With this goal in mind, new sustainable management practices and products are currently being developed, but their effects on the soil ecosystem are difficult to predict (Francis & Porter, 2011; Powlson et al., 2011).

Moreover, the geological properties of each agricultural region bring unique challenges to apply tested pesticides or protocols elsewhere (Tudi et al., 2021). Vineyards are particularly linked to this last point, as the conditions of the soil greatly determine the quality of the wine produced (Van Leeuwen & Seguin, 2006). However, the industry still relies on a substantial use of synthetic pesticides to ensure the production of grapes. These modify the soil ecosystem below and restrict the ecological services they could bring to viticulture, such as nutrient bioturbation and fertilisation, potentially improving the quality of the final product. However, more research is needed to better understand the effect agricultural pesticides have on the soil ecosystem in vineyards, as part of the greater goal to understand this connection for all types of crops.

Pesticides have cascading effects on the vineyard ecosystem, either because they are transferred to other parts of the environment through physical processes, such as runoff (Schriever & Liess, 2007) or leaching (Gonzalez et al., 2010), or because they spread in the food chain (Baudrot et al., 2020). Most studies have focused on understanding the effects the most commercialised pesticides have on the ecosystem, but much less is known on the combined effects when multiple pesticides are applied, or when these pesticides are rare or designed for a particular type of crop or agricultural region (Hough, 2021). To understand better the impact of such practices on the environment, we need to understand their impact on species composition. In this light, the use of eDNA can bring substantial advantages to monitor the current status of vineyard soils from a biological perspective and to better understand the effects the pesticides applied have beyond their target goal.

In this project, we compared the effects of conventional and organic management regime on the below-ground soil arthropod community using eDNA. We explored the effects of each regime on the arthropod community as a whole and by each individual pesticide, in order to quantify the relative impact each practice and pesticide has on the soil community. We profited from the extensive dataset provided by each farmer to study which pesticides are associated with the presence or absence of soil arthropods, to unravel potential effects they might be having on the soil arthropod community.

Soil arthropod community assessment

We found clear differences between Canton at community level (Figure 2A), suggesting that each has a unique community composition, regardless of the management regime. We observed a skewed dominance of certain orders in Valais compared to Vaud (Figure 1), regardless of the management regime. This might be explained by the soil physicochemical differences. Valais vineyards are drier and rockier than the Vaud vineyards. The significant correlation between the NMDS1 component, altitude and longitude (Figure 6C, 6D) indicates that the nature of the soil has a stronger effect on the soil arthropod community compared to the management practised (Figure 6B). These results advocate treating the two Cantons separately, to be able to disentangle the effect of management strategy from the effect of soil properties and climate on soil arthropod communities.

Management regime and pesticide use

The PCA revealed clear clustering by management types (Figure 3). We observed a close clustering of organic parcels indicating the practices in organic management are more alike than within conventional parcels, excluding the data on which pesticides are used. We interpret this result as the effect of stricter phytosanitary rules on organic labelled vineyards. This separation was also clear in the NMDS ordination of pesticides applied (Figure 4A). Organic parcels cluster closer together than conventional ones, consistently since 2016, indicating that organic parcels follow similar practices in terms of which pesticides are applied and in what amount, but are non-overlapping with conventional parcels. In fact, only when studying management through the pesticides applied we were able to find clear differences between management instead of between Cantons (Figure 4A). To better understand this separation in chemical products, the visualisation by active compounds revealed a detailed divergence between regimes. All parcels receive high sulphur and copper dosages, but conventional parcels receive a greater variety of fungicides and insecticides (Figure 4B). Additionally, they use herbicides, which organic parcels avoid completely. However, as a consequence they have to mow more frequently. The amount of sulphur and copper used reveals the main threat for vines in the sampled area is fungal infection. In vineyards, these two compounds, pure or bonded to other elements, are primarily used to prevent and control powdery and downy mildew infections, which is a prevalent risk to vineyards across the world (Dagostin et al., 2011).

Folpet and Glyphosate are a fungicide and herbicide respectively which are heavily used in conventional parcels (Figure 4B). The first is known to have no to low toxicity on insects, birds and mammals, but is highly toxic for aquatic species (Cabras et al., 1997). The second is more known and at the centre of controversy for its known negative impact on the ecosystem (Duke, 2018). It has a declared risk for terrestrial and aquatic species but the limited research on arthropod species, e.g. (Evans et al., 2010; Pereira et al., 2020) shows no clear risk for them. The information provided by the farmers provides an indication of which compounds are most likely to have an impact on the soil arthropod diversity, but this is only based on previous research that usually studies pesticides individually, potentially missing the impact of combined pesticide use. As such, the eDNA approach could unravel unknown effects of pesticides which were considered harmless to the soil ecosystem.

Alpha diversity and management practices

In terms of alpha diversity, we found conventional management vineyards to have an overall lower diversity compared to the organic ones (Figure 2D, Suppl. Table 2). This finding was consistent across regions, and confirms that conventional management practices are associated with lower soil arthropod diversity regardless of the type of soil or geographical location, which is interesting given the soil driven differences in terms of community composition (Figure 2A, Figure 6B-D). Nevertheless, we identified several conventional parcels where the alpha diversity measure was as high as for the organic parcels, suggesting other factors are taking part in the variability within conventional soils.

We found lower alpha diversity between stems than between lines, suggesting that, contrary to what we expected, compact soil between lines withholds more diversity than the spongier soil found between stems, where the soil compaction is lower. This could be because the products applied are deposited more easily between stems than between lines or because the compact soil between lines brings better conditions for arthropods in winter. However, our experimental setup was not oriented towards answering this type of question.

In fact, one of the potential limitations of this study is that sampling of the soil was done during early winter, when the arthropod activity in and on the soil is usually reduced (Langraf et al., 2021). As such, the amount of extracellular eDNA retrievable from the soil drops and is probably the lowest of the year (Valentin et al., 2021), but the cold temperatures and reduced activity favours the persistence of eDNA in the soil (van der Heyde et al., 2022). Certainly, a whole-year sampling would dramatically increase the resolutive power of the eDNA approach and improve our understanding of how vineyard management influences the soil arthropod community. We took a snapshot of the soil arthropod community in winter conditions, but we consider sampling should also be extended at least in summer to contrast the results found in this study. The pesticides described are applied along the year and there could be seasonal effects that we are not capturing here, such as in spring and summer, when the vine plants are the most sprayed to prevent pests and infections, and abundant precipitation enhances deposition of pesticides in the soil (Chen et al., 2019).

When modelling alpha diversity against the number of different pesticides applied, we only considered conventional parcels, as the only pesticides applied on organic management parcels were mostly fungicides and no herbicides (Figure 5). Number of herbicides was significantly linked to alpha diversity when Stem and Line samples were merged, but only marginally significant when kept apart (Suppl. Table 2). This result suggests that within conventional parcels, increased use of herbicides could be associated with a reduction of diversity in the soil arthropod diversity. The fact that insecticides were not significantly correlated could be because their use targets particular species of arthropods, which are already not present (or equally present). The poor correlation found between alpha diversity and the management variables suggests that such comparison is probably not the best for answering complex agricultural impact questions involving pesticide use. In line with the NMDS ordination, the distinct arthropod communities for each Canton suggests that the type of soil could be having

a major influence on the potential species composing the community, stronger than the detected effect of the pesticides applied.

Soil arthropods and pesticides

By using the eDNA approach, we have identified candidate pesticides that could be influencing the soil arthropods further than previously thought, or if so, confirming its toxicity. This is a wide spectrum approach that can be very useful for soil biomonitoring practices for agricultural purposes. We assessed the role of the pesticides applied, and the relative use of each pesticide in each parcel, using the *envfit* function, which calculates regressions on the NMDS ordination for each pesticide independently. When considering all data together, we detected fungicides, herbicides and insecticides as significant pesticides explaining the dissimilarity composition between arthropod communities. The vectors represented in Figure 7A point to soil communities from Valais, and as such, we can interpret that greater use of these pesticides is associated with the community shifts observed between the two Cantons in terms of soil arthropods. We highlight two herbicides, Linuron and Terbuthylazine; and one insecticide, Tebufenozid. Linuron is a wide spectrum herbicide (Maier-Bode & Härtel, 1981), Terbuthylazine is the active component of Lumax, a commercial herbicide that has been shown to persist in the soil and leak in the environment (Carretta et al., 2018). Tebufenozide is a moult-inducing insecticide that causes premature moulting in lepidoptera. It is very specific and considered of low toxicity (Addison, 1996) but interestingly, in this study we have found evidence pointing otherwise.

To better understand these differences, we reanalysed our data dividing parcels by Canton (Figure 7B, C). In Valais, our results indicate that this pesticide is linked to the arthropod community composition and aligns with the two insecticides detected. As mentioned before, the conditions are different between the two Cantons, and it is possible that distinct combinations of pesticides are being used to adjust for the climate and soil characteristics. To better understand these differences, we reanalysed our data but dividing the parcels by Canton. This suggests Tebufenozide (insecticide) could be interacting with Terbuthylazine (herbicide) and Linuron (herbicide) to enhance their biological impact in the soil ecosystem. Alternatively, they restrict the growth of weeds, which could also reduce the suitable habitats for soil arthropods (Freemark & Boutin, 1995).

We mainly found fungicides as significant pesticides using the *envfit* function (Figure 7). Despite not targeting the arthropod species, they are heavily used in the studied vineyards and could be acting as catalyzers of other pesticides used in the parcel and increase the toxicity of other insecticides and herbicides used (Cedergreen, 2014; Mesnage & Antoniou, 2018).

In this line, copper sulphate is used as fungicides in both types of management (Figure 4B), and was found significantly correlated to the arthropod community for both Cantons together (Figure 7A) and for Valais (Figure 7C). Much research has been done on the effects of copper accumulation in soil and its effects for the soil ecosystem (Karimi et al., 2021). Research done in vineyards found Glyphosate, the main herbicide used in the sampled conventional parcels (Figure 4B), to leak more in copper rich soils (Dousset et al., 2007). As Glyphsate was not significant, we cannot directly associate its leaking with copper as drivers of the soil arthropod

community. However, it is likely to have an effect on the soil as we identified herbicides reduce the alpha diversity on the studied parcels (Suppl. Table 2).
Interestingly, we detected Folpet (fungicide) within Canton Vaud. This fungicide inhibits cell division and is known to generate little resistance due to targeting basic cell metabolism (Arce et al., 2010) but it is known to be highly toxic for amphibians in the same environment (Adams et al., 2021). As such, our results indicate that its use in Vaud is correlated to the changes in arthropod community composition. However, we detected it has an opposite effect on the community compared to Laminarin (fungicide), which triggers a response in the vine plant to produce more defensive chemicals (Aziz et al., 2003). Altogether, this shows how the combined use of these fungicides could be reducing the soil diversity by targeting complementary biological processes.
Buprofezin is a common insecticide which inhibits the formation of chitin. It is used in the sampled conventional parcels (Figure 4B) to treat leaf-eating pests but it is banned in several countries because of its effects on non-target arthropod species (Liu et al., 2019). The fact that it is significant only for Vaud (Figure 7B) could indicate that the type of soil there facilitates the leaking of this pesticide in the soil and impact on the arthropods within. However, our results on insecticides could be misleading since insecticides are only used in four parcels in each Canton yet we observed a strong correlation for Buprofezin.

The insights provided by the approach presented here suggest the amount of fungicides identified, particularly the copper-based ones, could be indirectly enhancing the toxicity of other pesticides applied in vineyards and affecting the soil arthropod community. We suggest this can be the basis for further research on the effects of copper-based fungicides on the soil ecosystem.

Conclusions
In this project, we have studied the impact of vineyard management strategy on arthropods soil communities using eDNA high throughput sequencing, a sensitive method for detecting these species in the soil. We have described and compared the soil arthropod community composition and investigated the potential effects of management practices and pesticides used in the soil ecosystem. This study is part of a bigger research study, targeting bacterial, protist and arthropod communities in the same parcels as the present study. The consortium will also study the chemical properties of the soil and scan for the concentration of pollutants present. This information will provide a detailed view on how the pesticides used transfers into the soil. Combining the results from these studies will provide a better view at the effects management has across multiple levels of the ecosystem trophic network (Saint-Béat et al., 2015; Schmidt et al., 2021), and more robust conclusions can be reached regarding the cascading effects of pesticides deposited in the soil.

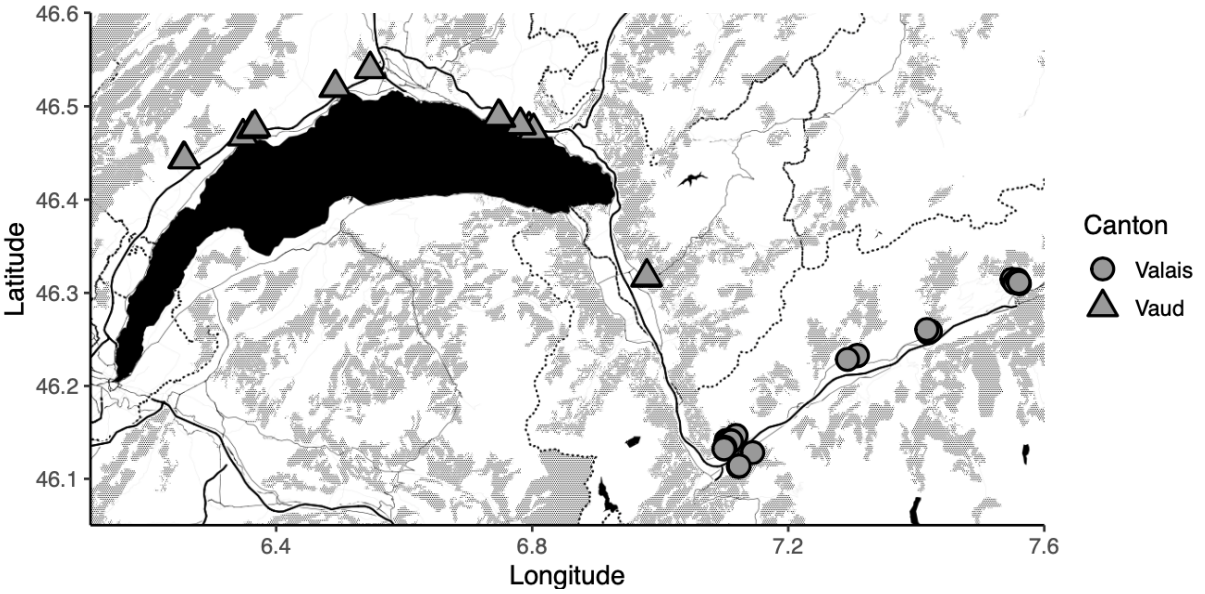
Acknowledgements

We thank the vineyards and their managers for granting access to sample their exploitations and provide the detailed surveys on the management done. We also thank Agroscope Changins for providing logistic support and coordinating the collaboration between ETH Zurich, Agroscope and University of Lausanne. We thank Claire Guerin, Molly Baur and Julie Guenat for the fieldwork assistance. We also thank Molly Baur for her assistance during the laboratory procedure. We thank Elias Barmettler for coordinating the pesticide and management databases. This project was supported by a Swiss National Science Foundation grant to LF (nr. 310030_192512). EM-C was supported by a fellowship in Life Sciences (Faculty of Biology and Medicine, University of Lausanne).

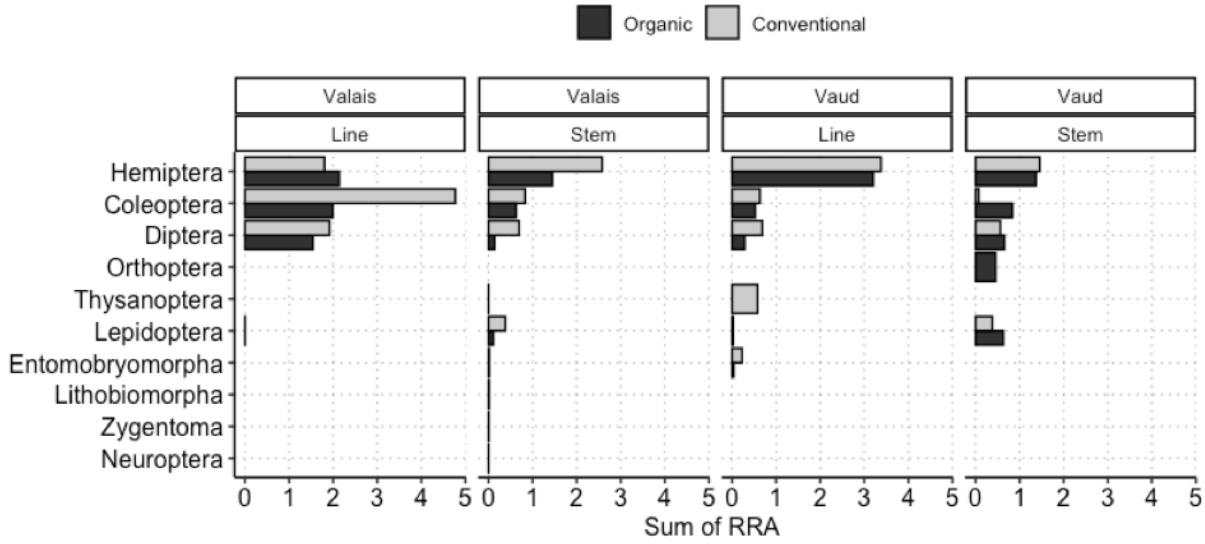
Conflicts of interest

The authors declare no conflicts of interest.

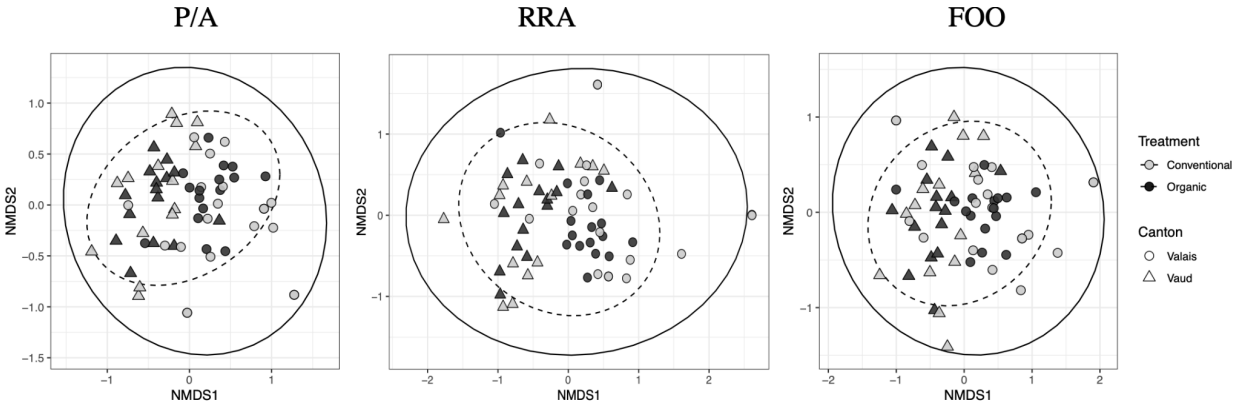
Supplementary material



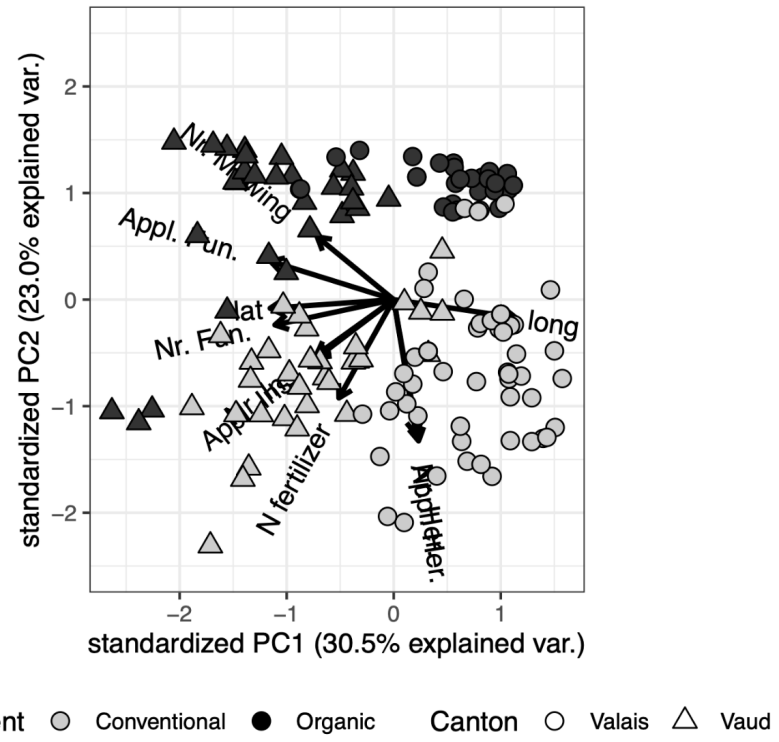
Supplementary Figure 1. Map of the locations of the parcels sampled. Colour stands for the Canton. All samples are taken within the Rhone river basin. The lake represented is Lake Geneva.



Supplementary Figure 2. Overview of the arthropod orders by Canton and by type of sample, i.e. S (stem, soil taken between vine stems) and L (soil taken between vine lines).



Supplementary Figure 3. NMDS comparisons between presence/absence (P/A), Frequency of Occurrence (FOO) and Relative read abundance (RRA) using Bray-Curtis dissimilarty matrix and keeping Stem and Line soil samples separated. In the three cases, organic parcels cluster closer together.



Supplementary Figure 4. PCA of management regime for each parcel as in Figure 3, but including latitude (*lat*) and longitude (*long*). Colour indicates the Canton and shape indicates the management regime. List of acronyms: *nr. mowing*, number of mowing events during one year; *appl.events*, number of times products were applied during one year; *prod.events*, number of different pesticides applied during one year; *fun*, fungicide; *ins*, insecticide; *her*, herbicide; *fertilizer.N.tot*, total amount of Nitrogen applied to a parcel as fertiliser, regardless of the type of fertiliser used.

Supplementary Table 1. Geographical location and management strategy for all the sampled vineyards. PI stands for conventional management and Bio stands for organic management.

ID	Canton	Town	Treatment	Altitude (m a.s.l.)
VD01	Vaud	Begnins	Bio	550
VD02	Vaud	Begnins	PI	541
VD03	Vaud	St Saphorin	Bio	477
VD04	Vaud	St Saphorin	PI	447
VD05	Vaud	Morges	Bio	434
VD06	Vaud	Morges	PI	428
VD07	Vaud	Rolle	PI	413
VD08	Vaud	Echandens	Bio	410
VD09	Vaud	Chexbres	Bio	534
VD10	Vaud	Chexbres	PI	534
VD11	Vaud	Perroy	PI	439
VD12	Vaud	Féchy	Bio	440
VD13	Vaud	Bourg en Lavaux	Bio	397
VD14	Vaud	Bourg en Lavaux	PI	408

VD15	Vaud	Aigle	Bio	432
VD16	Vaud	Aigle	PI	434
VS01	Valais	Salquenen	Bio	660
VS02	Valais	Conthey	PI	518
VS03	Valais	Salquenen	PI	660
VS04	Valais	Miège	PI	769
VS05	Valais	Miège	PI	668
VS06	Valais	Miège	Bio	695
VS07	Valais	St Léonard	Bio	575
VS08	Valais	St Léonard	PI	615
VS09	Valais	St Léonard	PI	657
VS10	Valais	Conthey	Bio	529
VS11	Valais	Salquenen	PI	656
VS12	Valais	Vétroz	PI	498
VS13	Valais	Charrat	Bio	463
VS14	Valais	Charrat	PI	494
VS15	Valais	Charrat	PI	500
VS16	Valais	Fully Plamont	Bio	714
VS17	Valais	Fully	PI	567
VS18	Valais	Fully	PI	690
VS19	Valais	Fully Liaudisaz	Bio	511
VS20	Valais	Fully	PI	531
VS21	Valais	Fully	PI	530
VS22	Valais	Fully Louye	Bio	497
VS23	Valais	Fully	PI	522
VS24	Valais	Fully	PI	499

Supplementary Table 2. Model 1 predictions for each alpha diversity metric. In all cases, management regime and sample type were significantly different.

Alpha modelling	Treatment	Sample type	Predicted	Std. Error	Conf. Low	Conf. High
Richness	Org.	Line	5.796948	0.118497	4.595522	7.312467
Richness	Org.	Stem	3.500291	0.139815	2.661298	4.603781
Richness	Conv.	Line	3.753307	0.128161	2.919601	4.825081
Richness	Conv.	Stem	2.266307	0.161927	1.650008	3.112801
Shannon	Org.	Line	0.617878	0.27503	0.48538	0.734895
Shannon	Org.	Stem	0.313294	0.271695	0.211272	0.43727
Shannon	Conv.	Line	0.373487	0.255203	0.265519	0.495726
Shannon	Conv.	Stem	0.143982	0.297126	0.085884	0.231432
Simpsons	Org.	Line	0.658255	0.282627	0.525375	0.770205
Simpsons	Org.	Stem	0.356747	0.276842	0.243771	0.488274
Simpsons	Conv.	Line	0.409177	0.260607	0.293561	0.535791
Simpsons	Conv.	Stem	0.166254	0.298217	0.100029	0.263488

Supplementary Table 3. Results for the models on Shannon and Simpson diversity using the management variables shown in the methods section. For both, only the use of herbicides was significant. List of acronyms: nr. mowing, number of mowing events during one year; appl.events, number of times products were applied during one year; prod.events, number of different pesticides applied during one year; fun, fungicide; ins, insecticide; her, herbicide; fertilizer.N.tot, total amount of Nitrogen applied to a parcel as fertiliser, regardless of the type of fertiliser used.

	AIC	BIC	logLik	deviance
SHANNON	-86.9	-78.1	50.4	-100.9
Conditional model	Groups	Name	Variance	Std.Dev.
	Canton	(Intercept)	1.43E-09	3.78E-05
Number of obs: 26, groups: Canton, 2				
Dispersion parameter for beta family()=0.776				
	Estimate	Std. Error	Z-value	P-value
(Intercept)	2.510617	1.533877	1.637	0.1017
fertilizer.N.tot2	-0.001013	0.003683	-0.275	0.7833
prod.events.ins2	-0.016574	0.150876	-0.11	0.9125
prod.events.fun2	-0.011301	0.012974	-0.871	0.3837
prod.events.her2	-0.165903	0.093589	-1.773	**0.0763**
	AIC	BIC	logLik	deviance
SIMPSON	-87.4	-78.6	50.7	-101.4
Conditional model	Groups	Name	Variance	Std.Dev.
	Canton	(Intercept)	1.35E-09	3.68E-05
Number of obs:26, groups: Canton, 2				
Dispersion parameter for beta family()=0.715				
	Estimate	Std. Error	Z-value	P-value
(Intercept)	2.630845	1.553847	1.693	0.0904
fertilizer.N.tot2	-0.001011	0.003735	-0.271	0.7866
prod.events.ins2	0.012398	0.150892	0.082	0.9345
prod.events.fun2	-0.013445	0.013149	-1.022	0.3065
prod.events.her2	-0.153847	0.093413	-1.647	**0.0996**

Chapter 4 - Spatiotemporal patterns of benthic macroinvertebrates in a natural glacier-fed stream using kick-net and eDNA sampling

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Author contributions

EM-C, NB, SL and LF designed the study and supervised all analyses. EM-C conducted the eDNA fieldwork and NB conducted kick-net fieldwork. EM-C carried out eDNA laboratory work and NB the taxonomic identification of kick-net samples. EM-C conducted bioinformatics and data analyses and prepared the figures. EM-C and NB wrote the first draft, with input from all other authors.

Abstract

The rapid melting of glaciers poses new challenges for hydrological management to balance sediment disposal and ecosystem biodiversity. In hydropower-impacted glacier-fed streams, there is an urgent need to better understand the biological dynamics within to offer sustainable solutions. In these ecosystems, benthic macroinvertebrates are used as water quality indicators, and in these streams, they are characterised by a high seasonality and spatial heterogeneity, which makes them complex to study. Moreover, the harsh conditions in alpine environments during winter has led to a bias on the collected data towards the summer months. In this light, recent new evidence suggests that the best conditions for macroinvertebrates to thrive could not be during summer as previously thought, when temperature is higher, but during winter, when the river is free of sediments as the glacier stops melting. In this project, we studied the seasonality and spatial heterogeneity of an undisturbed alpine glacier-fed river in Switzerland, sampling the macroinvertebrate community year-round. We aimed at understanding how macroinvertebrates use the spatial heterogeneity in the river system to cope with the changes in environmental conditions driven by the melting glacier. We combined the use of traditional kick-net sampling with modern eDNA techniques in order to both compare and complement the sampling strategies. We hypothesised: (i) the macroinvertebrate community would be more diverse during the winter months, aligning with lower sediment loads; (ii) tributaries, which are sediment free, would host a distinct macroinvertebrate community and have higher biodiversity during the summer months compared to the main course. We found more abundant taxa collected with kick-net were also more likely to be detected with eDNA. Regarding the seasonality, we did not find clear evidence for greater biodiversity during the winter months in the main course. However, we identified a reduction of total abundance of macroinvertebrates with increasing sediment load, produced by the melting of the glacier. Spatially, we found the community remained overall similar across seasons in the main course of the Val Roseg river, but experienced a clear shift within the tributaries at the start of the melting season. As we found the communities in the tributaries were most dissimilar to the main course, we propose the increase in sediments in the water marks the tipping point in the ecosystem. This suggests the lack of sediments in tributaries could be driving the macroinvertebrate community shift. However, we did not find evidence for colonisation patterns from tributaries or as spatial refugia. For hydropower-impacted streams, these findings indicate the macroinvertebrate community is sensitive to flushing of sediments, and advocate for developing strategies to offer sediment-free conditions for macroinvertebrates during the melting season.

Introduction

Alpine river systems are unique aquatic ecosystems characterised by high seasonal variability (Hannah et al., 2007). In the alps, river systems are usually fed by glaciers, which creates a strong seasonality in the water conditions due to glacier meltwater (Milner et al., 2009). In summer, rise in temperature melts the glacier and sustains the flow but adds a high sediment load in the water. The rise in sediments is due to glaciers being viscous fluids that flow downwards, eroding the rock beneath. There are other factors causing the erosion and consequent transport of sediments, like the increased sensitivity to weathering forces after the glacier retreats and exposes more ground. These sediments have a huge impact on the river benthic community, as they increase the turbidity and eroding power of the river, while reshaping the river course due to sediment accumulation (Brittain & Milner, 2001). In winter, low temperatures stop the glacier from melting, the surface of the river is frozen and the flow is reduced to a minimum. However, these systems are also fed by tributary rivers with groundwater origin or snowmelt-fed rivers, which are free of sediments. Their flow might be stopped during some weeks in winter or summer, but they offer different physicochemical conditions throughout the year compared to glacier-melt fed rivers, especially in terms of sediment load (Gabbud et al., 2019a).

A characteristic of alpine river systems is the presence of glacial floodplains. These are vast flat openings created by the retreat of a glacier (Malard et al., 1999, 2000; Tockner et al., 1997). Generally, their morphology is subject to frequent disturbances due to the varying hydrological regime driven by snow and glacier melt and the unconsolidated detrital character of the floodplain substrate, i.e. grains worn away from rocks. As such, channel stability is very weak and the braided stream is constantly changed, together with the multiple microhabitats within. They are important for natural resource management and play a crucial role in regulating water flow and soil stability. For example, glacial floodplains can store water during the wet season, releasing it gradually throughout the year (Müller et al., 2022). This helps to regulate water flow and prevent downstream flooding. They also act as carbon sinks, storing large amounts of carbon in the soil and vegetation (Tockner et al., 2002).

This ecosystem is home for species which are adapted to the harsh conditions of high altitudes and regular flooding, therefore highly specialised and often found nowhere else on earth (Brittain & Milner, 2001). Organisms inhabiting such ecosystems have adapted to deal with the acute seasonality, and many groups of species have developed specialised characteristics in order to proliferate in such environments. In fact, snow/glacier meltwater alters dramatically the physicochemical properties of the water flowing along the year and, subsequently, of the river habitats (Slemmons et al., 2013). In particular, benthic macroinvertebrates are a well-studied group of organisms and are thought to be greatly affected by these seasonal changes (Brown et al., 2007; Milner et al., 2023). They have to cope with the seasonal changes in the physical and chemical parameters of alpine rivers, such as temperature, flow rate, water quality and sediment load, which drive the composition and diversity of these macroinvertebrates (Giulivo et al., 2019). Previous research indicates an increase in biomass and taxonomic diversity of alpine benthic macroinvertebrates during summer together with the warmer

conditions, which are usually attributed to the higher temperature and sun exposure, enhancing biomass production within the river and boosts biological activity (Gabbud et al., 2019b). Nevertheless, most of the sampling expeditions take place during the warm periods, and little data is available for these ecosystems during winter time, when conditions are harshest. This has created a data bias towards summer months and has driven ecologists to associate warmer periods with greater biodiversity and abundance (Robinson et al., 2003). In parallel, research has also focused on potential windows of opportunity in autumn and spring, which have been studied over the last two decades.

Interestingly, recent research sampling alpine rivers during winter months has proposed an alternative scenario, as they found unexpectedly high diversity and activity during winter (Gabbud et al., 2019b). Their findings could be the base to a revolution in alpine river ecology, but more experiments are needed in order to disentangle the community dynamics along the year, i.e. comparing summer and winter communities. Historically, temperature has been considered to be the main driver behind community development, but the new insights point to a change of paradigm, suggesting sediment load could be of greater importance as biodiversity drivers on these alpine ecosystems. In this line, the new theory recently proposed suggests the peak of biodiversity and biomass of macroinvertebrates in glacier-fed streams is not in the summer months as previously hypothesised, but in winter (i.e. "Summer is in winter") (Gabbud et al., 2019b). The concept refers to the seasonal reversal of the biodiversity peak found in hydropower-impacted glacier-fed rivers. They found greater biodiversity in winter and an almost complete absence of life in summer, an opposite pattern compared to lowland water courses. This dynamic could also apply to undisturbed glacier-fed rivers, as similar environmental conditions occur and could be favourable for macroinvertebrates in winter months, despite the colder water.

Currently, alpine river systems and more specifically glacial floodplains are under great pressure because of their hydroelectric potential, as new constructions are being considered due to the climatic emergency (Brown et al., 2015; Gabbud & Lane, 2016). In existing hydroelectric exploitations, management authorities control the hydraulics of the system in order to balance hydroelectric output and ecosystem functioning, for example, by maintaining a minimum constant flow (Hirsch et al., 2014; Kuriqi et al., 2020). However, one of the factors which has been highlighted as problematic for this balance is the disposal of sediment accumulated in the alpine water intakes, which divert water from the river, modifying the natural water and sediment regimes. This is particularly problematic for glacier fed rivers, as they carry a high sediment load (Gabbud & Lane, 2016). Currently, dams are opened to rapidly increase the water discharge and carry out the sediment accumulated on the bottom, a procedure known as flushing (Crosa et al., 2010; Folegot et al., 2021). This carries downstream the sediments accumulated, however it changes deposition-erosion dynamics, destabilising river morphology and disturbing the macroinvertebrate communities and altering the whole ecosystem (Doretto et al., 2019).

Recent studies focusing on the impact of water intakes flushing suggests the changes in erosion-deposition dynamics of the river bed associated with the flushing events reduces the

biodiversity downstream of the dam (Espa et al., 2019; Lane et al., 2020). These events occur mainly during the summer months, when both the sediment load and water flow are greater due to the glacial meltwater. The benthic community is thus disturbed by water intake flushing, reducing the biodiversity during the summer months, when environmental conditions should be most favourable. The sudden change in flow, temperature or sediment load could be driving the macroinvertebrate benthic communities in river ecosystems, altering the expected seasonality in the river. As such, water carrying a high concentration of sediments (i.e. glacier milk) has a great eroding force and could act in the same way in natural river ecosystems as it does because of alpine dam flushing, i.e., reducing biodiversity and biomass of benthic macroinvertebrates. Furthermore, alpine floodplains are composed of multiple water channels, composing a braided system, all fed by the glacier meltwater. These may not be fed all year round and can completely dry out in winter or summer. Together with tributaries, they have lower water depth and velocity and usually a reduced sediment load carried from the glacier into the river (Gabbud & Lane, 2016). This is a key characteristic for creating a distinct habitat for macroinvertebrates, which provides refuge from the harsh habitat conditions within the main course, i.e. glacier-fed, part of the river.

Altogether, alpine dam flushing and glacier fed rivers converge on both having an ecological impact on the river benthos because of sediment erosion. Despite their dynamics being increasingly studied, the link between the geomorphology of alpine river systems and the distribution and abundance of benthic macroinvertebrates is still poorly understood. Because of this, research is now focusing on studying the impact of dam flushing on the river benthos, with an emphasis on benthic macroinvertebrates, as they are relatively easy to monitor and their presence and relative abundance serve as water quality indicators (Gresens et al., 2009). Thus, understanding of how macroinvertebrates respond to these disturbances, both in a natural setup and in an artificial one, will contribute to preserving the benthic community, water quality and power outlet. To answer them, systematic annual studies should be carried out to target the seasonal patterns of macroinvertebrate communities, accounting for spatial heterogeneity of habitat conditions and their suitability.

Collecting data on benthic macrofauna in alpine river systems is not an easy task. Traditional sampling methods, such as kick-net sampling, have been widely used to study benthic macroinvertebrates in alpine rivers (Hieber et al., 2005). This method involves physically turning over rocks and collecting the organisms that are present on the river substrate using a mesh placed downstream. However, this methodology requires entering the river and performing fieldwork under harsh climatic conditions, which sometimes can be dangerous or unfeasible. Furthermore, the procedure can be biassed by the operator sensitivity, and it is therefore hard to standardise. After kick-net sampling, taxonomic identification of the collected community is a tedious work that requires great taxonomic expertise to correctly classify each individual, and is one of the major drawbacks of this methodology (Pereira-da-Conceicoa et al., 2021). The morphological similarities between taxonomic groups also complicates their identification, and can limit the taxonomic resolution of identification, for example: to family or order level. In recent years, advances in molecular biology and ecology have led to the development of novel techniques for studying benthic macroinvertebrate communities in alpine

river systems. The use of environmental DNA (eDNA) metabarcoding is a non-invasive technique, which allows for the simultaneous detection and identification of multiple species from environmental samples (Taberlet et al., 2018). In aquatic environments, this method can be applied by filtering water through a fine mesh using a pump or directly collecting water to capture organic material released by organisms present in the river. The technique has proven to be a powerful tool for monitoring the biodiversity of benthic macroinvertebrates in alpine rivers (Brantschen et al., 2021). It can be used to better understand the seasonal variability of these communities and how environmental disturbances affect them. As such, eDNA metabarcoding techniques provide important complementary information to kick-net techniques and can be used to validate the results obtained from traditional methods and emancipate from their limitations. Together, these techniques provide a comprehensive picture of the benthic macroinvertebrates in alpine rivers, helping to shed light on the ecological processes and relationships that drive these unique and dynamic ecosystems.

In this project, we studied the seasonality of the benthic community in a complex alpine river system and the role of the physicochemical conditions on shaping its dynamics. We selected a pristine alpine river system at 2000m in Val Roseg (Grisons, Switzerland) and monitored the macroinvertebrate benthic community every two months during a whole year in order to assess both the changes of the community in the floodplain over the year and the role of the glacier melting and tributary rivers in defining seasonal biotic patterns. Samples were collected along the main channel and in tributaries using the two techniques described above, i.e. kick-net sampling and eDNA. We aimed at: (i) compare the two methodologies to identify their strengths and weaknesses, (ii) understanding the dynamics of the macroinvertebrate community along the year and which factors drive it, (iii) put community shifts in perspective of the physicochemical characteristics of each particular stream, in the light of its water source, i.e. glacier melt or groundwater, and (iv) disentangling the role tributary rivers play in a glacier fed river system for the benthic macroinvertebrates. Ultimately, we aimed at (v) putting the results found for pristine glacier-fed river systems in perspective to dam flushing.

Overall, this ambitious project aimed at better understanding the dynamics of macroinvertebrates in alpine rivers. We expect our findings to be comparable to other river systems and to improve management practices targeting macroinvertebrate biodiversity. The study of benthic macroinvertebrates in alpine rivers is a challenging but important aspect of freshwater ecology and conservation. In this line, the results of this project will serve as a compass for future studies in order to better integrate the available techniques at reach in order to improve the monitoring of river ecosystems.

Materials and methods

Study site

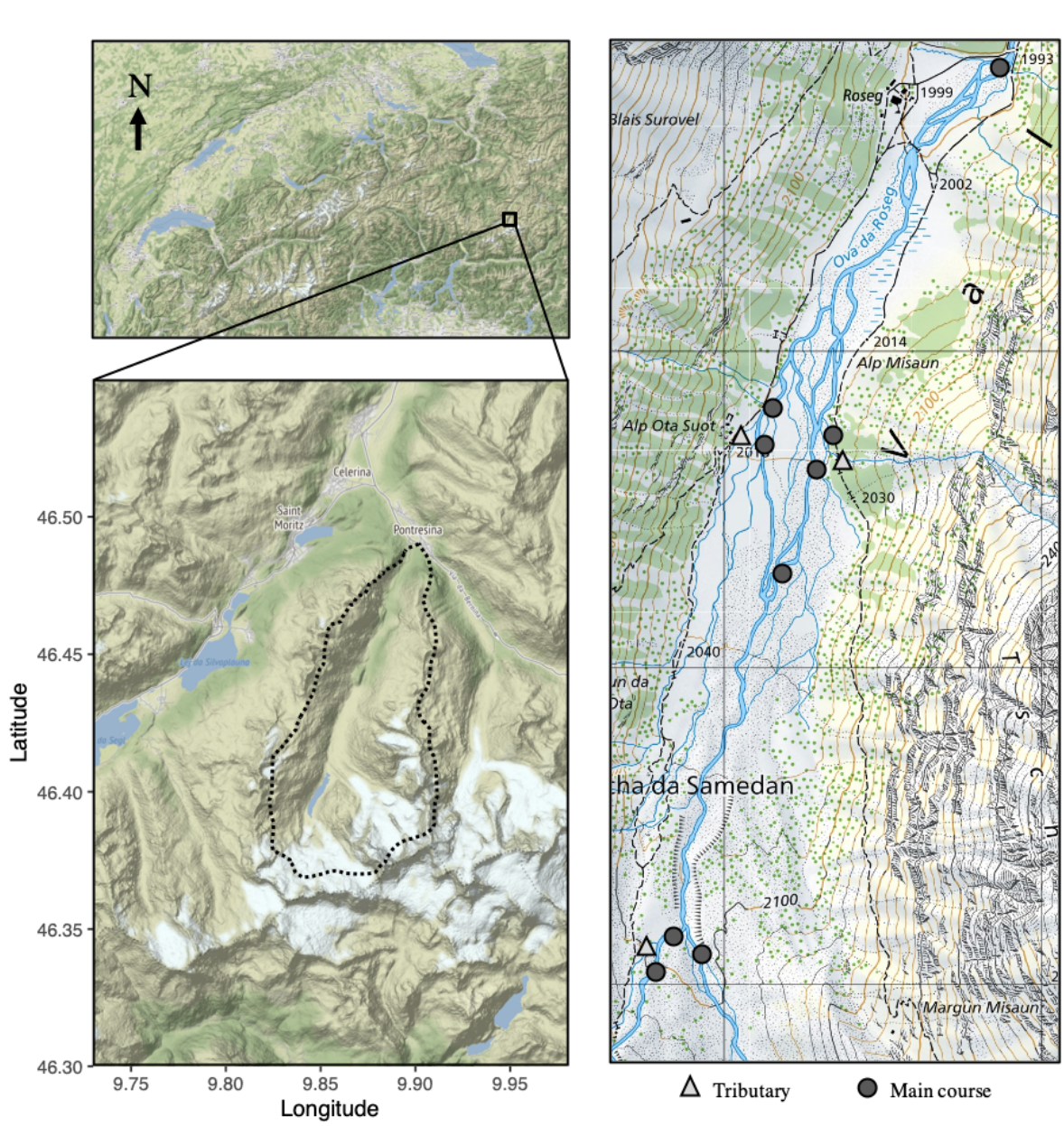

Figure 1. Map of the study region, with the Val Roseg boundaries highlighted in red. Top left image shows the location of Val Roseg within the alps and the Swiss plateau. Bottom left image shows a close-up of the study area comprising the Roseg Valley river catchment area (dashed line). Right image shows the Roseg floodplain in detail, with the dots indicating the sampling locations in the floodplain. Left images were generated in R. Right map was extracted from SwissTopo (map.geo.admin.ch).

The Val Roseg is located in the south-eastern part of the Swiss Alps, specifically in the south-eastern part of Canton Graubünden, on the border with Italy (Figure 1). The Roseg River originates on the Bernina massif at 2160 m a.s.l, from the natural proglacial lake called *Lej da Vadret* (or Lake Roseg), which collects the meltwater discharge of the Roseg glacier and other minor glaciers (Figure 1). The Lej da Vadret was formed naturally in the mid-20th century due to the damming action of the central moraine, with expected consequences on the distribution patterns of organic matter and the benthic community in the river, due to its retention capacity

of sediment loadings and the consequent decrease in downstream turbidity (Brittain & Milner, 2001; Burgherr et al., 2002; Tockner et al., 2002). The Roseg River is a second-order tributary of the Inn River, which is in turn tributary of the Danube River, and covers a distance of 11.3 km with a difference in altitude of 405 m, at the end of which joins the Bernina River (*Ova da Bernina*), before finally flowing into the Inn River (Burgherr & Ward, 2000; Tockner et al., 2002). The Roseg River drains a catchment area of 66.5 km^2, with an altitude ranging from 1755 m a.s.l (confluence with the Bernina River) to 4049 m a.s.l (Piz Bernina). The precipitation data collected between 1951 and 1980 indicate a mean value of 1600 mm/year, half of which fell as snow, while the annual mean flow is 2.76 m^3/s (over the period 1955-1997) (Burgherr & Ward, 2001; Tockner et al., 2002).

The river is mainly fed by the two glaciers, and therefore presents a typical glacial hydrological regime, with a peak flow in summer and a strong daily variability of the discharge. In winter the river flow depends mainly on krenal contributions and on the constant minimum flow provided by the lake, therefore the volume of water flowing through the valley decreases considerably, leading to the drying out of many channels in the floodplain (Malard et al., 1999). Over the past two decades, the structure of the river system has changed considerably under the stresses of climate change, which are leading to a rapid retreat of glaciers.
The floodplain of the Val Roseg has been described in detail (Malard et al., 1999; Tockner et al., 1997). It lies between 1995 and 2060 m a.s.l, is between 100 and 500 metres wide and covers an area of 0.67 km2 (Malard *et al.*, 1999; Zah *et al.*, 2001). Despite the intense daily discharge variation in glacier-fed streams and the instability of the material that characterises the floodplain, the Roseg floodplain is exceptionally stable, and some channels have remained unchanged for more than 50 years. dynamic and its morphological structure tends to change considerably over time. Along the floodplain, the river takes on a very complex braided structure, with a wide variety of channels with very different physical and chemical characteristics, forming a heterogeneous network of aquatic habitats (Tockner et al., 1997).

Sampling locations and experimental design
We selected twelve sampling sites to cover the floodplain, the tributaries and the two main sources of the Roseg river, i.e. Tschierva glacier and Roseg lake (Figure 2). Samples were collected between 2021-2022, in October, December, February, April, June and August. In a few cases, we were unable to collect samples due to the low temperatures or due to the scarcity of the flow. The experimental design consisted on sampling the junctions between the tributaries and the main course, in order to cover both and have an idea of the relative contribution of the tributaries in the main course. We selected three tributaries, i.e. T1, T2 and T3, but there are other tributaries feeding the floodplain, which we did not sample for logistic reasons. We added three sampling points away from the junctions in order to act as control samples of the main course at its source, i.e. Glacier site, at the middle of the floodplain, i.e. Main site, and at the end of the floodplain, i.e. Main end (Figure 2). Samples were labelled as part of the main course or as tributaries. Two sampling methodologies were used at each point: kick-net sampling and eDNA filtering.

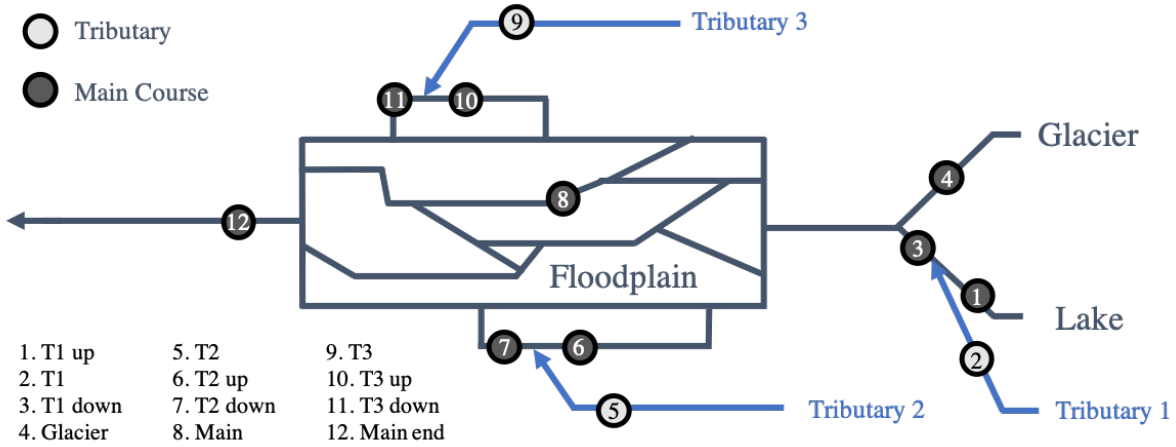

Figure 2. Schematic representation of the studied river system showing the 12 sampled locations, the sampled tributaries and the floodplain. The braided representation of the floodplain is not accurate and has only aesthetic purposes. The actual shape of the floodplain can be seen in Figure 1.

Kick-net sampling

For each sampling site, macroinvertebrates were collected using the kick-sampling method, following the standard procedure described by (Stark, J.D., 2001), in duplicates. A 25 cm x 25 cm kicknet with mesh gaps of 500 um was held downstream of the point to be sampled. The substrate material upstream of the net (0.35 m2, *i.e.* 30-40 cm x 100 cm surface area) was disturbed with the aid of a foot for the duration of approximately 30 seconds, allowing the raised material to flow independently into the net, carried by the current (Hauer, F.R., 2007). After roughly removing the larger grains and plant matter unintentionally collected, the contents of the net were transferred to bottles filled with 70% EtOH and refrigerated (95% in winter). Macroinvertebrates were later sorted using a stereoscopic microscope, counted and identified to the family level by means of the identification key drawn up by (Tachet et al., 2000), and with the help of the online portal *Perla* (DREAL, 2021).

In all cases, the downstream site was sampled first and the upstream site second, to avoid influencing the results or eDNA cross contamination, i.e. *T down* first. Important differences in habitat and microhabitat conditions can occur at the level of the individual sampling site, yet the two sets of data were collected by selecting habitats that were as similar as possible, specifically favouring riffle habitats. This was done to maximise the comparability of results between different sites, minimising data variability (Stark, J.D., 2001). Given the strong seasonal fluctuation in water discharge, and thus the variation in wetted width and water velocity, priority was given to keeping microhabitat conditions as constant as possible throughout the year and as consistent as possible between different sites.

Environmental variables

We measured three environmental variables at each sampling point: temperature (°C), electrical conductivity (µS/cm) and suspended sediment load (mg/L). These variables are often used to assess the glacial influence on the development pattern of the macroinvertebrate

community, and are often associated with the environmental harshness gradient that describes glacial river habitats (Castella et al., 2001; Dangles, O., 2012; Ilg & Castella, 2006; Lods-Crozet et al., 2001; Niedrist & Füreder, 2018).
Temperature and electrical conductivity were measured with a multi-parameter portable metre (WTW MultiLine Multi 3630 IDS), calibrating the respective probes daily before taking measurements. For the sediment load, a 500 mL sample of water was collected at each sampling site. In the laboratory, the water was filtered through PES 0.22m membrane filters with the help of a pump filtration system. The filters were then dried completely in the oven at 105°C (for approximately ~2h30) and the dry sediment mass derived with an analytical balance.

eDNA sampling
At each sampling location (Figure 2), eDNA water samples were taken in triplicates following the methodology of (Pont et al., 2018), with modifications. In brief, 30L of water was pumped through each eDNA capsule (0.45um, Waterra, Canada) to capture DNA from aquatic organisms, which is retained in the filter within. A total of 90L of water was pumped for each sampling location. Capsules were then sealed on one end and 40mL of NAP buffer (see (Camacho-Sanchez et al., 2013) for details) were added using a syringe before sealing the other end to preserve the eDNA until extraction could be carried out at the University of Lausanne.

eDNA extraction
Extractions were performed in a pre-PCR laboratory dedicated to low DNA-content analyses, using a protocol modified from (Pont et al., 2018). The filtration capsules were shaken thoroughly for 2 min to maximise DNA yield from the filter. The content of the capsule was then transferred to 50ml Falcon tubes using a syringe. All the material for this procedure was sterilised between samples. Tubes were then centrifuged at 7500 rpm for 1h at 4°C. Supernatants were discarded and 1440 µl of ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen) and 40 µl of proteinase K were added. After vortexing and resuspending the pellet, the mixture was transferred to 2 ml Eppendorf tubes and incubated for 2h at 56°C. The DNA extraction was pursued following the NucleoSpin Soil Kit protocol (Macherey-Nagel). Elution was done with 2 x 50 µl of SE buffer. Negative controls were included at all steps. The extractions were tested with qPCR to identify the best PCR conditions both in terms of dilution fold and PCR cycles. qPCR reagents and conditions were the same as in PCR amplification (see below), with the addition of SybrGreen (Thermo Fisher Scientific). Based on the results, all samples were subsequently diluted 10-fold before PCR amplification.

DNA metabarcoding
DNA extracts were amplified using a generalist arthropod primer pair (*Arth02*, (Taberlet et al., 2018)). *Arth02* targets the 16S mitochondrial rDNA (76-168 bp) of all arthropod species. Each biological replicate was amplified in 3 PCR replicates (i.e., 9 replicates per sampling location and season). Thermocycling conditions were as follows: denaturation at 95°C for 10 min, followed by 55 cycles of 30 s at 95°C, 30 s at 49°C and 1 min at 72°C, with a final elongation step of 7 min at 72°C. Each 96-well PCR plate contained blanks, negative extraction controls, negative PCR controls, and positive controls (DNA assemblies of a mix of 8 arthropod species, see Suppl. Table 1). Blanks correspond to empty wells and allow to estimate the proportion of

tag switches (i.e., false combination of tags, generating chimeric sequences) occurring during the sequencing process (Schnell et al., 2015). Amplifications were confirmed on a 1.5% agarose gel, and PCR products were subsequently pooled per PCR plate. Pooled amplicons were purified using a MinElute PCR Purification Kit (Qiagen). Purified PCR products were quantified using a Qubit Fluorometer (Life Technology Corporation). To reduce tag jumps, we followed the Tagsteady library preparation as in (Carøe & Bohmann, 2020). Final libraries were quantified, normalised and pooled before 150 paired-end sequencing on an Illumina MiniSeq sequencing system with a High Output Kit (Illumina, San Diego, CA, USA).

Bioinformatic data analyses

The bioinformatic processing of the raw sequence output and first filtering was done using the OBITools package (Boyer et al., 2016). Forward and reverse reads were assembled with a minimum quality score of 40. The joined sequences were assigned to samples based on unique tags combinations. Assigned sequences were then de-replicated, retaining only unique sequences. All sequences with less than 100 reads per library were discarded as well as those not fitting the range of metabarcode lengths. This was followed by two different clustering methods. First, pairwise dissimilarities between reads were computed and lesser abundant sequences with single nucleotide dissimilarity were clustered into the most abundant ones. Second, we used the Sumaclust algorithm (Mercier C, 2013) to further refine the resulting clusters based on a sequence similarity of 97 %. It uses the same clustering algorithm as UCLUST (Prasad, D.V., 2015) and it is mainly used to identify erroneous sequences produced during amplification and sequencing, derived from its main (centroid) sequence. Sequences were taxonomically assigned to taxa with a database for Arth02 generated using the EMBL database (European Molecular Biology Laboratory).

Further data cleaning and filtering was done in R (version 4.0.2) using the metabaR package (Zinger et al., 2021). Sequences that were more abundant in extraction and PCR controls than in samples were considered as contamination and removed. Operational taxonomic units (OTUs) with similarity to the reference sequence lower than 97 % were also eliminated from the dataset. Removal of tag-leaked sequences was done independently for each library. This approach allowed us to discard single OTUs instead of whole PCR replicates. However, PCR replicates with too small reads count were also discarded. Each taxonomic assignment was manually inspected using the BLAST algorithm of GenBank to account for potential mis-assignation, because we first used a relatively low similarity threshold. For cases with multiple candidate species, the geographic range was considered to select the correct species or the OTU was assigned at a higher taxonomic level.

Remaining PCR replicates were merged by sample location and sequence, keeping the mean relative read abundance (RRA) and the P/A (presence/absence) for each OTU. We also grouped the OTUs by species, family and order.

Statistical analyses

All downstream analyses were carried out using R software (Version 4.0.2). First, we calculated a principal component analysis (PCA) to visualise the differences between environmental conditions across the sampled locations for all sampled locations and seasons. Second, we calculated the coverage of the sequencing efforts for each sample and season.

We then compared the retrieved taxa with the kick-net sampling data, and visualised which methods covered each taxon across seasons. In order to compare the similarity between the two methodologies, we transformed the eDNA data into presence/absence, and the kick-net sampled individuals into proportions of individuals. We modelled the likelihood of eDNA detection based on the proportion of individuals sampled for each season separately using a generalised linear mixed effect model (Binomial family) (Figure 6):

$$P/A \sim Proportion\ of\ individuals * Season + (1|Sample) \qquad Model\ 1$$

Model 1 included only eDNA data from taxa which had been found using kick-net sampling, as the goal of this methodological comparison would have been misleading due to the greater diversity of taxa detected with eDNA, which included species which are not present in the benthos of the river sampled with kick-net, but rather come from upstream organic material shed by organisms, which is impossible to detect through the kick-net macroscopic approach.
We also calculated three alpha diversity measurements for the two methodologies, i.e. Richness, Shannon and Simpson index. Third, we calculated the dissimilarity matrix (Bray-Curtis distance using RRA data grouped at order level) for each sample and visualised the dissimilarity between individuals using a non-metrical dissimilarity scaling (NMDS) all together and divided by season. We followed the same procedure for the kick-net data and also compared the two dissimilarity matrices using the Mantel test. Fourth, we used generalised linear mixed models (GLMM), with the *glmmTMB* package, to investigate to what extent the variables (scaled and centred) influenced the likelihood of eDNA being detected. eDNA data (present/absent) were fitted to the explanatory variables extracted calculated from the kick-net sampling data, i.e. transforming the number of individuals for each taxon into proportion of individuals, with the assumption that relatively more abundant taxa would be more likely to be detected through eDNA. We also used GLMM to investigate the influence of the main environmental variables, i.e. sedimental load, conductivity and temperature, on the Shannon alpha diversity metrics calculated for both kick-net (Beta binomial family) and eDNA (Tweedie family) sampling methodologies:

$$Shannon \sim Sediment\ load + Conductivity + Temperature \qquad Model\ 2$$

Ultimately, we merged the community compositions of the tributaries and the above-junction samples and compared them to the below-junction sample in order to investigate the relative contribution of tributaries on the main course arthropod composition. We calculated this for each junction and season to investigate the dynamics along the year.

Results

Environmental conditions

We visualised the differences between environmental conditions using a PCA (Figure 3). We found samples from the same season shared similar physicochemical conditions. Sedimental load was attributed to the warmer months, i.e. June and August. Similar to temperature, which

was attributed to June, August and October. Conductivity was higher for the colder months, i.e. December, February and April. Tributaries were distributed in the PCA ordination along the conductivity-temperature axis, as they are exempt from the sediments released from the melting glacier. However, we identified two tributaries in august with high sediment load (Suppl. Figure 7). This was probably due to a landslide which occurred between the samplings of June and August and affected T1 and T2. After visualising the environmental data, we categorised the arthropod community data into the two melting regimes in Val Roseg. We found the biggest change of environmental conditions occurred between February and April, aligning to the melting of the glacier and snow (Supl. Figure 2). As such, we labelled October, December and February as "Freezing" seasons and April, June and August as "Melting" seasons.

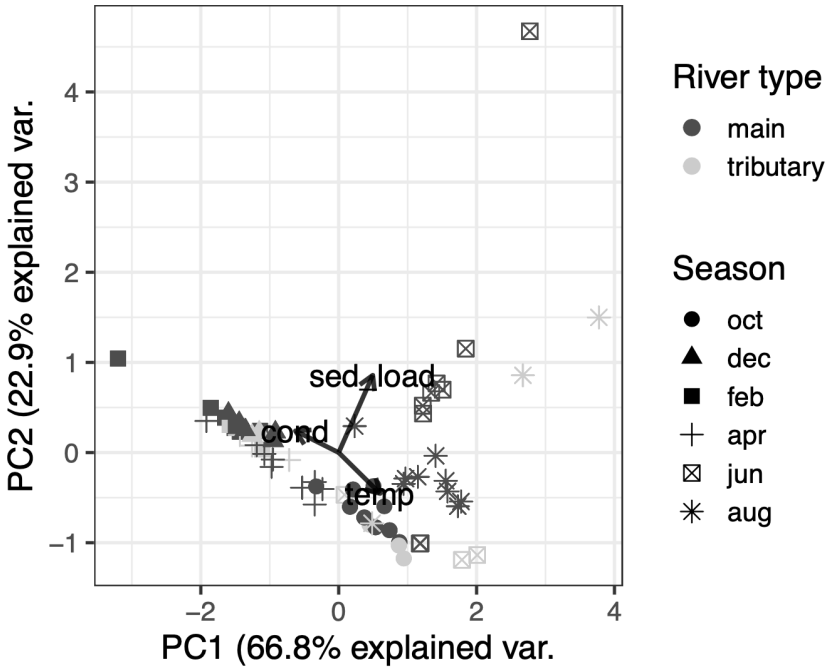

Figure 3. PCA visualisation of the environmental variables. Colour indicates if the sample was taken in the main course or in a tributary. The three arrows correspond to the computed environmental variables, i.e., sedimental load, conductivity and temperature.

eDNA and kick-net sampling

After quality filtering, we retained a total of 11,409,147 reads, distributed across 257 distinct sequences. The three most abundant orders were Diptera, Ephemeroptera and Collembola (Suppl. Figure 1), which were consistently found across sampling seasons. For kick-net sampling, a total of 149,276 individuals from 24 different families were collected and identified to the family level. These included arthropods, molluscs, annelids and platyhelminthes (Suppl. Figure 2).

eDNA sample coverage

The three samples from T1 junction in october were not sequenced and T1 samples in february were not collected due to the scarcity of the flow, which impeded filtering any water, so we

couldn't include these 4 samples in the analyses. Out of the 68 samples collected and sequenced, we were able to obtain sequences from 61 of them (Figure 4). Among the samples with sequence yield, we found a great amplitude of reads obtained for each replicate (Figure 4), and between samples, some yielded up to 100 times more reads than others. In those samples, the variability of reads was also higher. We did not observe any clear signs of tributary samples yielding less reads than main course samples.

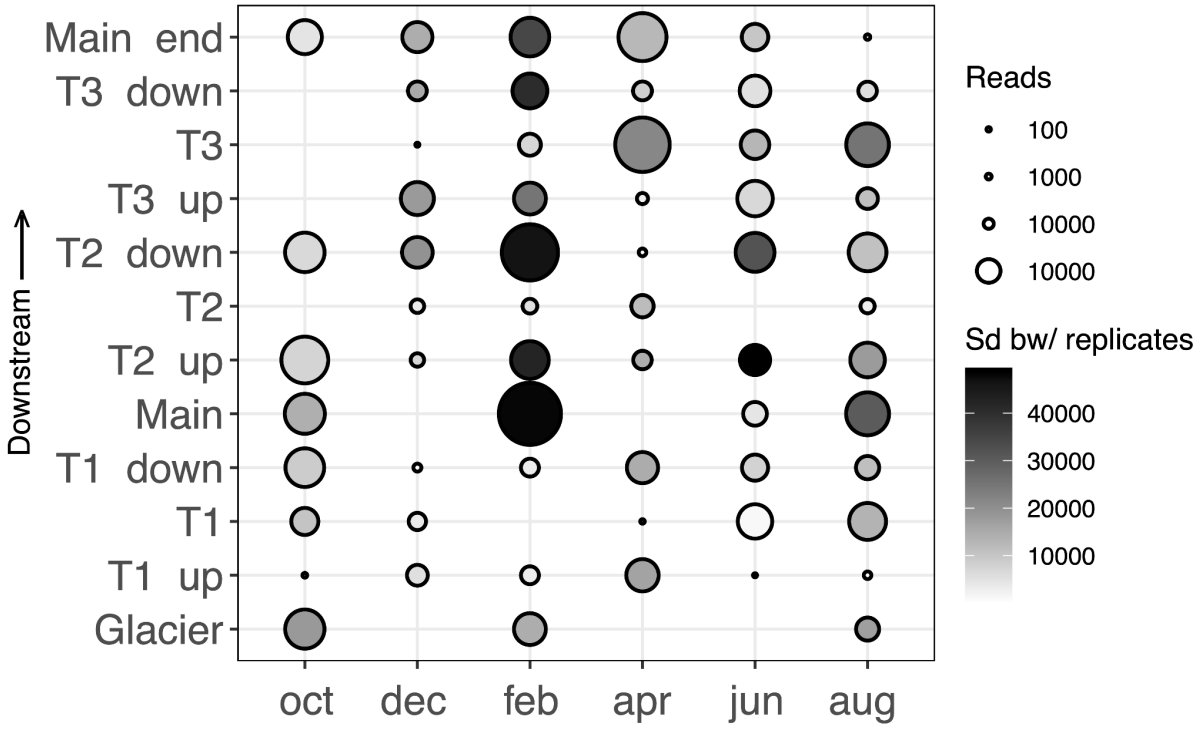

Figure 4. Coverage of the samples across seasons in terms of retrieved eDNA reads. Size stands for the number of reads obtained among replicates and colour indicates the standard deviation between the sampled triplicates (i.e. one filter capsule each). Although there is some variability across samples in terms of number of reads, most of the samples retrieved reads to some extent. T1 samples in October have not been sequenced yet. *Sd bw/* stands for standard deviation between replicates

Comparison between kick-net sampling and eDNA metabarcoding

We visualised the differences in taxonomic coverage between the two methodologies in Figure 5. We kept Ephemeroptera, Trichoptera, Plecoptera (EPT) and Diptera as separate orders and grouped all the others because of their relative higher abundance across the sampled seasons (Suppl. Figure 2). In Figure 5, we considered a single count as the combination of a single family in a particular sample. As such, we were able to distinguish between orders found using both methodologies or only one of them. Diptera was the order which was found most consistently across seasons and methodologies, but it was also the most abundant one. Overall, kick-net sampling detected more EPT orders compared to eDNA. However, the clear

dominance of eDNA among the *Other* orders shows that this methodology can detect taxa which are not physically present in the riffle habitats selected for kick-net sampling.
When comparing the similarity between the data collected with the two methodologies (Figure 6), the proportion of individuals significantly explained the likelihood of eDNA detection (Model 1, all seasons together, $p=0.0162$), but this was not shared across all seasons.

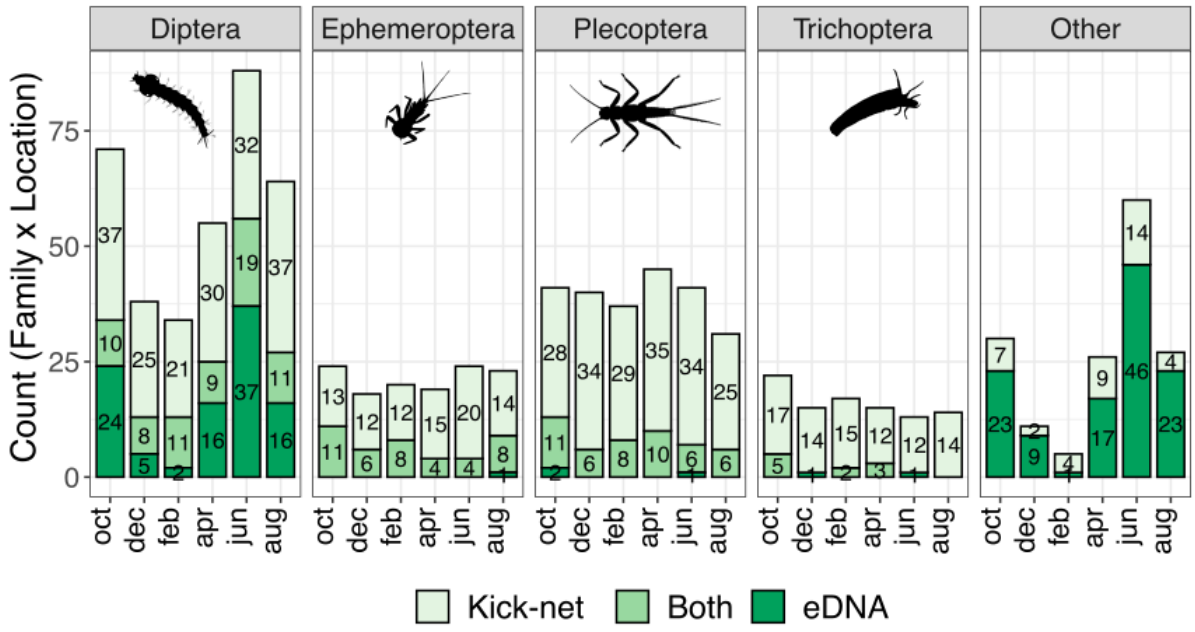

Figure 5. Comparison between methodologies in terms of families found in each location across seasons. We grouped all orders that were not Diptera, Ephemeroptera, Plecoptera or Trichoptera as *Others*. The first four orders mentioned are the most common groups in alpine river systems and were clearly more abundant in the kick-net sampling.

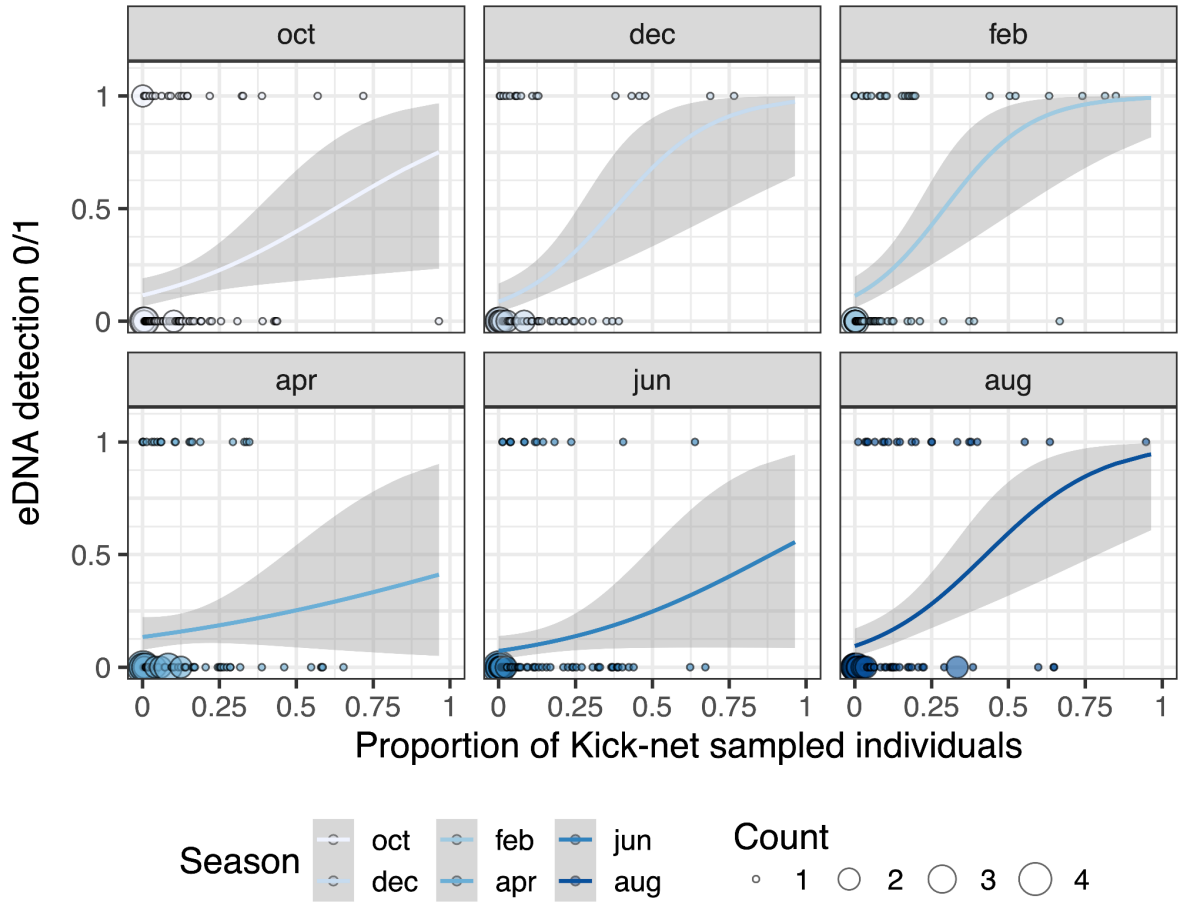

Figure 6. Modelled prediction of eDNA detection based on the proportion of kick-net sampled individuals (Model 1). Size of the dots indicates count of occurrences, and colour scale indicates the season. The coloured line indicates the model's predicted values with its confidence interval in grey. For this figure, we only retained sequences of taxa found through kick-net sampling, as the objective here was to compare the two methods and if the results were relatable.

Seasonality of the arthropod community

We visualised the arthropod community detected with eDNA on an NMDS ordination (Figure 7A). We did not find clear differences to identify each season distinctively, but when grouping the samples by the melting regime of the glacier, we found the communities of the main course of the river were close on the ordination whereas the tributary communities revealed a more acute shift between the freezing and melting dynamics of the water system (Figure 7B). For this multivariate analysis, we excluded 6 samples which contained uniquely Diptera DNA, which would not contribute to the data visualisation on the NMDS. These samples were considered as overamplified given the kick-net sampling detected more than a single order but the eDNA only detected Diptera. We created the same visualisation but using the kick-net sampling data only (Figure 8). We further studied the dissimilarities by dividing the ordination by season, to compare the main course samples with the tributary samples (Suppl. Figure 4). The same figure was produced but using the kick-net sampling data (Figure 9, Suppl. Figure 5). We used the Mantel test to test for the correlation between the arthropod community

detected using kick-net and eDNA sampling using the dissimilarity matrices calculated and found they were significantly correlated (*R=0.18, p=0.006*).

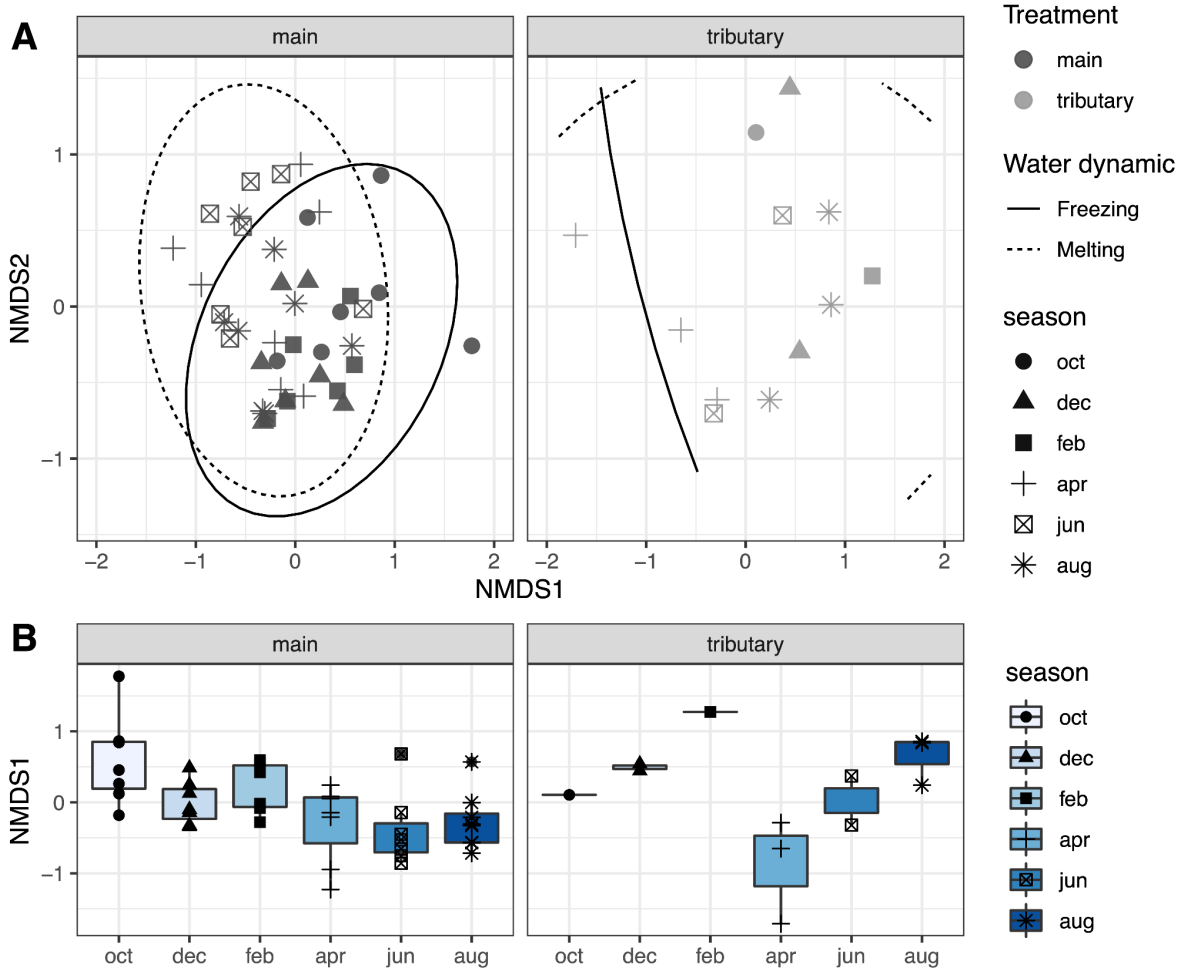

Figure 7. A) eDNA NMDS ordination of the arthropod community composition for each sample and season calculated grouping the sequences by order and visualised having main course samples and tributary samples separate. Ellipses indicate the two melting regimes along the year, i.e. water freezing or water melting, as it simplifies the main driver of the community in terms of water conditions with 95% confidence interval. For the tributaries, ellipses go beyond the represented area. B) eDNA NMDS1 from Figure 7A grouped by season and also divided between samples collected in the main course and samples collected from the tributaries.

Tributary contribution to arthropod composition

We visualised the contribution of the three sampled tributaries to the main course of the river for each sampled season through their dissimilarity. The results are shown for both the eDNA and the kick-net sampling methodologies (Figure 9). However, we were unable to retrieve arthropod sequences from some tributaries, and the visualisation with the eDNA approach remains incomplete.

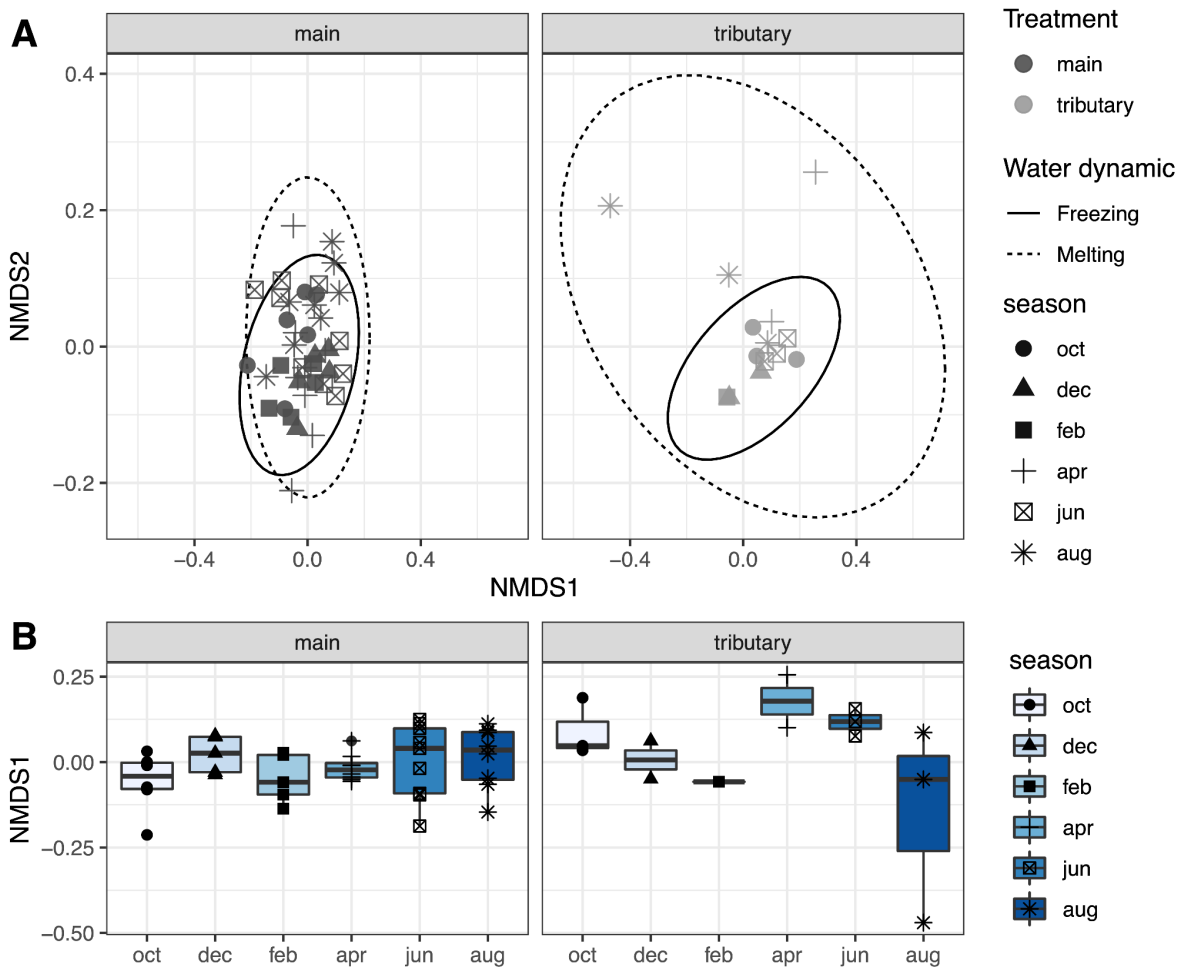

Figure 8. A) Kick-net NMDS ordination of the arthropod community composition for each sample and season calculated grouping the sequences by order and visualised having main course samples and tributary samples separate. Ellipses indicate the two melting regimes along the year, i.e. water freezing or water melting, as it simplifies the main driver of the community in terms of water conditions, with 95% confidence interval. B) Kick-net NMDS1 from Figure 8A grouped by season and also divided between samples collected in the main course and samples collected from the tributaries.

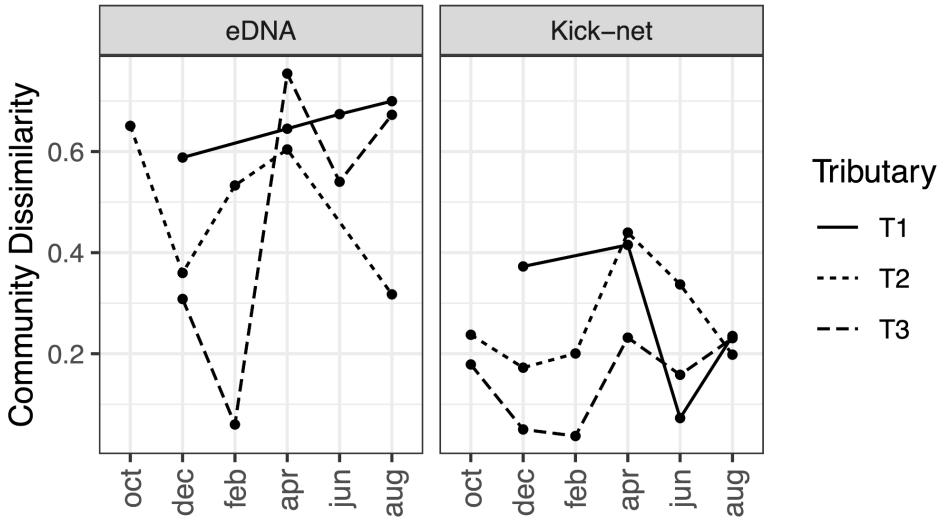

Figure 9. Community dissimilarity comparing the above vs. the below junction for each tributary and each season based on the eDNA and kick-net sampling data, grouped by taxonomic order. The dashed lines indicate each distinct tributary river sampled.

Discussion

Macroinvertebrates in river systems have been used as water quality indicators because they are very sensitive to changes in the physicochemical parameters of the water. As such, they serve as a proxy for measuring the impact a certain disturbance is causing in the environment (Hieber et al., 2005). Such disturbances occur not only in human-engineered river systems, but also in natural ones, which can affect the flow, nutrient concentration, eroding power or turbidity (Milner et al., 2009). Their high sensitivity makes macroinvertebrates good reference species to understand the role certain environmental conditions have on the river's biological community.

In glacier-fed systems, the melting of the glacier comes with an acute change in the water physicochemical conditions. Warmer months lead to higher sediment load and water flow as the glacier melts (Suppl. Figure 3), which can act as an abrasive for the river benthos, limiting the growth of primary producers, such as algae and biofilm, and changing habitat suitability for the macrozoobenthos (Malard et al., 2000; Tockner et al., 1997). In fact, habitat conditions in Alpine river systems are very heterogeneous in space and time; and multiple environmental conditions can co-occur within the same river based on the local environmental conditions. For example, river branches or disconnected ponds can offer refugia from the conditions in the main channel, or tributary rivers can offer sediment-free water, hosting species which otherwise would be unsuitable for the glacier meltwater section of the river. Conditions change depending on the relative contribution of the different water sources feeding the alpine streams, which translates into a very dynamic development of life with important seasonal fluctuations and significant variability in the aquatic community (Milner et al., 2001). Such seasonality has led alpine river macroinvertebrates to adapt their behaviour and biological cycles in consequence. This strengthens the need to account for this spatial and seasonal heterogeneity

when designing studies which involve surveying river macroinvertebrates (Brown & Hannah, 2008).

In glacier-fed streams, the harsh environmental conditions found were thought to make these ecosystems unproductive and with little diversity (Dangles, O., 2012; Füreder, 1999). Yet, they offer a wide range of habitat conditions throughout the year that provides the macrozoobenthos with narrow windows of opportunity to develop when the optimal conditions are found. Hence, year-round surveillance provides an excellent opportunity to detect where and when these windows occur, and which species are benefitting. By doing so, we also identify the environmental variables which best correlate to the biodiversity shifts, improving biomonitoring procedures and sampling strategies for similar ecosystems. Moreover, the harshness of alpine river systems in winter has complicated data collection and skewed ecological studies within the summer. To improve our understanding of glacier-fed streams, we have monitored the shifts in species abundance throughout the year and aimed to identify the potential window of opportunity for macroinvertebrates in the Val Roseg.

In this project, we studied the seasonality of macroinvertebrates and their spatial heterogeneity within the river system and tested the "Summer is in winter" hypothesis. To properly sample the arthropod community, we have combined two approaches, i.e. Kick-net and eDNA sampling. We have focused our experimental setup in order to capture the year-round changes in the community along the alpine floodplain of the Val Roseg and to identify the role tributary rivers play for the macroinvertebrates.

Kick-net sampling vs eDNA metabarcoding

The two techniques have been tested on a year-round basis in order to identify the benefits and limitations of each. Sampling in Val Roseg allowed us to test the feasibility of the techniques within a great variety of environmental conditions. In winter, snow accumulation complicated the access to the sampling points, and compromised the success of the two methodologies. For eDNA, the low temperature of the water meant that the filtration system would often freeze. Similarly, the collected material from kick-net would often freeze on the sorting trays before being transferred into ethanol. For kick-net sampling, one tributary was not sampled in December because the flow was too scarce for sampling (but not for eDNA sampling). In summer, the difficulties were linked to the glacier meltwater. Kick-net sampling was compromised by the flow of water, which was too high in some sections of the main course and impeded the sampling in the exact same location as in previous months. For eDNA, the sediment load was a major problem for water filtration, as the filters of the capsules would clog with sediments before the required volume of water had been pumped through. Despite these technical complications, both methods were resilient with the seasonality of the Val Roseg and we managed to obtain data from all kick-net samples and from most of the eDNA ones (Figure 4). The samples which did not amplify for eDNA were not linked to a particular season, sediment load or sampling location, which suggests that the lack of amplified arthropod DNA is due to the low initial concentration of their DNA in the extraction or because of co-extracted inhibitors which impeded the PCR reaction. Since our water eDNA samples had a very low concentration of arthropod eDNA, we had to adjust the amplification conditions for these

samples to 10X dilution and 55 cycles to obtain enough reads per sample. We reduced the effect of potential overamplification by increasing the filtering thresholds for tag-jumps and spurious sequences in the bioinformatics data processing. Low DNA concentration is a recurrent problem with water eDNA sampling (Roussel et al., 2015), and one of the promising solutions to tackle this issue is the use of capture probe enrichment technologies (Alfano et al., 2021; Seeber et al., 2019), which captures the target DNA within the sample to increase its concentration without PCR added biases.

In terms of the detected arthropod taxa with both methodologies, we identified about one third of the taxa detected with Kick-net in our eDNA samples (Figure 5). This ratio was higher for Diptera, probably because of their relatively high abundance across seasons according to kick-net data, despite the peak of Ephemeropta in April (Suppl. Figure 2). However, it was overall lower for the EPT orders, which are the groups usually used as water quality indicators. It was not surprising to identify many other taxa with the eDNA compared to kick-net (Figure 5). Kick-net sampling targets a particular environment within the river, i.e. the sedimentary benthos in riffle sections of the river, the habitat of EPTs. In contrast, eDNA captures a much greater surface of the river, which expands beyond the water, and can detect the signal of soil organisms which are not physically in the river, such as springtails (e.g. Collembola, Suppl. Figure 1). The extended range of species detection provides a better description of the taxa in the area but can also add confounding effects, given the DNA is transported downstream from its source, which can lead to false positive detections. This is a complex issue for freshwater eDNA methodologies as the distance DNA can travel and still be detected depends on many biotic and abiotic factors.

When comparing the similarity of the two techniques, we found a significant correlation between the proportion of individuals counted with kick-net sampling and the likelihood to be detected using eDNA (Figure 6). However, this trend was not significant for the months of April and June. Sediment load could be affecting the similarity between the two techniques, but since the correlation was significant for August, when the sediment load was still high, we cannot directly link sediments to poorer performance of the eDNA methodology. In this sense, purpose-built experiments should be done targeting the impact sediment load has on eDNA detectability.

One of the methodological constraints that we had to balance between the two techniques is the asymmetry in taxonomic resolution. Kick-net sampling is extremely accurate in estimating relative abundances of each taxon but requires expertise and time to provide taxonomic identification at species or genus level. On the contrary, eDNA is very accurate in terms of taxonomic resolution but the quantitative proportions are highly influenced by how eDNA is captured from water and the extraction and amplification process. Because of this, we decided to calculate both dissimilarities by grouping taxa at order level instead of family. This was done in order to standardise the limited taxonomic resolution of the kick-net sampling with the eDNA potential PCR biases, for which we suspect the relative read abundance is not accurate despite the greater taxonomic resolution of the technique. Alternatively, choosing a more specific primer, e.g. targeting only EPT orders, could have improved the coverage of the eDNA methodology and the comparability of the two methodologies, since kick-net sampling recovered mainly these orders as they are abundant in the sampled locations. In this sense, the

use of Arth02, targeting all arthropods, might not be the best for methodological comparisons, but it provides a better glimpse of the arthropod community as a whole, of which EPT orders are just a part.
Hence, methodological comparisons between these two antagonistic techniques are complex because we aim at capturing a complex ecological signal from a small sample to represent the whole ecosystem, and try to identify global drivers of the ecological changes observed. Of course, the noise generated by both methodologies goes in opposite directions, and compromises are to be made in order to draw reliable conclusions in terms of ecosystem functioning.
Overall, we consider eDNA can be used as a substitute for kick-net sampling when the goal is the ecosystem as a whole, but eDNA will not be able to provide such a detailed local picture of the macrozoobenthos as if sampling with kick-net. As mentioned, the water filtration method samples water transporting organic material from above the pumping location. As such, taxa detected are not restricted to the immediate location of the pumping. This has to be considered when designing studies involving river eDNA. In our case, the triplet design at the junctions of tributaries and the main river was very interesting because we were able to compare the relative contribution of tributaries using the sample collected downstream of the junction as control for the two samples collected above it.

Seasonal and spatial heterogeneity of the arthropod community
We decided to study the data dividing the sampling months into two categories: "freezing" and "melting", as a proxy for the melting regime in the overall system. This dynamic summarises the physicochemical shifts in the water (Suppl. Figure 3). When visualising the arthropod communities, we detected a high similarity within the main course of the river for both methodologies, i.e. the main river community was more stable and overall similar along the year (Figure 7A, 8A). Using eDNA, we observed a directional trend of community shift, visualised using the first component of the NMDS ordination (Figure 7B), although it was not significant. For the Kick-net sampling, the first component of the ordination was also similar across seasons (Figure 8B). Together, this suggests the main course hosts an overall similar community composition in terms of taxonomic orders along the year, despite the detected shifts in the total abundance of benthic macroinvertebrates (Suppl. Figure 2). Contrary to the main course, the communities in the tributaries were more variable, and their ordination shifted between the months of February and April, which aligns to the start of the glacier melting season and then recovering the pre-melting composition (Figures 7B, 8B). This is an interesting finding as it suggests that the community in the tributaries changes at the start of the melting season, which could be explained by the increase in sediments in the main course (Suppl. Figure 7). From an alpha diversity perspective, this shift in communities within tributary rivers and the consistency within the main course is supported by both methodologies (Suppl. Figure 6). Statistically, sediment load was the only environmental variable significantly correlated to Shannon index, but only for the kick-net data (Suppl. Table 2). In line with the NMDS results, alpha diversity remains similar across the main course of the river but experiences a sudden increase between the months of February and April. This was clear for the kick-net data (Suppl. Figure 6B), but was delayed by two months for the eDNA data (Suppl. Figure 6A). This small difference could be related to further species being captured by the eDNA methodology that

were not sampled with the kick-net, as the soil arthropod community changes when snow melts, which occurs between April and June. Nevertheless, despite the shift in community composition in tributaries, it does not translate into a clear shift in the arthropod community of the main course, or else we would have observed a distinct clustering for the samples of February and/or April. This is probably due to the presence of other mitigating factors, that when combined, may overshadow the positive effect of tributaries in stabilising glacier-fed habitats, where we do detect a clear community shift.

When divided by season, we find the tributaries cluster separated from the main course using the eDNA approach during the "freezing" months, i.e. October, December and February, and overlapped during the "melting" months (Suppl. Figure 4). However, the gaps in the eDNA data complicates the study of the communities by season. With the kick-net data, the overlap occurs in all seasons. However, an interesting indication is the close clustering in February of the tributaries with the *Glacier* sample, i.e. the one closest to the source of the sediment release (Suppl. Figure 5). In the following seasons, tributaries are found further away in the ordination from the reference *Glacier* sample. As such, we interpret the conditions occurring between February and April are the key to the community shift detected in the tributaries.
The environmental conditions of February combine the greatest amount of light exposure with the least sediments and are associated with the greatest number of individuals (Suppl. Figure 7). As such, we considered it as the reference community for the "Summer is in winter" hypothesis. It is during that time, when the *Glacier* community aligns the closest to the tributary communities (Suppl. Figure 5). As winter fades, the sudden increase in sediments sets the end of the "Summer in winter", as the total abundance of arthropods starts to drop, between February and April (Suppl. Figure 2). The correlation between abundance of individuals and sediment load (Suppl. Figure 7) suggests sediments could have a detrimental effect on their abundance, and could be limiting the development of a new arthropod community that could take advantage of the milder conditions of true summer. However, sediments do not seem to impact the biodiversity within the main channel, rather the abundance (Suppl. Figure 6).

In parallel, the conditions in the tributaries remain closer to the conditions in February, and arthropods could be profiting from it by extending their particular summer in the tributaries, which host less life during the winter months but experience a sudden shift when the glacier starts to melt, as it remains free of sediments compared to the main course. This community shift within the tributaries was detected using both methodologies in the NMDS (Figure 7B, 8B) and using alpha diversity measurements (Suppl. Figure 6). Moreover, the modelling of kick-net data confirmed the role of sediment load on reducing alpha diversity (Suppl. Table 2).

Tributary role in the seasonal dynamics
We aimed at defining the community contribution of the tributaries on the main course. This approach is an interesting way to investigate the role of tributaries from an ecological perspective, as it integrates the main river communities and puts them in perspective to the tributaries. It was based on the assumption that the down sample of a junction would be a representation of the up sample and the tributary combined. However, the observed dissimilarity between the above communities and the below one is dependent on the relative

water contribution of the tributary to the main course in terms of flow, which varies along the year. We didn't account for this variable and consider that these methodological issues should be tackled through purpose-based experiments.
Comparing the community between the down sample and the other two informs on the relative impact tributaries have on the community composition downstream in the main course, and could be a proxy for the ecological relevance of tributaries as alternative habitats for the macroinvertebrates. We measured the dissimilarity for the two techniques, and found the results complex to interpret overall (Figure 9). The eDNA data missed some key points regarding the tributaries and the kick-net data only missed two values regarding T1. Focusing on the kick-net data, we observed a seasonal trend in terms of tributary to main course dissimilarity and identified the peak of dissimilarity for both methods was in April. This aligns with the results found in terms of ordination in Figures 7B and 8B and in alpha diversity measurements (Suppl. Figure 6, Suppl. Table 2). In both cases, the shift in tributary composition occurred between the months of February and April. Thus, it seems that the tributaries experience a strong shift in their community composition at the beginning of the melting season, in line with the rise of sediment load in the main course. As for the main course, despite no changes were observed in terms of community composition (Figures 7B and 8B), the number of individuals found did change, being reduced with increasing sediment load (Suppl. Figure 2 and 7).

These results reflect the spatial heterogeneity of the arthropod communities in the Val Roseg river, and how it is strongly linked to the acute seasonality of the environmental conditions in the river. Altogether, it reinforces the hypothesis that sediments have a strong effect on the benthic communities, because with the rise of sediments comes the greatest dissimilarity between the tributaries and the main course. It seems that the beginning of the melting period is particularly important for the community dynamics, and during that period, the connectivity with the tributaries is of key importance to offer sediment free water to the benthic macroinvertebrates. This seasonal pattern, which could very well be a local migration of individuals within the river, could help to the recovery of the community in the main course or maintain it during the melting period. In this context, mitigating factors, i.e. the proglacial lake, tributaries, floodplain stability and vegetation development, contribute to maintaining biodiversity throughout the year, stabilising glacier-fed stream habitats. In the case of the Val Roseg, tributaries had a distinct community composition, suggesting their distinct physicochemical properties allow for hosting a different community from the main course. They could be acting as sheltering environments from the sediment rich waters in the main course, but we did not observe any clear colonisation patterns. Unfortunately, the scope of this study was not designed to answer this question, but we consider that further research should explore it.

Conclusions and management implications

The implications of these findings expand beyond natural glacier-fed rivers, as the impact of sediments in the river benthic community occurs regardless of its conservation status. As such,

there is a clear parallelism between glacier melting and dam flushing in terms of sediment load in the water.
In alpine river systems, the rapid retreat of glaciers causes a major problem for hydraulic management organisations, as the amount of sediments released obstructs the water infrastructure downstream. To cope with river sediments, water intakes located at high altitude act as sediment traps in order to clear the water and reduce its eroding power when transferred to hydroelectric power plants. However, the accumulation of sediments is released frequently in sudden discharges, i.e. flushing, which occurs during the melting period, when the most sediments accumulate. This creates regular flow peaks with high sediment load on the river ecosystem downstream. As in Val Roseg, the macrozoobenthos has to cope with an acute seasonality in terms of temperature, erosion and water flow, driven by the melting of the glacier in the warmer months. The discharge of water causes a peak of erosion on the river bed downstream of the dam, but this impact cannot be directly related to a glacial-fed system because the change in flow is less drastic there. However, the sediments released in the river have the same eroding power. For this reason, we suggest research targeting the impact of dam flushing on hydropower-impacted streams could explore the benefits of creating mechanisms to maintain part of the river free of sediments from the flushing discharge to act as spatial refugia for the benthic community, as tributaries do for Val Roseg.

Altogether, the complexity of alpine rivers and their strong seasonality makes them a highly dynamic ecosystem difficult to study. The legitimacy of the "Summer is in winter" theory investigated in this study is yet to be confirmed, but we found strong indications that sediment load reduces the quantity of macroinvertebrates and increases the diversity within tributaries. Nevertheless, further studies should address the ecological extent of this inverted seasonality in alpine rivers and which are the key environmental variables responsible for it.

Acknowledgements

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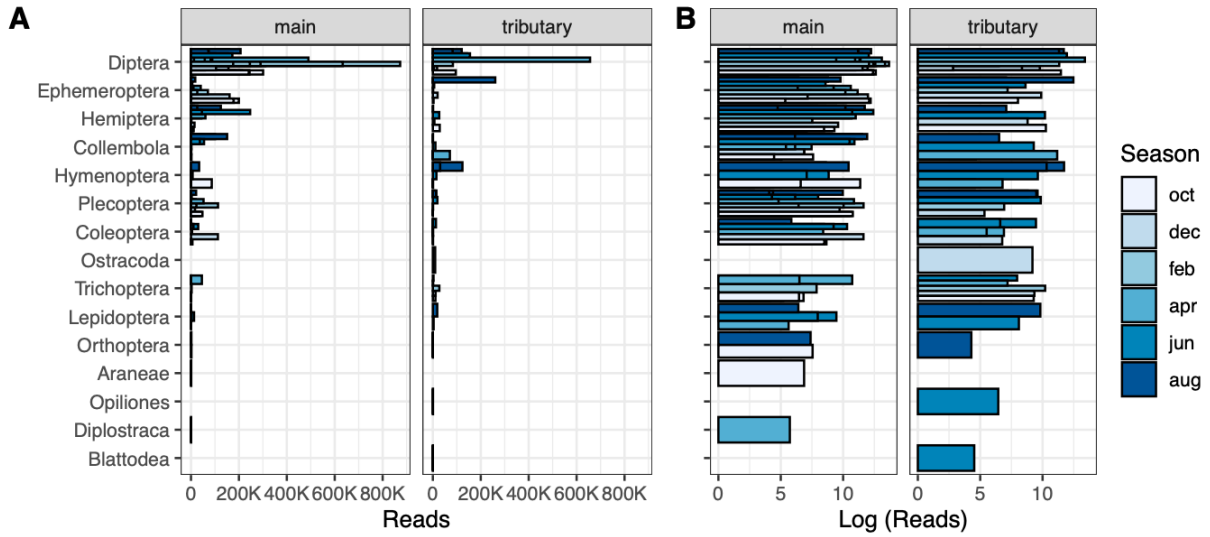
Conflicts of interest

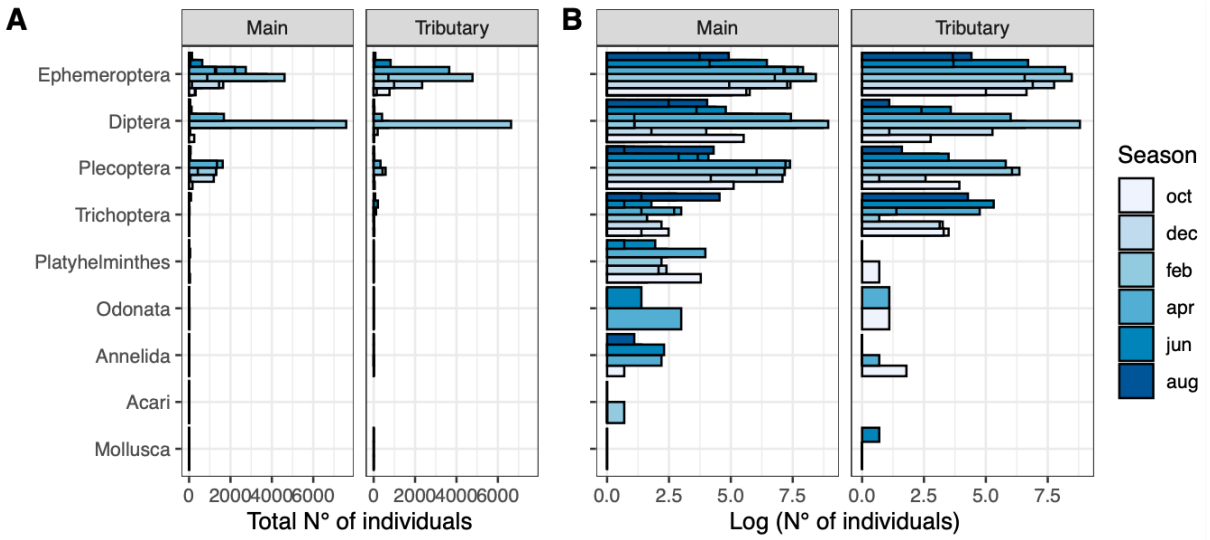
In this chapter, in the Material and Methods section, the sub-sections concerning the study site, kick-net sampling and environmental variables were taken directly from the Mémoire of Noah Barchi MSc thesis with his permission for use in this Chapter (Reference: Noah Barchi (2022). *Seasonal pattern of benthic macroinvertebrate community structure and composition in a*

natural glacier-fed stream in the Swiss Alps. MSc Thesis, Faculty of Geosciences, University of Lausanne). This chapter is not the manuscript of a paper.

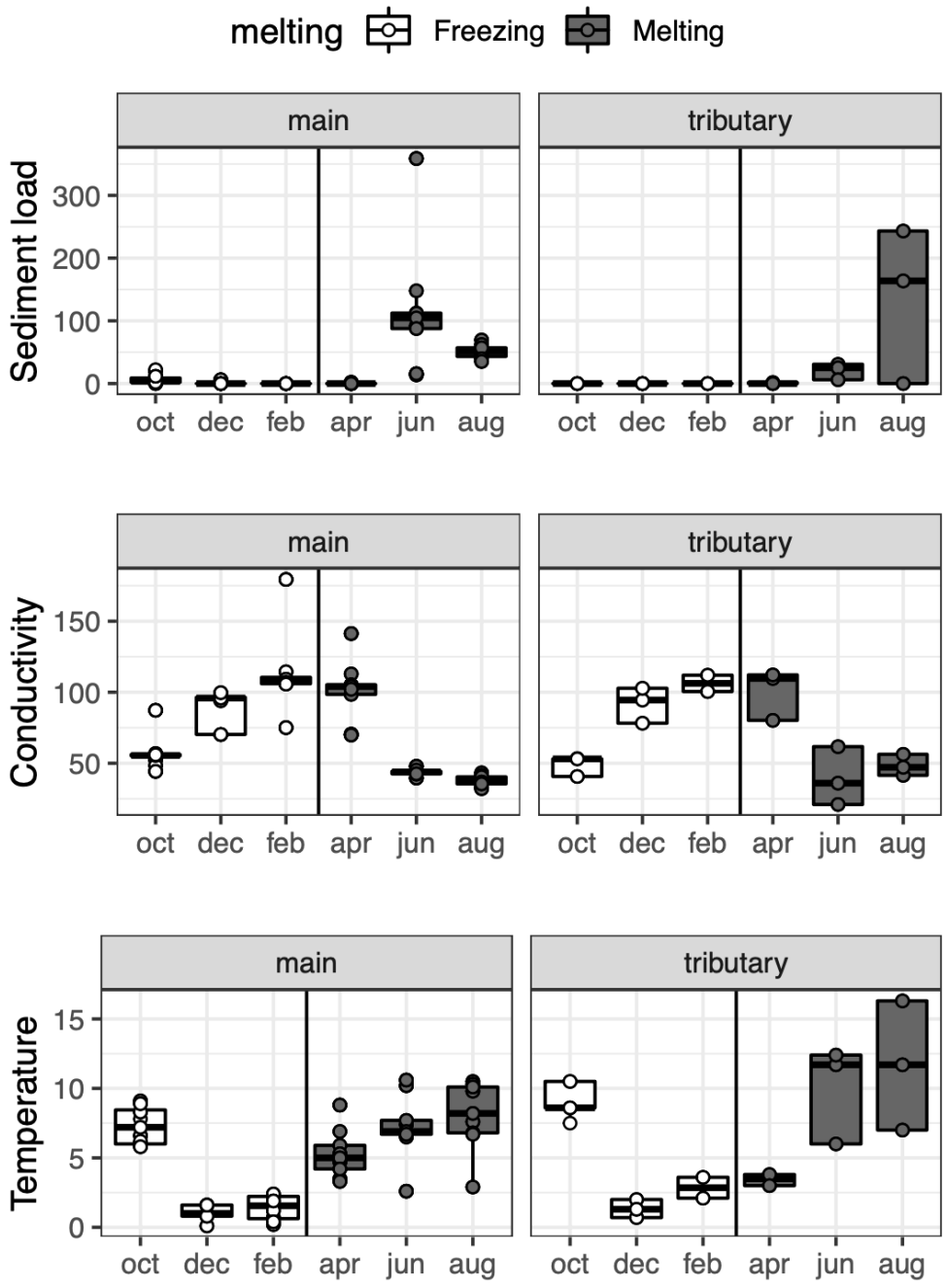
Supplementary material



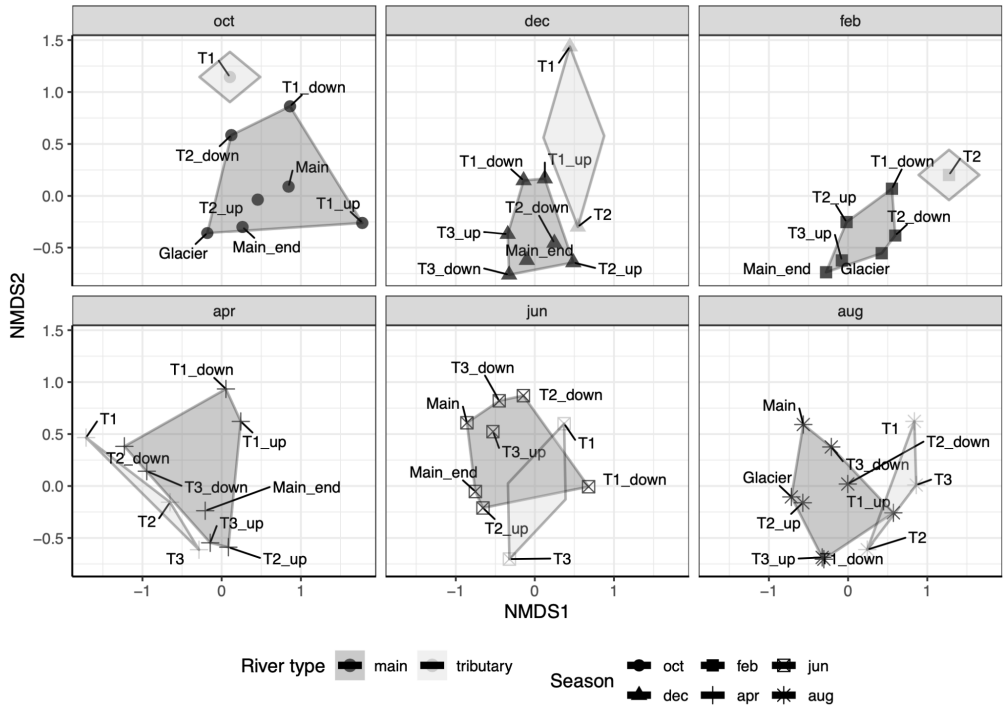
Supplementary Figure 1. A) Overview of the amount of reads per arthropod order and season. B) Log transformation of the reads per order and season. Data is shown divided between tributaries and main course samples. Orders are organised by abundance of reads.



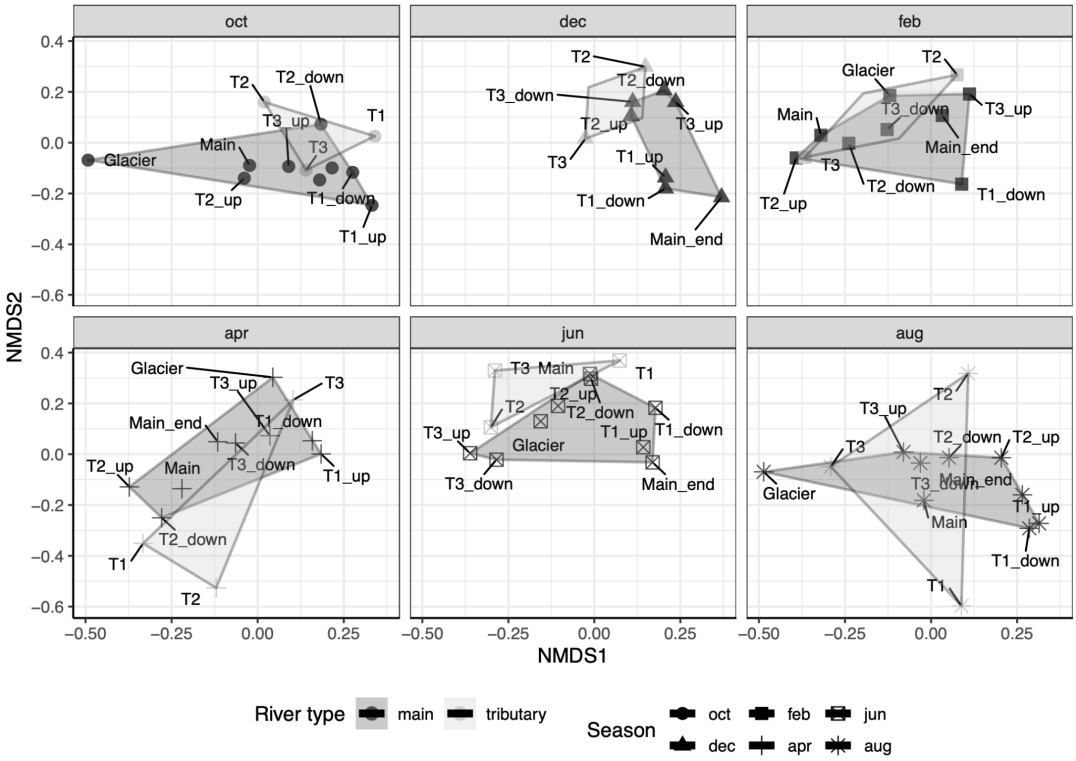
Supplementary Figure 2. A) Overview of the amount of individuals per arthropod order and season. B) Log transformed number of individuals. Data is shown divided between tributaries and main course samples. Orders are organised by abundance.



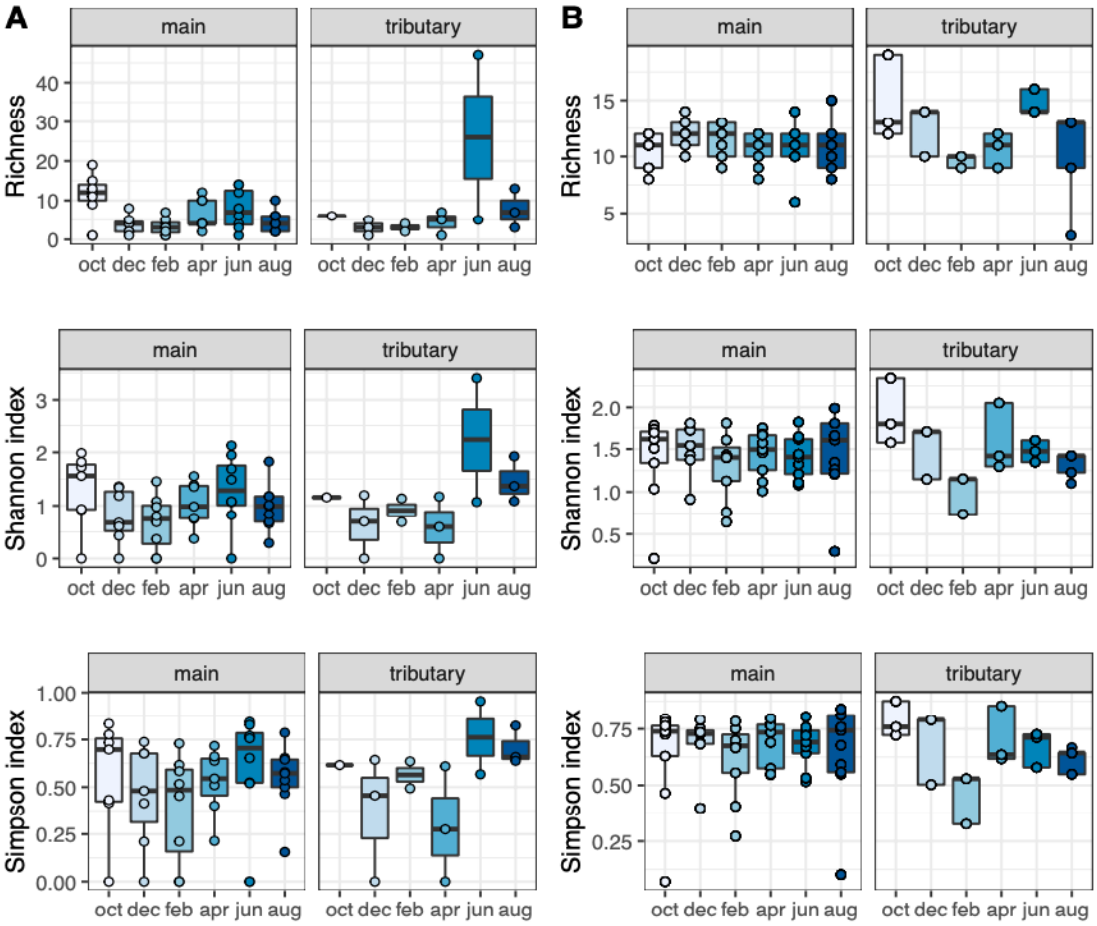
Supplementary Figure 3. Environmental variables shown in Figure 3 PCA divided by season and separated between the so-called "Freezing" and "Melting" stages.



Supplementary Figure 4. NMDS ordination from Figure 4A, i.e. using the eDNA arthropod community data, but divided by season and grouped by type of river, i.e. Main course or tributary. The shape corresponds to the season.



Supplementary Figure 5. NMDS ordination using the kick-net sampling arthropod community data, but divided by season and grouped by type of river, i.e. Main course or tributary. The shape corresponds to the season.



Supplementary Figure 6. Alpha diversity measurements for A) eDNA data and B) Kick-net sampling. In both cases, we used the data grouped at family level.

Supplementary Figure 7. Number of individuals sampled using kick-net sampling technique against the sediment load. The colour of the dots indicates the season. All samples from february recorded a sediment load of 0.

Supplementary Table 1. List of Arthropod species used for the positive controls.

Order	Species	Relative concentration
Orthoptera	*Acheta domesticus*	10.000000
Phasmida	*Timema douglasi*	5.000000
Coleoptera	*Harmonia axyridis*	2.500000
Lepidoptera	*Galleria mellonella*	1.250000
Hemiptera	*Pyrrhocoris apterus*	0.625000
Blattodea	*Blaptica dubia*	0.312500
Plecoptera	*Isoperla rivulorum*	0.156250
Trichoptera	*Silo pallipes*	0.078125

Supplementary Table 2. Shannon alpha diversity modelling against the environmental variables for the two methodologies.

eDNA				
Estimate	Std.	Error	z-value	Pr(>|z|)
(Intercept)	-1.124135	0.5035633	-2.232	0.0256*
cond	-0.0046316	0.0044664	-1.037	0.2997
temp	0.0415793	0.0416686	0.998	0.3183
sed_load	-0.0002386	0.0025472	-0.094	0.9254
Kick-net				
Estimate	Std.	Error	z-value	Pr(>|z|)
(Intercept)	0.645358	0.391178	1.65	0.099
cond	-0.004791	0.0034	-1.409	0.1587
temp	0.020349	0.029511	0.69	0.4905
sed_load	**-0.003212**	**0.001462**	**-2.197**	**0.0281***

Chapter 5 - Assessing environmental DNA metabarcoding and camera trap surveys as complementary tools for biomonitoring of remote desert water bodies

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Author contributions
LF, PC and OG designed the study and supervised all analyses. BN, SR, OG, PC, MB and CN conducted fieldwork. CA provided logistic support. CS carried out laboratory work. BN, EM-C, SR, FC and PC carried out species identifications and image coding based on camera trap data. EM-C conducted bioinformatics, data analyses and prepared the figures. EM-C and JS wrote the paper, with input from all other authors.

Abstract

Biodiversity assessments are indispensable tools for planning and monitoring conservation strategies. Camera traps (CT) are widely used to monitor wildlife and have proven their usefulness. Environmental DNA (eDNA)-based approaches are increasingly implemented for biomonitoring, combining sensitivity, high taxonomic coverage and resolution, non-invasiveness and easiness of sampling, but remain challenging for terrestrial fauna. However, in remote desert areas where scattered water bodies attract terrestrial species, which release their DNA into the water, this method presents a unique opportunity for their detection. In order to identify the most efficient method for a given study system, comparative studies are needed. Here, we compare CT and DNA metabarcoding of water samples collected from two desert ecosystems, the Trans-Altai Gobi in Mongolia and the Kalahari in Botswana. We recorded with CT the visiting patterns of wildlife and studied the correlation with the biodiversity captured with the eDNA approach. The aim of the present study was threefold: a) to investigate how well waterborne eDNA captures signals of terrestrial fauna in remote desert environments, which have been so far neglected in terms of biomonitoring efforts; b) to compare two distinct approaches for biomonitoring in such environments and c) to draw recommendations for future eDNA-based biomonitoring. We found significant correlations between the two methodologies and describe a detectability score based on variables extracted from CT data and the visiting patterns of wildlife. This supports the use of eDNA-based biomonitoring in these ecosystems and encourages further research to integrate the methodology in the planning and monitoring of conservation strategies.

Introduction

Large-scale biodiversity loss has been documented in all types of ecosystems around the globe due to anthropogenic and climate change effects (Butchart et al., 2010; Díaz et al., 2019; Rosenzweig et al., 2008; WWF, 2020). Reliable biodiversity surveys are therefore needed to assess species conservation status over time and to plan and monitor management measures (Pimm et al., 2014), including the identification of biodiversity hotspots (Brooks et al., 2006; Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). Desert ecosystems have been neglected in terms of scientific and monitoring efforts, resulting in knowledge gaps particularly for remote areas with difficult access, although they harbour diverse biological assemblages (Brito et al., 2014; Durant et al., 2012; Durant et al., 2014) and cover almost one fifth of the earth's land (Safriel et al., 2005). As climate change may impact environmental conditions in desert ecosystems disproportionately faster (Loarie et al., 2009), these unique systems should be placed at the centre of attention.

Biomonitoring aims to provide detailed data on species' distribution, abundance and diversity. Conventional, observer-based methods, such as visual censuses and systematic trapping are time- and labour-intensive and mostly focus on a limited number of taxa (Thomsen & Willerslev, 2015). Technology-based methods gain importance in conservation research (Stephenson, 2020), whereof we compare two in this study. Non-invasive camera traps (CT) with infrared sensors are widely employed for conservation research and monitoring, in particular for larger terrestrial mammals (Caravaggi et al., 2017; Salvatori et al., 2021). They are becoming less effort-intensive thanks to AI-based tools to sort CT datasets. Nevertheless, there are also limiting factors for unbiased detectability of species, such as movement range (Burton et al., 2015; Caravaggi et al., 2020) or body mass. Small mammals are usually underrepresented in CT because their size is insufficient to trigger the camera sensor (Leempoel, Hebert, & Hadly, 2020), but see (Littlewood, Hancock, Newey, Shackelford, & Toney, 2021). They offer valuable information on species' abundances, density and richness while allowing for multispecies monitoring (Rovero & Zimmermann, 2016). Environmental DNA (eDNA) approaches offer valuable biodiversity assessment tools given the simultaneous analyses of complex DNA mixtures that enable to detect species' presences, estimate diversities and relative abundances. Advances in DNA sequencing technologies facilitated an increase in eDNA studies over the last decade (Bohmann et al., 2014; Ruppert, Kline, & Rahman, 2019; Taberlet, Bonin, Zinger, & Coissac, 2018; Thomsen & Willerslev, 2015). In particular, DNA metabarcoding (i.e. PCR amplification of short but informative barcodes with universal primers and next generation sequencing (NGS) of DNA mixtures (Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012)) allows the simultaneous assessment of whole communities. Most of these studies focuses on aquatic organisms from freshwater ecosystems (Belle, Stoeckle, & Geist, 2019; Rees, Maddison, Middleditch, Patmore, & Gough, 2014). Water samples are well suited to collecting eDNA due to high distribution capabilities of eDNA in water bodies (Rodgers & Mock, 2015; Valentini et al., 2016). Sampling being standardisable and relatively fast, the method requires only single visits to study sites (or repeated visits for temporal monitoring). Water-borne eDNA reflects temporally accurate biodiversity information due to the limited persistence of free eDNA in water for days or maximally weeks

(Barnes & Turner, 2016). DNA degradation is the main cause impeding detection by eDNA-based techniques. Experimental studies have shown that the persistence of free aqueous eDNA (not bound to particles, i.e., sedimentary eDNA) depends on a number of factors, with e.g. higher temperatures, more solar radiation and neutral or acidic *pH* leading to shorter detection periods (Pilliod, Goldberg, Arkle, & Waits, 2014; Strickler, Fremier, & Goldberg, 2015). However, its persistence depends on dynamic interactions of various biotic (e.g. rate of DNA shedding, microbial activity) and abiotic factors, hindering the drawing of general patterns. While most studies have been carried out in temperate areas, but see (Coutant et al., 2021; Ishige et al., 2017; Mena et al., 2021; Sales, Kaizer, et al., 2020; Seeber et al., 2019), sampling eDNA from remote desert water bodies is particularly challenging because of DNA degradation, which is expected to be accelerated by extreme seasonal and daily temperature variations and high UV-B exposure found in this type of environment, technical difficulties caused by the filtration of typically turbid water samples (Egeter et al., 2018) and restrained accessibility. There are few studies to date using water samples to assess biodiversity in an arid or semi-arid environment, but see (Egeter et al., 2018; Seeber et al., 2019). Despite these challenges, eDNA methods may reveal valuable tools for general biodiversity assessments and the monitoring of iconic and threatened species in precious ecosystems with reduced accessibility.

eDNA from terrestrial animals has been mostly assessed by analysing scats (De Barba et al., 2014; Kartzinel et al., 2015; Swift et al., 2018), soil (Leempoel et al., 2020; Yoccoz et al., 2012; Zinger et al., 2019), stomach content samples (Kennedy, Lim, Clavel, Krehenwinkel, & Gillespie, 2019; Masonick, Hernandez, & Weirauch, 2019; Soininen et al., 2013), leeches blood meals (Abrams et al., 2019; Nguyen et al., 2021; Tilker et al., 2020; Weiskopf et al., 2018; Wilting et al., 2021) or carrion flies (Calvignac-Spencer et al., 2013; Gogarten et al., 2020; Rodgers et al., 2017; Schubert et al., 2015). Bulk tissue samples (mixtures of, for example, insects or other macroinvertebrate specimens) are also increasingly used not only to assess invertebrate diversity but also as an indirect way to sample vertebrate DNA (Lynggaard et al., 2019). However, animals also leave DNA traces in water while drinking or bathing, which means this water can be sampled and analysed to detect non-aquatic organisms. This has first been proven using PCR and Sanger sequencing in an experimental setting with coyote DNA (Rodgers & Mock, 2015). Further studies successfully analysed eDNA of terrestrial animals shed in water bodies sampled across different natural environments, from saltlicks in a Bornean tropical forest (Ishige et al., 2017), water bodies (Seeber et al., 2019; Ushio et al., 2017; Ushio et al., 2018) and ponds (Harper et al., 2019), stagnant and running water combined (Mena et al., 2021), to rivers and streams (Coutant et al., 2021; Sales, Kaizer, et al., 2020; Sales, McKenzie, et al., 2020). This approach is particularly relevant for desert ecosystems with extreme conditions, where waterholes are small and scattered, leading to a spatial concentration of terrestrial animals that must gather and use the few available water sources (Davis, Kerezsy, & Nicol, 2017; Razgour, Persey, Shamir, & Korine, 2018; Vale, Pimm, & Brito, 2015). Albeit the close association of water resources and desert species, there are also numerous adaptations to reduce their dependence and some species, such as gazelles, do not always comply with this expectation, as documented in the Trans-Altai Gobi (Nasanbat, Ceacero, & Ravchig, 2021).

While CT and eDNA are two key tools available for species monitoring, there is limited information available to help researchers choose the most appropriate method for their needs, to compare performance, and decide if and when methods can be used together (Stephenson, 2020). In order to enable inter-method comparability and their complementary use, comparative studies are therefore needed. Here, we compare CT and eDNA, with a focus on vertebrate terrestrial taxa in two desert ecosystems. While eDNA approaches are still relatively recent, CT have been used far longer but are undergoing increased attraction for conservation monitoring (Rovero & Zimmermann, 2016). The complementary use of these two methods can be appropriate for many situations and in particular for environments that are not favourable for observer-based monitoring. Analysing images of CT allows us to quantify relative densities of species per sampling site. Based on these data we can identify variables that best describe visiting patterns and assess whether they are mirrored by eDNA sequence data. We expect e.g. to find DNA of those taxa that visit regularly, in great numbers and shortly before sampling. The aim of the present study was threefold: a) to investigate how well waterborne eDNA captures signals of terrestrial fauna in remote desert environments, b) to compare two approaches for biomonitoring in such environments and c) to draw recommendations for future eDNA-based biomonitoring.

Materials and methods

Sampling sites

CT and water sampling were conducted at ten different sites in the Gobi Desert in Mongolia and four different sites in the Central Kalahari Game Reserve, within the Kalahari Desert in Botswana (Figure 1 and Table S1, Supporting Information). The water bodies were natural in Mongolia and artificial in Botswana.

The Great Gobi A Strictly Protected Area (SPA) in Trans-Altai Gobi was created in 1975, covers 44,000 km^2 and hosts emblematic species such as the snow leopard (*Panthera uncia*), the brown bear (*Ursus arctos*), the Asian wild ass (*Equus hemionus*) and the Bactrian camel (*Camelus ferus*). For a detailed description of the environmental conditions of this ecosystem, see (Nasanbat et al., 2021).

The Central Kalahari Game Reserve was created in 1961 and covers 52,800 km^2, where ecotourism is a fundamental source of income (Stone, Stone, & Mbaiwa, 2017). It hosts a great diversity of emblematic African large mammals including the African elephant (*Loxodonta Africana*), the cheetah (*Acinonyx jubatus*), the lion (*Panthera leo*), the brown hyena (*Parahyaena brunnea*) and the South African oryx (*Oryx gazella*).

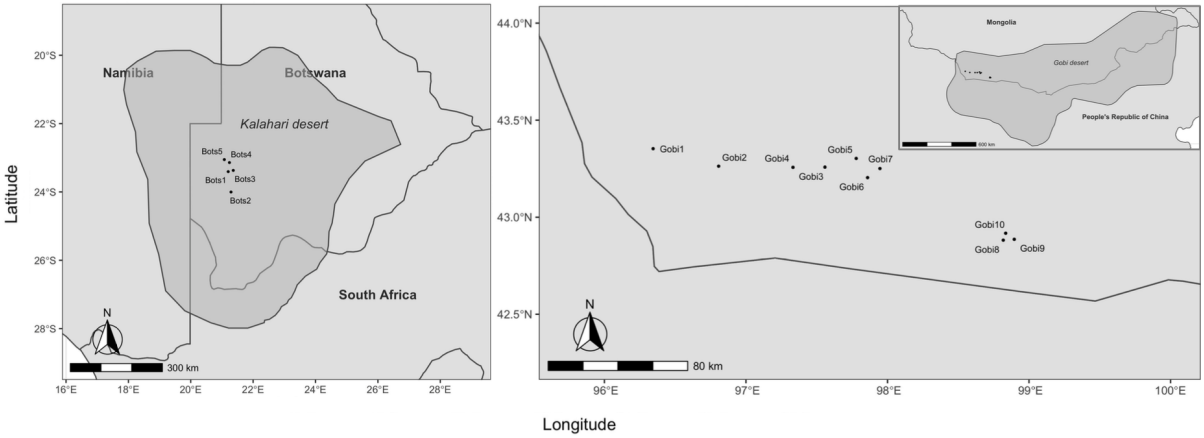

Figure 1. Sampling locations in (A) the Kalahari Desert and (B) the Trans-Altai Gobi Desert. Dark grey areas in each map indicate the extension of the Kalahari Desert and the Trans-Altai Gobi Desert respectively.

Water sampling

At each sampling location (Figure 1), one water sample was taken following the methodology of (Pont et al., 2018), with modifications. The water was filtered through a VigiDNA 0.45 µM crossflow filtration capsule (SPYGEN, Le Bourget du Lac, France), with disposable 200 mL sterile syringes for each filtration capsule. For the Mongolian samples, 10 L of water were filtered at each location. For the Kalahari samples, filtered volumes varied from 1 to 10 L (average 6.3 L) depending on water body size and water turbidity. To avoid eDNA degradation, water in the capsule was replaced by 80 mL of CL1 conservation buffer (SPYGEN, Le Bourget du Lac, France) and stored at room temperature. Sampling in Mongolia took place in August 2018 and in Botswana in May 2019 (for all details on sampling locations and samples see Table S1, Supporting Information).

DNA extraction

Extractions were performed in a pre-PCR laboratory dedicated to low DNA-content analyses, using a protocol modified from (Pont et al., 2018). The filtration capsules were shaken for one hour at 420 rpm and agitated manually for two minutes to ensure a maximum DNA yield from the filter. From each capsule, 45 mL were poured into three separate 50 mL Falcon tubes (15 mL each) and 33 mL of 96 % ethanol and 1.5 mL of 3M sodium acetate (*pH* 5.2) were added before overnight incubation at -20 °C. After precipitation, tubes were centrifuged at 7000 g for 30 min at 6 °C. Supernatants were discarded and tubes incubated at 56 °C for 10 minutes to evaporate residual ethanol. 720 µL of ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen, Hilden, Germany) and 40 µL of proteinase K were added and the mixture transferred to 2 mL Eppendorf tubes for at least two hours of incubation at 56 °C. The DNA extraction was pursued at step 6 of the NucleoSpin Soil Kit protocol (Macherey-Nagel, Düren, Germany). The three sub-samples were pooled in the extraction column. Elution was done with 2 x 100 µL of SE buffer. Negative controls were included at all steps.

The extractions were tested for inhibitors with real-time quantitative PCR (qPCR) applying different dilutions in triplicates. qPCR reagents and conditions were the same as in PCR

amplification (see below), with the addition of SybrGreen (Thermo Fisher Scientific, USA). Based on the results, all samples were subsequently diluted 10-fold before PCR amplification.

DNA metabarcoding

DNA extracts were amplified with two primer sets. The first primer pair targets a fragment of the mitochondrial DNA (mtDNA) 12S rRNA gene in vertebrates (Vert01 (Taberlet et al., 2018), corresponding to 12SV5F/R in (Riaz et al., 2011)), the second targets a fragment of the mtDNA 16S rRNA gene of mammals (Mamm02 (Giguet-Covex et al., 2014; Taberlet et al., 2018)). Human-blocking primers were added to the PCR mixes to prevent amplification of human DNA contaminants (for details of all primers see Table S2, Supporting Information). The total PCR volume was 20 µL, including 2 µL of template DNA and 1U AmpliTaq Gold 360 mix (Thermo Fisher Scientific, USA), 0.16mg/ml of bovine serum albumin (BSA, Roche Diagnostics, Basel, Switzerland), 2 µM of human-blocking primer and 0.5 µM of each tagged forward and reverse primer (i.e. primers with eight variable nucleotides added to their 5' end, allowing further sample identification, see (Taberlet et al., 2018)). Each sample was amplified in 12 replicates per primer in three separate PCR plates. Thermocycling conditions were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 49 °C and 57 °C for Vert01 and Mamm02, respectively, 1 min at 72 °C, with a final elongation step of 7 min at 72 °C. Each 96-well PCR plate contained 12 blanks, eight negative extraction controls, eight negative PCR controls and eight positive controls (DNA assemblies of species not present in the studied regions). Blanks correspond to empty wells and allow to estimate the proportion of tag switches (i.e., false combination of tags, generating chimeric sequences) occurring during the sequencing process (Schnell, Bohmann, & Gilbert, 2015). Successful amplification was confirmed on a 1.5 % agarose gel and PCR products were subsequently pooled per PCR plate. Pooled amplicons were purified using a MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Purified PCR products were quantified using a Qubit 2.0 Fluorometer (Life Technology Corporation, USA).

Library preparation was performed using a TruSeq DNA PCR-Free Library Prep Kit (Illumina, San Diego, CA, USA) with an adjusted beads ratio of 1.8 to remove small fragments. After adapter ligation, libraries were validated on a fragment analyzer (Advanced Analytical Technologies, USA). Since larger and smaller fragments besides the target size remained after this step, additional post library bead purifications were performed. To remove large and small fragments from Mamm02 libraries, a ratio of 0.7 was used followed by a ratio of 1.1. For Vert01 libraries, a ratio of 1 was used to remove small fragments. Final libraries were quantified by qPCR, normalised to 1 nM and pooled before 150 paired-end sequencing on an Illumina Miniseq Sequencing System with a Mid-Output Kit (Illumina, San Diego, CA, USA).

Bioinformatic data analyses

The bioinformatic processing of the raw sequence output was conducted using the *OBITools* package (Boyer et al., 2016). The subsequent steps were followed separately for each library. Forward and reverse reads were assembled with a minimum quality score of 40 and assigned to samples based on unique tag and primer combinations, allowing two mismatches with primer, and identical sequences were clustered. All sequences with less than five reads per library were discarded as well as those not corresponding to primer specific barcode lengths,

i.e., 56-132 bp for Vert01 and 53-84 bp for Mamm02(Taberlet et al., 2018). This was followed by two different clustering methods. First, pairwise dissimilarities between reads were computed and lesser abundant sequences with single nucleotide dissimilarity were clustered into the most abundant ones. Second, using the *sumaclust* algorithm, we reduced remaining clusters based on a sequence similarity of 97 % (Mercier, Boyer, Bonin, & Coissac, 2013). Sequences were then assigned to a taxon using a reference database in two steps. First, *in silico* PCRs were performed with the *ecoPCR* software (Ficetola et al., 2010) on the whole EMBL repository to build reference databases for both metabarcodes (4,455 Mamm02 sequences; 16,292 Vert01 sequences, Supporting Information). Taxonomic assignments with these databases were performed using a 95 % sequence similarity threshold. Unassigned sequences after this step were discarded from downstream analyses. Second, each taxonomic assignment was manually inspected, and each sequence queried using the BLAST algorithm of GenBank to account for potential mis-assignation, because we used a relatively low similarity threshold. This is more likely to occur for the Vert01 metabarcode, because the amplicons can be very similar between close species. For cases with multiple candidate species, the geographic range was considered to select the correct species.

Further data cleaning and statistical analyses were conducted in R (version 4.0.2). Sequences that were more abundant in extraction controls as well as in negative and positive PCR controls than in samples were considered contaminants and removed as well as known common contaminants that were not expected in the study areas (Furlan, Davis, & Duncan, 2020). To account for tag switching, we considered the leaking of a sequence to be directly linked to its abundance. We performed Wilcoxon signed-rank tests to assess the relationship between samples and blanks. Removal of tag-leaked sequences was done independently per library. Dysfunctional PCR replicates with too small reads count were also discarded. Absolute sequence read counts were transformed to relative read abundance (RRA). Sequences not present in at least two PCR replicates were discarded from downstream analyses. Finally, RRA values were grouped across replicates to obtain a mean value per sample (for a reference data cleaning workflow, see (Axtner et al., 2019)). Environmental DNA was considered both as presence/absence and as RRA data for comparison with CT data. One location in Kalahari (Bots1) had to be excluded from the analyses due to insufficient amplification, possibly due to a problem during sampling, storage and/or the DNA extraction step.

Camera trapping and image coding

Camera traps were set up on sampling sites (Figure 1) between 40 to 70 days before water sampling, in such a way as to cover a maximum of the water bodies and shorelines. In the Trans-Altai Gobi Desert, we used Reconyx HyperFire HC600 (Reconyx, Holmen WI, USA), with trigger time of 0.2 s, recovery speed of 0.9 s, sensitivity set to “medium” and detection range/field of view of 30 m/42°. We also used a Scout guard 565F model camera, with a trigger time of 1.2 s, a recovery speed of 1 s and a detection range/field of view of 10 m/52°. In the Kalahari Desert, we used Reconyx Professional HP2X HyperFire 2, with a trigger time of 0.2 s, a recovery speed of 1 s and a detection range/field of view of 24 m/40°.

We retained images spanning up to 40 days before water sampling to be able to compare between locations. Images were manually examined and the number of individuals per taxon, the time and date of visit recorded in hourly intervals (to minimise the risk of counting several

times the same individuals). We coded all individuals in the pictures, regardless of their interaction with the water body, assuming that their presence implied a need for water. Animals were identified to the lowest possible taxonomic rank (species or genus). For each taxon we recorded *body mass* extracted from PanTHERIA (Jones et al., 2009; Pigot et al., 2020), the *total number of visits*, the number of *days of last visit before sampling* and the *mean frequency of visits* as potential explanatory variables.

Based on these variables, we built an equation to evaluate the quantity of DNA of a given species in a given location, under the assumption that taxa frequenting a water body more often would be more likely to be detected and yield more reads. To this end, we used the maximum number of individuals recorded in a single picture for each taxon *i* at each station *j*, within each hour interval *t* (N_{it}). First, we calculated a CT based DNA detectability score with:

$$D_{ij} = m_i \sum_t \frac{N_{ijt}}{t} \qquad \textit{Equation 1}$$

where m_i is the body mass of species *i*, N_{ijt} the maximum number of individuals recorded in a single picture of species *i* at station *j* and at time *t* [days] before water sampling.
This equation gives a value of the cumulative DNA detectability (D_{ij}) for each taxon in each location at a particular time, which we then summed up over the 40 days before water sampling to obtain a cumulative DNA detectability score through time. This approach assumes that the eDNA added by a taxon in the water remains constant until its next visit and reaches its maximum concentration at the end of the recording period. We assume that the quantity of released DNA is proportional to species body mass m_i.
The second index (Dr_{ij}) is based on Eqn. (1) but considers the relative cumulative quantity of DNA in the water body at the end of the recorded period.

$$Dr_{ij} = \frac{\Sigma D_{ij}}{\Sigma D_j} \qquad \textit{Equation 2}$$

The above-mentioned indexes do not account for the presence and constant turnover of eDNA of other taxa over the days preceding a visit, i.e., the pool of eDNA in the water body. We recalculated the scores from Eqn. (2) to account only for the last 5 days before sampling ($Dr5_{ij}$), to reduce the pooling effect. Our three detectability formulas were tested using the Mamm02 dataset only. Overall, we excluded the Vert01 data because the amplification of mammals and birds using this primer yields mainly mammal sequences and very few bird sequences (see Figure S2 for an overview on bird detection).

Statistical analyses
We investigated the individual effect of all CT-derived variables (*days of last visit before sampling*, *total number of visits* separately, *body mass* and *mean frequency of visit*) on the eDNA data, both qualitatively (presence/absence) and quantitatively (logit RRA).

The relative read abundances (RRA) were transformed to avoid zero values using Eqn. (3) with a sample size (S) of 12 samples per site (Smithson & Verkuilen, 2006):

$$RRA' = \frac{RRA*(S-1)+1/2}{S} \qquad \textit{Equation 3}$$

A logit-transformation was subsequently used to achieve normality:

$$logit\,(RRA') = log\frac{RRA'}{1-RRA'} \qquad \textit{Equation 4}$$

The non-linear correlations between some of the CT-derived variables suggested a more complex role of each variable to explain detectability (Figures S1 and S2). We aimed at disentangling from our hypothesis which variables were best explaining the observed eDNA detection. First, we used Kendall rank correlations to test separately the effect of each variable. Second, we used generalized linear mixed models (GLMM), with the *lme4* package (Bates, Mächler, Bolker, & Walker, 2015), to investigate to what extent the variables (scaled and centred) influenced the likelihood of eDNA being detected. A logistic regression was performed on the presence/absence eDNA data and a normal regression for the RRA data (logit RRA) on the CT-derived variables. eDNA data (present/absent) were fitted to the explanatory variables extracted from CT data with a binomial distribution (Model 1, Supporting Information). Third, we used linear mixed-effect models (LMM), with the *lmer* package (Kuznetsova, Brockhoff, & Christensen, 2017), to investigate the influence of the variables on the RRA data (Model 2, Supporting Information). For both model selections, we used Akaike information criterion (AIC) to select the model with the best fit, i.e., the lowest AIC value, to reduce overfitting or underfitting the model (Burnham & Anderson, 2002). We chose the qualitative approach (Table 1, Model 1) to further investigate eDNA detection probability based on CT-derived variables because of its better explanatory power and ecological significance compared to the quantitative approach (Table 1, Model 2). We recalculated the predicted values of Model 1 for the three variables separately (*days of last visit before sampling*, *total number of visits* and *body mass*) and combined through the cumulative detectability (D_{ij}, Eqn. 1).

Results

DNA metabarcoding

After all quality filtering steps, we retained 1,254,585 reads of 93 different OTUs for the Vert01 assay that were assigned to 37 taxa. 747,628 reads of 51 different OTUs were assigned to 36 taxa for the Mamm02 assay (all species detected by eDNA can be found in Table S4, Supporting Information). We detected 18 taxa in the Trans-Altai Gobi and 21 in the Kalahari. Vert01 and Mamm02 primers are overlapping for some taxa, i.e., these taxa can be amplified by both primer sets. Bird species detected with both eDNA and CT can be found in Figure S2. While most mammal taxa were detected by only one primer pair, nine were shared between assays in Kalahari and 16 in Gobi (Figure 2). Despite not being a prior goal of this project, we

compared primer specificity and found that eight mammal taxa in Gobi and one in Kalahari were detected exclusively with the Vert01 primer set. The numerous presences of birds in the Vert01 dataset contributed to the variable detection score between primers, as we did not detect bird sequences in the Mamm02 results.

Camera traps

We identified 38 taxa in Kalahari and 22 in Gobi with CT (Figure 3, Table S3, Table S4, Supporting Information). One camera from Kalahari could not be recovered and this location was therefore excluded from all analyses (Bots2). Using the variables retrieved from the images, we assessed the correlations between them to better understand the visiting patterns of the recorded species. We observed a negative exponential correlation between *total number of visits* and *days of last visit before sampling* ($R^2 = 0.35$, p-value < 0.001, Figure S1A, Supporting Information) and between *total number of visits* and *mean frequency of visits* ($R^2 = 0.31$, p-value < 0.001, Figure S1B, Supporting Information).

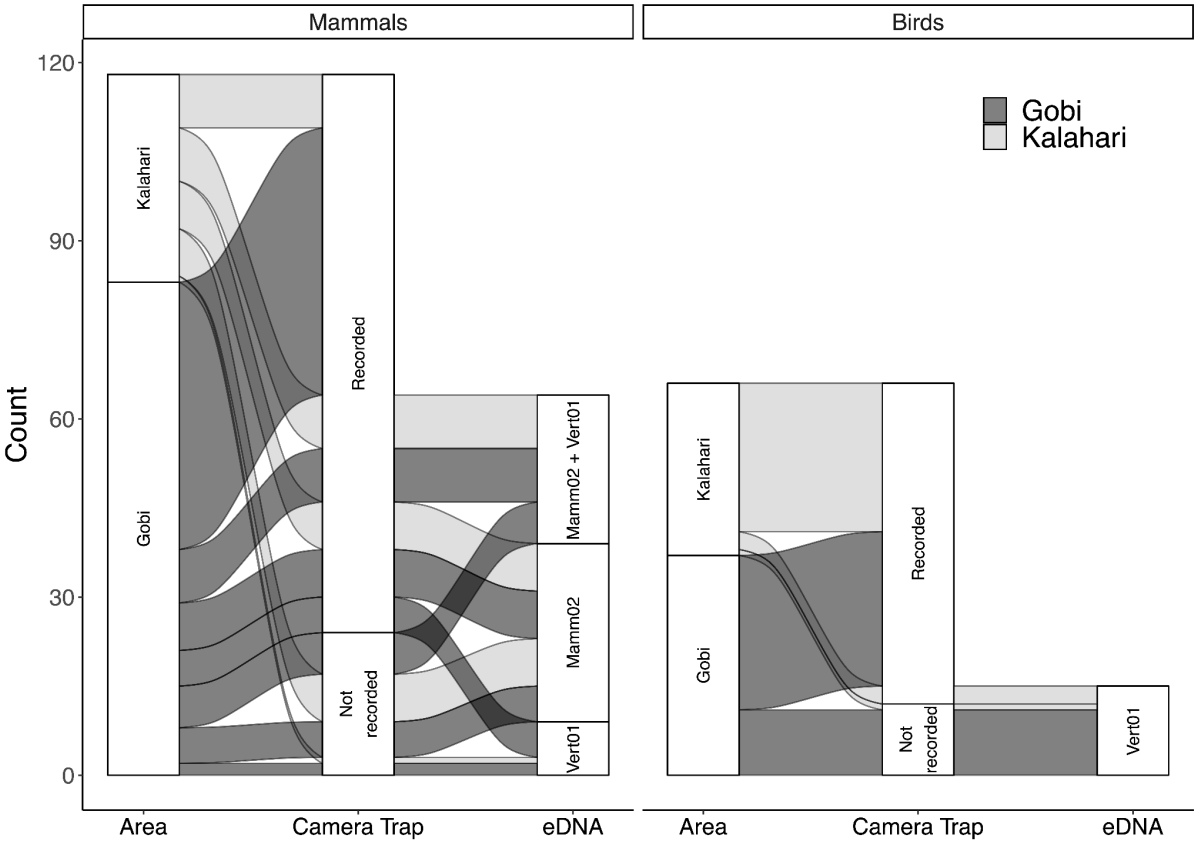

Figure 2. Comparison between areas of study, detection methods and primers used for birds and mammals separately. The Y-axis represents the number of different species in each particular location.

Comparison between eDNA and camera trap data

In total, 84 taxa were identified combining data from CT and eDNA, 59 in Kalahari and 31 in Gobi. Some species were present in both areas. Detailed overview on the performance of

detection methods can be found in Figure 2 and 3, as well as primer differences within the successfully amplified species. Note that each occurrence in Figure 2 indicates a single species for each particular location.

Detectability score and eDNA

CT results were used as a reference to compare the detectability score of the eDNA approach in these environments. Using the score from Eqn. (1), we separated the cumulative curves by positive and negative eDNA results (Figure 4). D_{ij} increases drastically if there were visits to the water body the day before sampling or the same day. We found significant correlations with eDNA data for the raw CT variables and with the detectability score D_{ij} (Eqn. 1), both for the quantitative (RRA) or the presence/absence measure. Relative detectability approaches (Dr_{ij} and $Dr5_{ij}$) poorly explained the eDNA results compared to the absolute D_{ij} approach (Table 1).

	presence/absence	logit(RRA')
D_{ij} (Eqn. 1)	***p < 0.001***	***p < 0.001***
	AIC = 186.62	*R^2 = 0.136*
Dr_{ij} (Eqn. 2)	*p = 0.372*	*p = 0.879*
	AIC = 238.85	*n. s.*
$Dr5_{ij}$	*p = 0.526*	*p = 0.157*
	AIC = 60.79	*n. s.*
Days of last visit before sampling	***p < 0.001***	*p = 0.065*
		n. s.
Total number of visits	***p < 0.001***	***p < 0.001***
		R^2 = 0.29
Mean frequency of visits	***p < 0.001***	***p < 0.001***
		R^2 = 0.133
Body mass	***p < 0.01***	***p < 0.001***
		R^2 = 0.12

Table 1. Results of the logistic regression for 0/1 eDNA data (categorical approach) and linear regression for the RRA data (quantitative approach). We used logit transformation on the RRA

data and removed 0 and 1 values from the dataset to test for the linear regression, as shown in Equation 3 and 4. Significant p-values are shown in bold, n. s. stands for *not significant*. R^2 values show the Adjusted R^2.

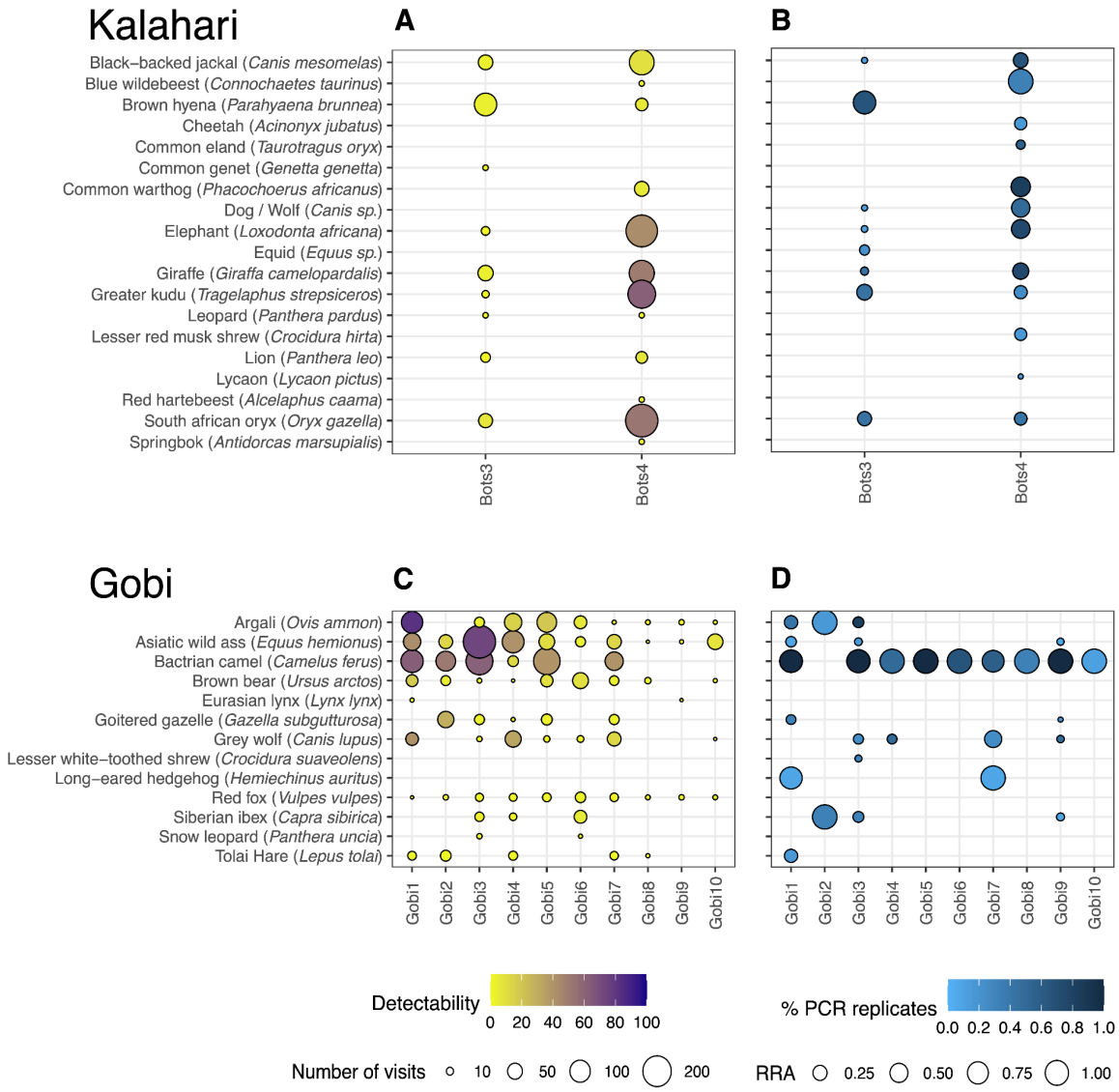

Figure 3. Bubble plot representing the detected mammalian species by each method. A) Species recorded with camera traps in the Kalahari Desert. B) Species detected with eDNA in the Kalahari Desert. C) Species recorded with camera traps in the Trans-Altai Gobi Desert. D) Species detected with eDNA in the Trans-Altai Gobi Desert.

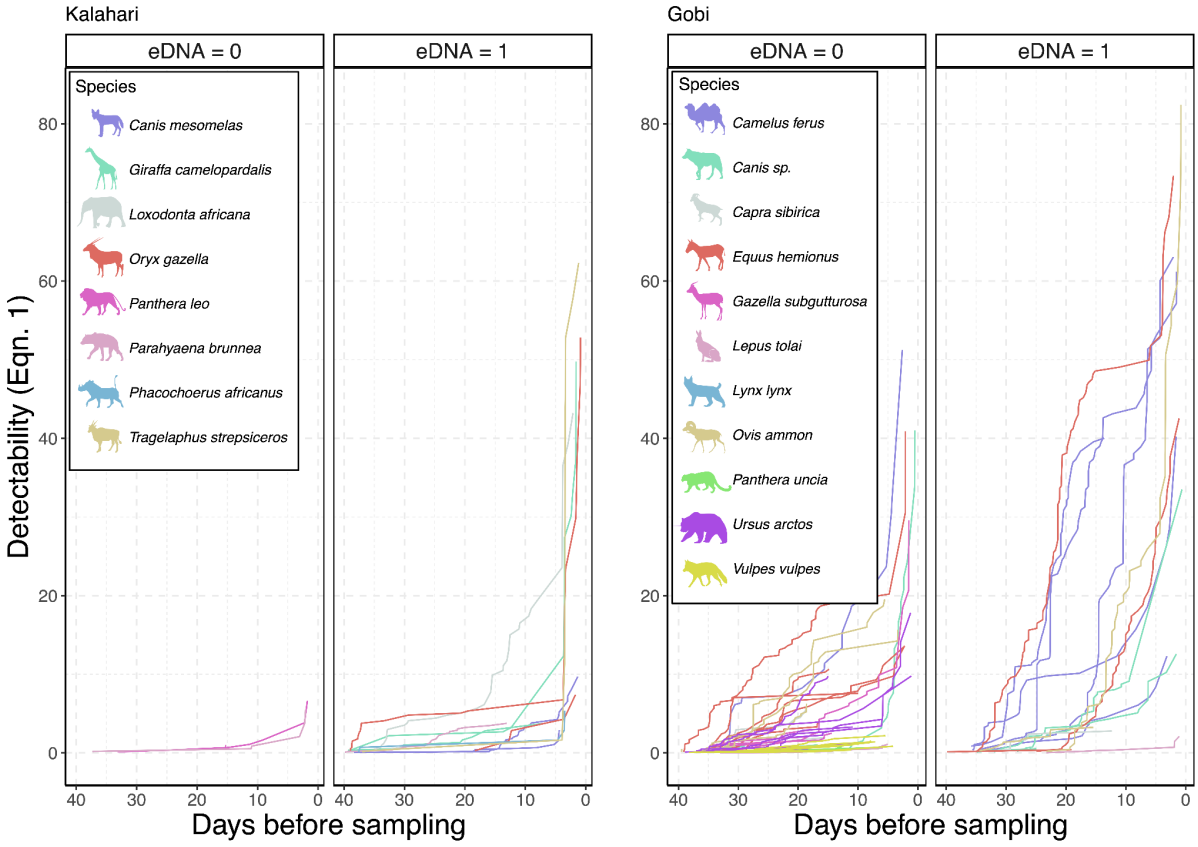

Figure 4. Detectability curves for each of the sampled areas calculated using Eqn. (1). Lines in the "0" box indicate that no eDNA could be recovered. Lines in the "1" box yield eDNA. This figure does not show species which were recorded only once, but these were included in Figure 3. Each species is represented by a silhouette.

Modelling eDNA detection

The explanatory variables showed significant correlations with eDNA results when tested individually, except *days of last visit before sampling* for RRA (Table 1). We first used eDNA presence/absence data as our model response variable (Model 1). Variance in Model 1 was significantly explained by *days of last visit before sampling*, *total number of visits* but not *body mass (log transformed)*, which also had the lowest AIC score. In Model 2, the quantitative response variable (logit RRA) was not significant for any possible combination (Model 2, Supporting Information).

The lower fit of the RRA data led us to further explore the presence/absence approach (Model 1) regarding detectability by eDNA. Figure 5 illustrates the relationship between CT data and eDNA detection for the cumulative detectability score (D_{ij}) (Figure 5A) and for each of the three variables independently (Figures 5B, 5C, 5D), which were used to build the detectability score equation (Eqn. 1).

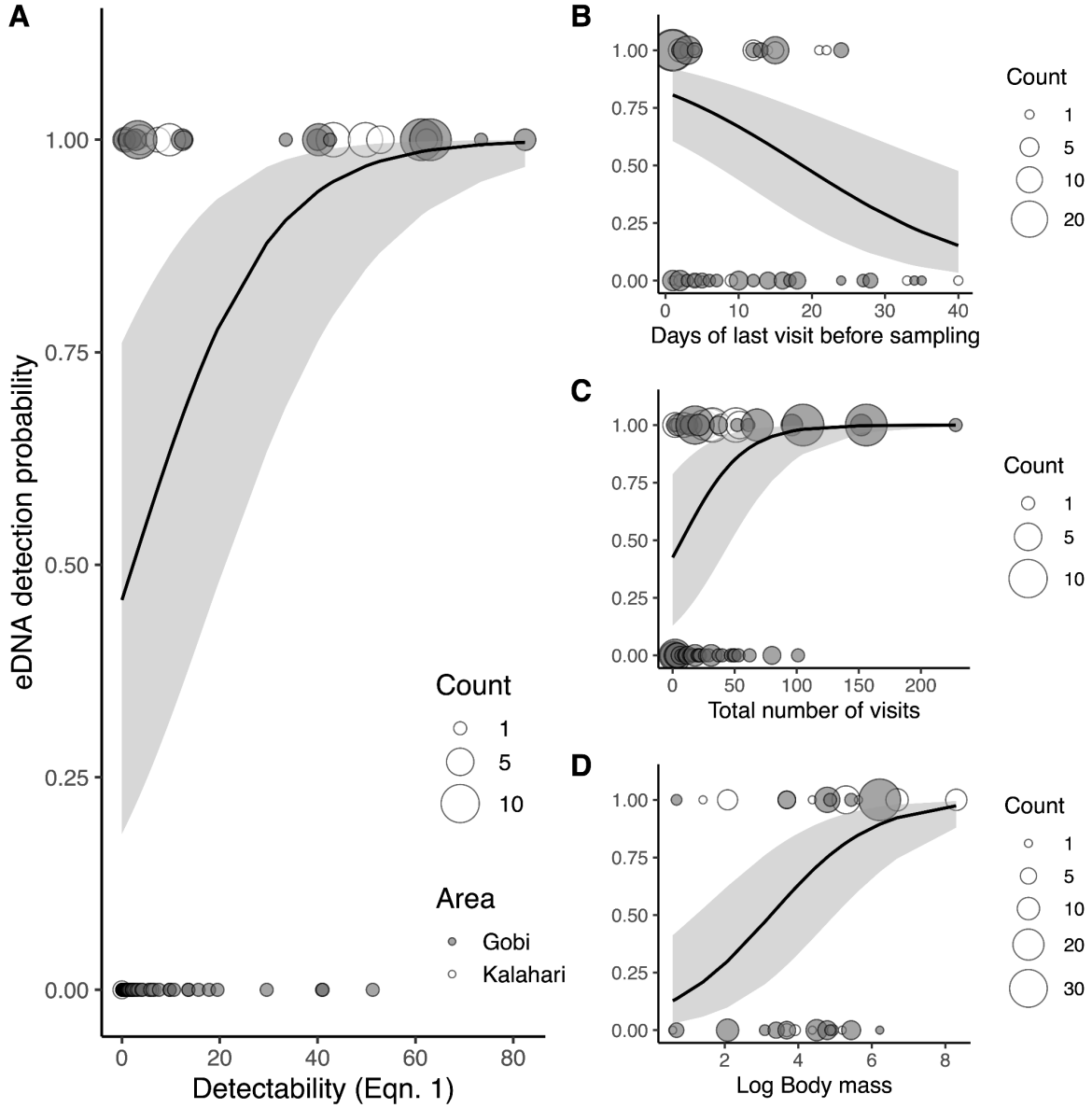

Figure 5. A) Modelled prediction of eDNA detection for cumulative detectability (Eqn. (1). B, C, D) Modelled prediction for each variable involved in Eqn. (1) separately. All with p-value below 0.001. Size of the dots indicate count of occurrences and grey scale indicates the area. The black line indicates the model's predicted values with its confidence interval in grey.

Discussion

Studying fauna in terrestrial environments using eDNA is generally more challenging than in aquatic habitats because the presence and concentration of eDNA is less homogeneous across the area of sampling (Leempoel et al., 2020; Lyet et al., 2021). To study terrestrial and semiaquatic environments using eDNA, sampling water can be therefore more advantageous over other sample types (Harper et al., 2019; Rodgers & Mock, 2015). Biomonitoring in arid

or semi-arid ecosystems represents an additional challenge given the harsh environmental conditions and often vast spatial areas. However, the rare and spatially dispersed water bodies attract organisms and operate as DNA pools that record and temporarily preserve the information of visiting animals, thus representing unique opportunities for sampling. In this study, we successfully show the detection of terrestrial fauna using desert water. We analysed the correlation between CT image data and eDNA sequence data and showed the interplay between the visiting patterns of species and the probability to detect them using eDNA. We detected terrestrial organisms in all our water samples with both primer sets, which illustrates the capacity of our assays to detect terrestrial fauna using water-borne eDNA from desert ecosystems. While CT is widely used for biomonitoring, only few studies compare CT and eDNA data for terrestrial animals (Leempoel et al., 2020; Lyet et al., 2021; Mena et al., 2021; Sales, Kaizer, et al., 2020; Sales, McKenzie, et al., 2020) and fewer explored the potential of water-borne eDNA for arid and semi-arid ecosystems. Seeber *et al.* (2019) used samples of water bodies in two African ecosystems to study the presence of mammal species, comparing a hybridisation capture approach to conventional PCR. Furthermore, Egeter *et al.* (2018) sampled water in three Saharan water bodies for a mainly methodological DNA metabarcoding study focusing on the water filtration process. We observed clogging of the filters during filtration in the Kalahari due to the turbidity of the water resulting in variable volumes (Table S1, Supporting Information), although we used the filter pore size as recommended by (Egeter et al., 2018). Alternatively, Abrams *et al.* (2019) and Weiskopf *et al.* (2018) investigated the suitability of leech-derived eDNA as a survey tool for vertebrate species by comparing it to CT detections. Taking the analyses one step further, Tilker *et al.* (2020) combined the data obtained with these approaches to identify species responses to environmental factors.

DNA metabarcoding relies on “universal” primers that are designed for restrained taxa or groups. Variable numbers of mismatches between primers and templates are the presumed main cause for the preferential amplification of certain taxa and the under- or non-representation of others (Piñol, Mir, Gomez-Polo, & Agustí, 2015; Piñol, Senar, & Symondson, 2019). The multiplexing of primers and barcodes is an attempt to minimise these effects (Alberdi, Aizpurua, Gilbert, & Bohmann, 2018; Galan et al., 2018; Jusino et al., 2019; Krehenwinkel et al., 2017). However, in our study, while we chose the Vert01 primers to target birds and reptiles in addition to mammals, mammal sequences were also the most amplified with Vert01 primer, duplicating eDNA detections. The redundancy of the information raises the question of the utility of primer multiplexing in this context (Figure 2).
The presence of trace DNA of humans and domesticated animals in eDNA studies represents a common issue, the possible sources of such contaminations being numerous (Furlan et al., 2020). We removed the obvious cases (pig, cow), however, we kept two taxa in the Kalahari data set which are ambiguous and unconfirmed by CT. The detection of *Equus* sp. DNA in one Kalahari sample could be explained by the presence of horse, zebra or donkey or the transport of such DNA, for example by a predator. In addition, in the Kalahari samples we amplified DNA assigned to *Canis* sp. (dog/wolf). The sequences differ from the ones found in the Gobi samples, therefore excluding a cross-contamination, but its source remains unclear.

Previous CT vs. eDNA studies found that smaller animals are less likely to trigger CT and risk being overlooked in this type of biomonitoring studies (Leempoel et al., 2020; Lyet et al., 2021; Sales, Kaizer, et al., 2020). Smaller species probably release less DNA into the water than larger species and are hence less likely to be detected due to the presence of other species' DNA. Mena *et al.* (2021) conducted a comparative study of different traditional survey methods (pitfalls, grids, mist nets, CT) and aquatic eDNA for the detection of terrestrial mammals in tropical forests. The overall results point out the benefits of eDNA surveys, in terms of detection scores, labour-effort and costs, but depend very much on the species and sampling area. It must be noted that in the present study we analysed 40 days of CT data and compared it to the results of only one water sampling event for each locality (Figure 2 and 3). This has to be kept in mind when evaluating the performance of the eDNA assays, since a 40-days period is well beyond the persistence of free eDNA in water according to literature (Barnes & Turner, 2016). In the light of these shortcomings, we consider it encouraging to have detected using eDNA more than half of the mammals recorded with CT (Figure 2). We also acknowledge the limitations of taking a single sample per water body, as it may be underrepresenting the wildlife diversity. Additional samples from the same water body would likely increase detected species numbers, and we advocate that future studies aim to determine the optimal number of samples per location.

In this study, DNA was already present in the water prior to the placement of CT and the *a priori* DNA composition is unknown. We used the RRA approach as a proxy for species abundances, but it could be biased by the variable *body mass* of a species or by its behaviour (e.g. drinking, bathing, defecating), which affects the release of DNA (Harper et al., 2019). We did not find different detection scores between methods for nocturnal/diurnal species, but this is due to the capacity of CT to detect nocturnal species, contrasting to line transects (Coutant et al., 2021). For Gobi, the coverage of species is biased by the overwhelming presence of Bactrian camels (*Camelus ferus*). These animals are big and recurrent in the area, as confirmed by CT. To overcome this limitation, we suggest adding blocking primers for dominant species, if known beforehand. The usage of blocking primers is recommended for this type of study (De Barba et al., 2014; Vestheim & Jarman, 2008). Egeter *et al.* (2018) used Vert01 primers without human blocking primers and obtained 68 % of total reads assigned to hominids. In our study, we had fewer human sequences (9.36 % of sequences for Mamm02 and 37.5 % for Vert01), indicating a good performance of the blocking primer. A higher concentration of blocking primer than the one we used would increase the risk of co-blocking targeted taxa (Shehzad et al., 2012; Taberlet et al., 2018). In fact, the high abundance of camel and human DNA could explain the low detection rates of other less recurrent species, mainly carnivore species, despite the frequent CT recordings. For example, the locally rare and emblematic brown bear (*Ursus arctos*), whose residual population in the Gobi Desert numbers a few dozen individuals, was detected only once with the Vert01 primer pair (and therefore is not present in Figure 3D). This low detection of carnivore eDNA was also observed for Botswana and could indicate that further factors, such as drinking technique or contact with water (bathing), are involved besides the species body mass, e.g. (Lyet et al., 2021). We therefore encourage future metabarcoding studies to investigate the factors associated with successful eDNA detection of carnivores.

The negative correlation between the variables *total number of visits* and *days of last visit before sampling* (Figure S1A, Supporting Information) was expected because species visiting a water body many times are also likely to have visited it recently, and it could only be biased by migratory or nomad species that visit a water body in great numbers but low frequency. We excluded this potential confounding effect because taxa with high *total number of visits* were also the ones with the lowest *mean frequency between visits* (Figure S1B, Supporting Information). Hence, we were able to use these variables as predictors to calculate and model eDNA detection probabilities.

We tested several equations to combine the explanatory variables retrieved from CT data into a comprehensive index to account for the expected detectability of species in each location, both using a categorical (0/1) and quantitative (RRA) approach (Table 1). When visualising the increasing detectability score (Eqn. 1) by eDNA detection (Figure 4), there was a sudden increase for some species at the end of the monitored period. Such increase is due to the visits occurring not long before water sampling, which have a major impact on the overall score through time. Dr_{ij} and $Dr5_{ij}$ could not be visualised across time because only single values per species and location can be obtained. We found better correlation between RRA and $Dr5_{ij}$ compared to Dr_{ij} (Table 1), probably due to lower DNA degradation, but none were significant, which highlights the complexity of defining detectability scores. However, these scores are a simplification of reality, as they do not account among other factors for DNA decay (Barnes & Turner, 2016). Using RRA as a proxy for species relative abundance must however be taken with caution because of the biases that DNA extraction, amplification and sequencing imply (for an overview of the biasing factors, see (Fonseca, 2018). Furthermore, the categorical approach homogenised the coverage of each species and, in practice, increases the weight of low RRA species in our test (Deagle et al., 2019). This is of particular interest when using RRA data, as it provides a more realistic proxy in terms of abundances. Overall, the better correlation of RRA for $Dr5_{ij}$ suggests the RRA approach to be a better proxy for species detection when water samples are taken frequently. The categorical approach is recommended when the sampling is done only once (as in the present study) or sparsely. In addition, these scores only make sense when comparing CT and eDNA data. Still, the characteristics of the sampled area need to be accounted for when drawing detectability scores. For instance, Lyet *et al.* (2021) sampled river water to detect mammal species and they defined their detectability score based on the camera trap detection rate and the pluviometry of the day. Nonetheless, our results are promising, and optimising the accuracy of these scores will improve cross-validation of both methodologies, both for comparative studies and when using eDNA as a complementary tool to CT. However, the complexity of interacting ecological factors complicates building a simple equation to reliably infer eDNA detection probability. In this line, a purpose-built experiment should be carried out to tackle this matter, with a limited number of species in a controlled environment.

Another goal of this study was to draw guidelines for future studies aiming to use eDNA as a biomonitoring tool in desert environments. We built models based both on eDNA 0/1 data (Model 1) and RRA data (Model 2) data. All variables used in Model 1 except *body mass* were significant, suggesting that the categorical transformation of our eDNA data is more advisable

when the goal is species detection rather than its relative abundance. We used this model as a reference to calculate detection probabilities for each variable and area independently, in order to disentangle the effect of each and visualise them (Figure 5). Interestingly, the positive eDNA detections based on *Final cumulative detectability* are clearly divided into two groups (Figure 5A). This suggests that our detectability score fails to properly reflect the true detectability of some species, which could be explained by the poor correlation observed for *days of last visit before sampling* (sparse distribution of non-detection occurrences, Figure 5B) and *body mass* (Figure 5D). In this line, these last two variables should be studied more in depth to properly understand their impact on eDNA detectability. The good fit of *total number of visits* (Figure 5C) is, as mentioned before, influenced by *days of last visit before sampling* (Figure S1B).

Modelling with RRA data (logit transformed) was more ambitious because we also had to cope with the issue of PCR-introduced biases, which were minimised when transforming our eDNA data to a categorical approach. The best model fit was obtained with *total number of visits* and *days of last visit before sampling* (Model 2) but none of these variables were significant. The RRA per taxa is assumed to be correlated to the released DNA i.e. the initial biomass of taxa in a sample (Deagle et al., 2019). It was difficult to correlate RRA data to visiting patterns to the water body. In fact, the degradation of DNA in the water combined with the continuous turnover of new DNA creates a complex multivariate dynamic system of DNA concentration and quality in the water body which is captured only once at the moment of sampling. In our study, the 40-days-range of CT monitoring exceeds the time free eDNA remains detectable in water. This could explain the lack of significance for the explanatory variables of Model 2. We tested this same model only with data from the last 5 days before sampling ($Dr5_{ij}$) and we obtained a better fit but remaining non-significant. Nevertheless, the proposed DNA detectability scores calculated from CT data successfully represented the detection of species through eDNA, surpassing 75 % of positive detection for D_{ij} scores above 25 (Figure 5A). Furthermore, species that visited the water bodies more than 25 days before sampling were never detected (Figure 5B), which indicates the maximum dayspan between sampling events. However, these numbers apply only to our particular study system. DNA degradation and its detectability through DNA metabarcoding are very sensitive to environmental conditions of the sampled area, and future studies should target the effects of additional biophysical (such as *pH*, temperature, UV- radiation, water body size and depth) or biological variables (such as bacterial activity). Increasing the resolution and ecotype range of this kind of study will contribute to defining the probability of species detection through eDNA and contribute to improving sampling strategy for future research.

The direct comparison of detection success is strongly biassed by the different survey lengths of this study and we found, therefore uncontested, CT to detect more taxa than the eDNA approach. Both methods have undoubtedly pros and cons, and it is crucial to weigh those and adapt the sampling strategy to the respective study system. Our results on detectability suggest that CT is preferential over eDNA for monitoring species when eDNA sampling cannot be made at regular intervals or to cover long monitoring periods. It ultimately also depends on the targeted taxa, being preferential when you study medium- to large-sized organisms which can be easily differentiated morphologically (Mena et al., 2021). Species abundances and densities

can also be deduced from image data more easily. However, to rely on cameras implies the risk of losing data (over 40 days, 1/14 cameras were lost) and demands repeated visits which may be complicated in remote areas (at least installation and recovery of cameras), where eDNA sampling offers valuable study opportunities (McInnes, Bird, Deagle, Polanowski, & Shaw, 2021). Also, CT does not offer the same options as eDNA in terms of possible population genetic studies (Bohmann et al., 2018; Nguyen et al., 2021; Sigsgaard et al., 2020; Sigsgaard et al., 2016; Tilker et al., 2020; Wilting et al., 2021) and the extension to other phyla (as e.g. invertebrates, plants and bacteria). eDNA sampling is appealing due to its sensitivity, standardisation and non-invasiveness, as well as the independence from taxonomic experts for taxa identification. eDNA techniques may enable the detection of elusive species and the taxonomic differentiation of morphologically similar as well as of cryptic species (Thomsen & Willerslev, 2015). In terms of quantifying the abundance of species, the eDNA approach has to cope with the dynamics of DNA in the water body released by the visiting individuals. They create a continuous turnover of available DNA that can easily bias the inference from amplified DNA to species relative abundances. Furthermore, genetic data at this scale is per se unable to provide certain population dynamic parameters (sex, age, absolute numbers of individuals). As we have seen in our data, regular sampling in short intervals is necessary to provide a complete picture in terms of species richness. Alternatively, eDNA surveys can be used e.g. on a broad geographic scale to get a first glimpse of the biodiversity of the area that can be locally refined with CT (Sales, McKenzie, et al., 2020). In fact, the combination of the two methods is increasingly used in biomonitoring studies (Sales, Kaizer, et al., 2020; Sales, McKenzie, et al., 2020), which is advantageous due to their complementary strengths. This could be particularly advantageous when the study system includes both large and small taxa of interest. The water samples are going to be dominated by the DNA of large taxa, which are easily recorded by CT, but they complicate the amplification of small taxa. Dividing methodological efforts, for instance by including blocking primers of the large dominant species, will increase the detection of the other smaller species, which are more likely to be missed by CT.

Our results show that water bodies concentrate information about large remote regions that are difficult to access and monitor using conventional, observer-based methods. They function as eDNA reservoirs containing information about valuable ecosystems. In light of the manifold risks facing wildlife – particularly species in remote and threatened ecosystems – further cross-method tests are needed, to validate their parallel application and support their integration into conservation monitoring strategies.

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Conflicts of interest

PT is co-inventor of a patent related to the Vert01 primers for vertebrate identification using degraded template DNA. This patent only restricts commercial applications and has no impact on the use of this locus by academic researchers. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the paper.

Supplementary material

Additional supporting information may be found in the online version of the article at the publisher's website. https://doi.org/10.1002/ edn3.274

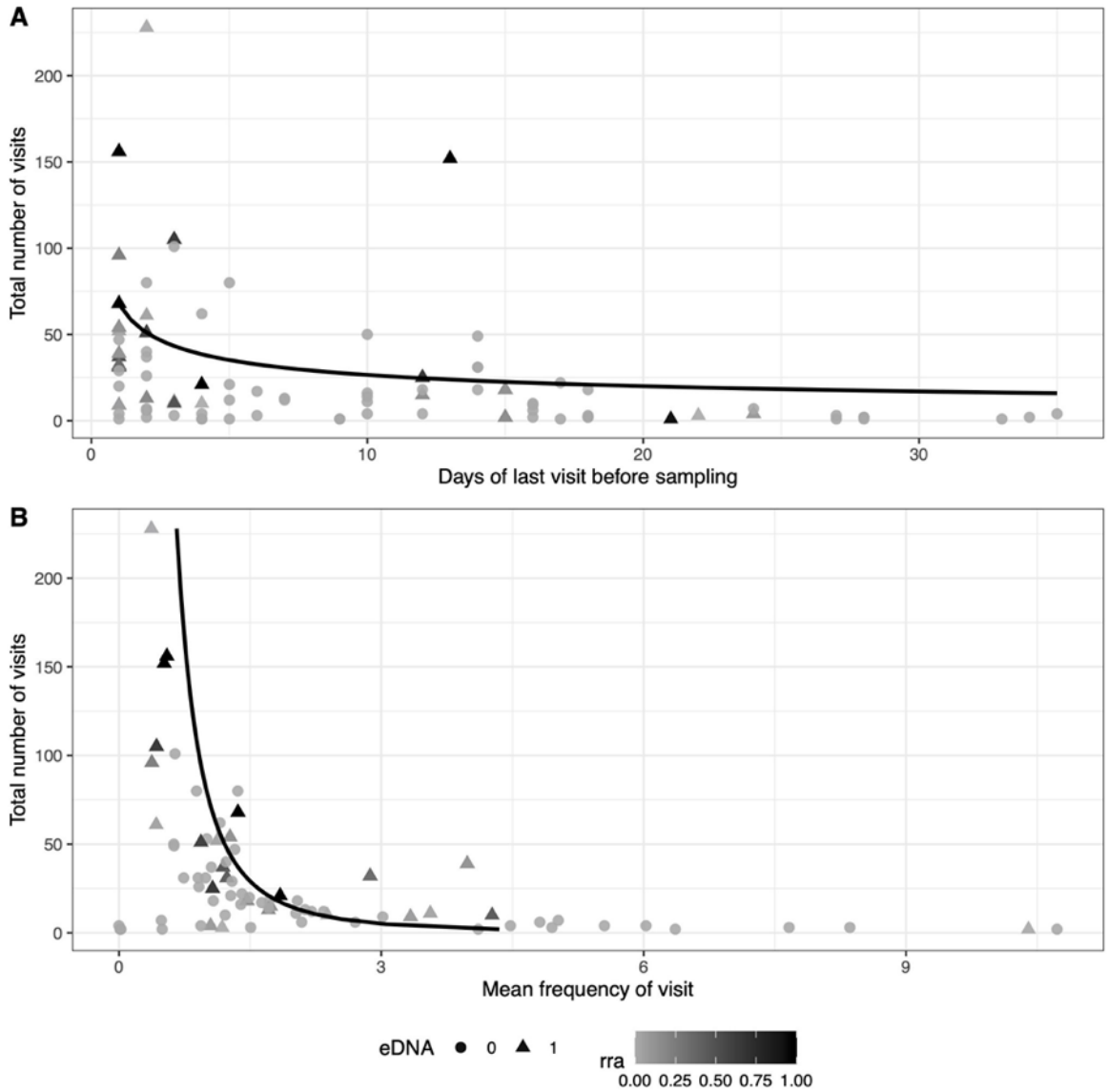

Figure S1. A) Correlation plot between *total number of visits* and *days of last visit before sampling*. The shape indicates if sequences were retrieved or not. Grey tones indicate RRA of sequences retrieved per species and location (logit.rra), the line shows the modelled negative exponential regression (R^2 = 0.35, p-value < 0.001). B) Correlation between *total number of visits* and *mean frequency of visits*. Grey tones indicate the area, the shape indicates the detection through eDNA. The line shows the modelled negative exponential regression (R^2 = 0.31, p-value < 0.001).

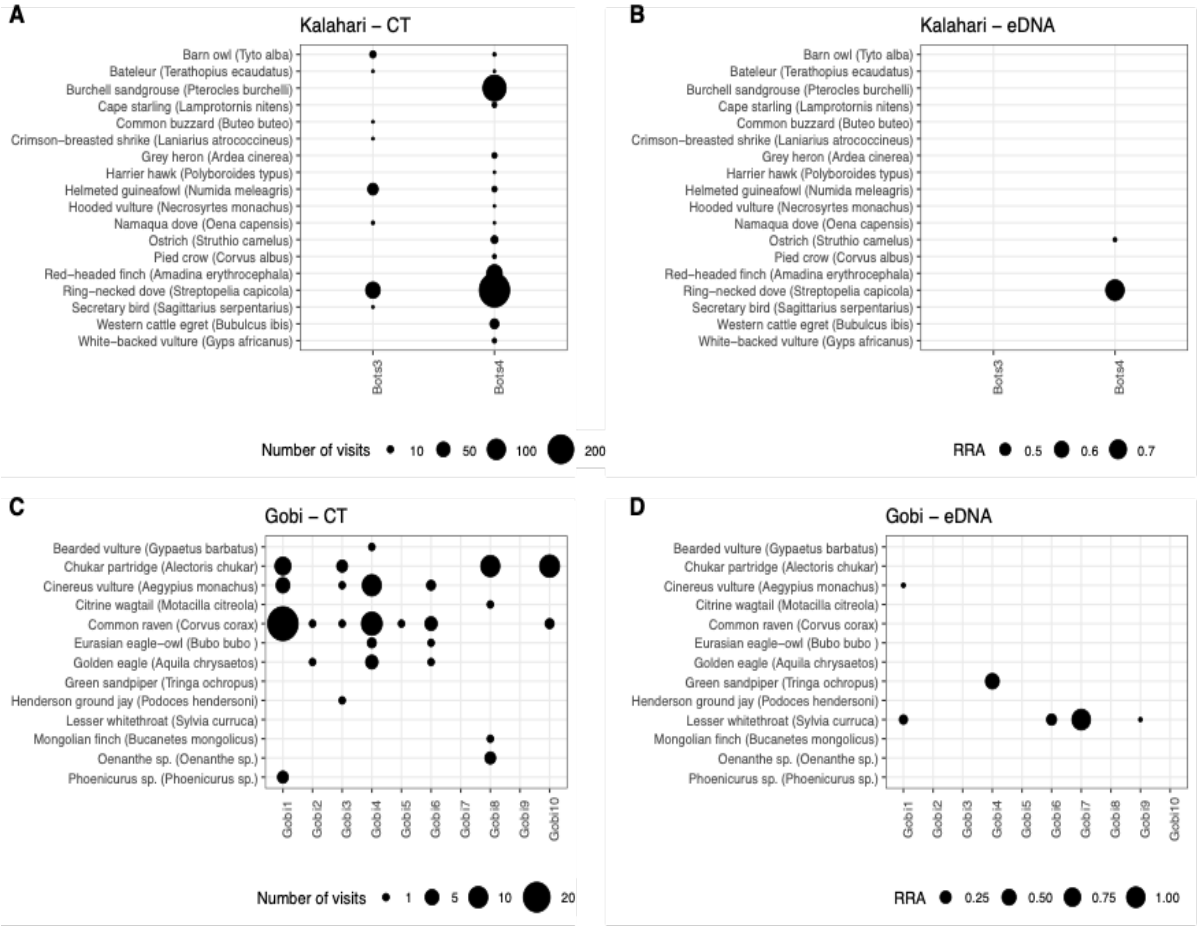

Figure S2. Bubble plot representing the detected bird species by each method. A) Species recorded with camera traps in the Kalahari Desert. B) Species detected with eDNA in the Kalahari Desert. C) Species recorded with camera traps in the Trans-Altai Gobi Desert. D) Species detected with eDNA in the Trans-Altai Gobi Desert. Species detected with eDNA outside their geographical range were considered as contamination and excluded from the figure.

MODEL 1. eDNA (0/1) ~ days of last visit before sampling + number of visits + body mass + (1 | species) + (1 | area/location). Significant p-values are shown in bold				
AIC	BIC	logLik	deviance	df. Resid
116.7	139.1	-51.4	102.7	172
Fixed effects				
	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-61.92	19.96	-3.102	**0.0019*** **
Days of last visit before sampling	-0.5525	0.159	-3.453	**0.0005*** **
Number of visits	0.1408	0.007	1.995	**0.045***
Body mass	8.253	5.059	1.631	0.102
Random effects				
	Variance	Std. Dev		
species	1.03 e+03	3.21 e+01		
location:area	7.44e+02	2.728 e+01		
area	5.55 e-10	2.35 e-05		
Number of obs: 179, groups: species, 25; location:area, 12; area, 2				

MODEL 2. logit.rra ~ days_of_last_visit_before_sampling + number_of_visits + (1 | species) + (1 | area/location). Significant p-values are shown in bold				
AIC	BIC	logLik		
442.88	461.05	-214.44		
Fixed effects				
	Estimate	Std. Error	t value	Pr(>|z|)
Intercept	-1.6859	0.4405	-3.828	**0.00346** **
Days of last visit before sampling	-0.333	0.3789	-0.879	0.3819
Number of visits	0.4888	0.3254	1.502	0.1370
Random effects				
	Variance	Std. Dev		
species	1.793	1.339		
location:area	0	0		
area	0	0		
Residual	3.926	1.981		
Number of obs: 103, groups: species, 15; location:area, 7; area, 2 Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				

Summary of results

Chapter 1 - Density-dependent resource partitioning of temperate large herbivore populations under rewilding

We found strong diet overlap between them as expected from their long-term population dynamics. The diet of horse and cattle remained mostly unaltered and it was the one of red deer that changed the most across the years. Between the four years, niche overlap decreased with red deer population size, the most abundant species. This relationship was strongest in species interactions which included red deer. When calculated as total energy expenditure, we also found that our results in niche overlap are more linked to the shifts in red deer than to the total herbivore energy fluctuation. We suggest red deer changed their diet in response to their own population size, reducing their niche overlap with increasing red deer population. In this case, resource competition translated into shorter vegetation height, reducing resource availability and forcing herbivores to consume different plant taxon. We conclude that in this temperate ecosystem without strong population regulation by predators or humans, inter- and intraspecific resource competition are key factors structuring community composition and dynamics from small to large herbivores, with a competitive advantage of the smaller species, but with also several opportunities for resource partitioning.

Chapter 2 - Niche overlap between two large herbivores across landscape variability using dietary eDNA metabarcoding

Our findings indicate diet composition is non-homogeneous across the landscape, both within and between species. Red deer showed greater diet variability and lower niche overlap within species compared to bison. We detected a reduction of niche overlap for red deer with increasing predation risk, leading to more dissimilar diets, suggesting their feeding behaviour is affected by wolf presence. This correlation was not found for bison, which are rarely predated by wolves. Higher habitat quality was associated with higher niche overlap only within bison, probably due to their suboptimal feeding strategy as browsers. These results show the importance of integrating environment-induced diet variation in studies aimed at determining the landscape usage or niche overlap of a species. Niche overlap can therefore be a powerful tool for inferring predation risk for red deer, and estimating predator abundance or its perception. This approach has enormous potential for wildlife management in areas where predator estimates are unknown or difficult to quantify.

Chapter 3 - The role of pesticides and vineyards management on soil arthropod community using eDNA

The two types of management used distinct pesticide combinations along the retrieved years, but both heavily relied on fungicides. We found the soil arthropod community of organic vineyards to have greater diversity compared to conventional ones. The community differences between regions indicated that the nature of the soil was a stronger driver of the sampled community than the management applied. However, we were able to identify several active compounds significantly correlated to the shifts in the arthropod community that could be having a negative impact on the soil ecosystem, highlighting the need to focus on these pesticides when designing new sustainable management practices. Our study provides an

ambitious but scalable approach to monitor pesticide impact on the soil biological community to improve vineyard ecosystems but further research is needed in order to better understand the combined effects of pesticides on the soil ecosystem.

Chapter 4 - Spatiotemporal patterns of benthic macroinvertebrates in a natural glacier-fed stream using kick-net and eDNA sampling

We found more abundant taxa collected with kick-net were also more likely to be detected with eDNA. Regarding the seasonality, we did not find clear evidence for greater biodiversity during the winter months in the main course. However, we identified a reduction of total abundance of macroinvertebrates with increasing sediment load, produced by the melting of the glacier. Spatially, we found the community remained overall similar across seasons in the main course of the Val Roseg river, but experienced a clear shift within the tributaries at the start of the melting season. As we found the communities in the tributaries were most dissimilar to the main course, we propose the increase in sediments in the water marks the tipping point in the ecosystem. This suggests the lack of sediments in tributaries could be driving the macroinvertebrate community shift. However, we did not find evidence for colonisation patterns from tributaries or as spatial refugia. For hydropower-impacted streams, these findings indicate the macroinvertebrate community is sensitive to flushing of sediments, and advocate for developing strategies to offer sediment-free conditions for macroinvertebrates during the melting season.

Chapter 5 - Assessing environmental DNA metabarcoding and camera trap surveys as complementary tools for biomonitoring of remote desert water bodies

We found significant correlations between the two methodologies and described a detectability score based on variables extracted from Camera trap data and the visiting patterns of wildlife. This supports the use of eDNA-based biomonitoring in these ecosystems and encourages further research to integrate the methodology in the planning and monitoring of conservation strategies.

General discussion

Ecosystem biomonitoring is experiencing a revolution thanks to the advances in environmental DNA (eDNA) sampling and high throughput sequencing. eDNA is defined as the genetic material shed into the environment by organisms through mucus, urine, faeces and other biological materials. This genetic material can then be collected and extracted from environmental samples. We can now sample ecosystems with lower effort while detecting more taxa than ever before, thanks to the cost reduction in laboratory and sequencing parts (Taberlet et al., 2018), which has enlarged the reference databases to identify more species and more accurately from environmental samples (Berry et al., 2021; Marques et al., 2021). Now, not only are we able to search for certain species in their environment without having to directly observe or capture them, because of the non-invasiveness of the technique, but we can obtain much more information about the functioning of the ecosystem (Seymour et al., 2020). As such, we can identify the assembly of species present in the sampled environment or the trophic interactions between species (Taberlet et al., 2018). Experimentation with eDNA has upscaled the limits for the ecological information that can be retrieved from a single environmental sample. This has opened a new research field where ecologists can now answer ecosystem questions through a molecular approach, and geneticists can now implement their molecular skills beyond the laboratory and directly in the environment (Barnes & Turner, 2016; Morisette et al., 2021). Altogether, they have led the field of eDNA to consolidate as an important tool for ecological studies.

We are now closer to achieving the ultimate goal of eDNA, which is to correctly infer the presence and relative abundance of species in the ecosystem from the collected environmental samples, but many constraints still exist. One of the main challenges is the interdisciplinarity of the field (Ruppert et al., 2019). It requires a good ecological fieldwork foundation, for an accurate eDNA sampling strategy, good molecular laboratory knowledge, to maximise the ecological information extracted from the environmental sample, and good bioinformatics and statistical expertise, to put the two previous steps in perspective and clean the generated datasets from the environmental and sequencing noise accumulated.
Furthermore, there are other challenges to consider when aiming to use eDNA for ecological inference. The sampling strategy has to be adjusted to maximise the resolutive power of the data collected, and to do so, experience is key to anticipate potential problems that we might encounter, from sampling to statistics. In parallel, working with eDNA requires specific lab conditions which reduce potential interference with amplified products or external contamination. This is because the use of generic primers comes with the risk of amplifying DNA which is exogenous to the sampled material. This is a recurrent problem in eDNA studies, and many protocols exist now to reduce this type of contamination and software to remove contaminants from the produced datasets (Zinger et al., 2021).

During the last decade, eDNA research has advanced towards the close integration of genetics and ecology to maximise the potential to answer questions from a whole ecosystem perspective. However, the complex nature of eDNA dynamics in the environment poses major challenges to this objective. Multiple factors drive the detection probability of a species or the

community composition of the target environment (Furlan et al., 2016), such as pH, temperature, UV exposure, PCR inhibitors or co-extracted non-target DNA. In parallel, eDNA persistence (Harrison et al., 2019), its transport (Valentin et al., 2021) and its relative concentration in the environment (Fonseca, 2018) complicate the ecological interpretation of the produced datasets. Despite this, an increasing number of studies have been tackling the intricacies of this methodology for many years, standardising the procedure to have replicable studies across distinct environments (Dickie et al., 2018). Every new advance gets us closer to achieving a standardised eDNA application for ecological studies, where the data obtained can be reliably put in perspective of the environment where it was sampled.

In this thesis, we have contributed to advancing the use of eDNA for ecosystem monitoring studies by both advancing on methodological aspects to increase its reliability and by testing its application on monitoring environmental disturbances caused by humans. Together, we have identified strengths and limitations to the current application of eDNA techniques and prepared the ground for future studies using eDNA to answer ecological questions from a whole-ecosystem approach.

Ecosystems and environmental DNA

Generally, the chosen ecosystems to study were selected based on the extensive knowledge collected throughout many years on their ecological dynamics. This *a priori* knowledge is indispensable to design the eDNA sampling strategy to capture the range of the ecological signal we are interested to study, i.e. the variance in the data as a response for the environmental driver under study (Carraro et al., 2021; Deiner et al., 2017; Mize et al., 2019).
In this thesis, we have sampled in six distinct ecosystems, i.e. temperate grasslands, primaeval forests, vineyards, alpine rivers and two remote deserts, each with a unique history. For each of them we had detailed environmental data from the past that we could use to put the retrieved eDNA data in perspective to the hypothesis being tested. For example, when studying the soil communities in vineyards, the data on the pesticides applied during the last five years and the management practices were key to contextualise the eDNA results. Without it, applying eDNA techniques on an understudied system might risk restraining the potential findings to a descriptive level, i.e. describing the species found. This is not less important *per se*, but studying potential environmental drivers might become unrealistic given the lack of ecological foundation to properly test hypotheses on the eDNA data produced.
Moreover, one of the conceptual limitations of eDNA is that it represents a snapshot of the ecosystem, capturing the biological community at the time of sampling. Still, this snapshot will span back in time depending on DNA persistence. This means the retrieved eDNA will vary depending on the month, day and even the time of the day (Jensen et al., 2022; Milhau et al., 2021; Souza et al., 2016). As such, having a good prior ecological understanding of the environment is essential to adjust sampling strategies to properly capture the fluctuations in the ecosystem of interest. However, for areas where there is little knowledge about the functioning of the ecosystems within, this does not imply they are unsuitable for eDNA research. These understudied environments require preliminary work to prepare the sampling design to account

for the uncertainty surrounding the ecosystem, to account for eDNA technical aspects and the ecology of the species and interactions under study. In this sense, the advances in monitoring technologies, can facilitate this task and provide a quick overview of the environment to reach the sampling stage with a better understanding. This will maximise the sampling efforts and yield data with the optimal resolution to be statistically powerful for hypothesis testing.

To overcome this time-dependence of eDNA, we suggest increasing the sampling effort in order to capture the temporal variation, which can range from seasonal to daily, because of the variability in DNA persistence and DNA released by the target species. By doing so, we can obtain a dynamic representation of the changes occurring, which is very useful when monitoring long-term or ongoing environmental disturbances but also key when the disturbances occur on a daily basis. In both cases, the distribution of the sampling effort will have to decide the optimal sample frequency that best captures the changes in the ecosystem. In this sense, automation will play an important role in the upcoming years. We expect new sampling methodologies designed to sample for eDNA *in situ*, possibly remotely, and preserve it until DNA extraction can be performed, increasing the amount and coverage of the samples at a lower cost, which will improve our resolution and understanding of the environment. Alternatively, molecular advances can provide a temporal scale to date retrieved sequence, and better inform of its source location. This could be achieved through eRNA, which due to its shorter lifespan, could become a more reliable temporal indicator (Yates et al., 2021). However, to this point, research is still understanding the intricacies of eDNA transportation and degradation in the environment, which is highly variable depending on the physicochemical properties of the environment (Fremier et al., 2019; Jo & Yamanaka, 2022; Valentin et al., 2021). Moreover, eDNA can come from intra- or extracellular sources (Barnes & Turner, 2016), which also changes its degradation, before it can no longer be retrieved in the laboratory process. For instance, depending on the environment and sampling strategy, the type of target DNA varies. For soil, target eDNA is mostly extracellular, i.e. no cell lysis during extraction. For water, filtering through a 0.45uM pore size means that we only target intracellular DNA. Also, since the filters are not negatively charged, we also prevent sampling any extracellular DNA. Despite this, the progress made over the last ten years has settled the basis for us to be able to explore the current boundaries of this revolutionary technique for ecology and ecosystem management.

Trophic interactions as management indicators

There are several ways to monitor how the environmental conditions affect the species interactions within the ecosystem (Brown et al., 2001). One of these are trophic interactions, i.e. the transfer of energy between individuals, as it provides direct information on the diet of the individual and indirectly on its behaviour and potential area of distribution. They are very interesting for eDNA studies because it provides direct information on the diet of the individual and indirectly on its behaviour and potential area of distribution (Banerjee et al., 2022). Sampling the scats of the target species for eDNA gives access to more information than just the diet. We can investigate, for example, the host species itself (Baeza et al., 2023), their

parasites (Bass et al., 2015) or their microbiome (Ficetola et al., 2019). For large herbivores, we found this information is even more accessible because their feeding behaviour leads to an abundance of scats in their habitat and their digestive strategy ensures the plant DNA remains at reach for eDNA detection.

In the first two chapters, we have used the dietary data retrieved to study herbivores as indicator species for management decisions altering their ecosystem in a measurable way. By sampling herbivore scats in two well-documented ecosystems, we were able to identify the composition of their diet and quantify each plant taxon relative consumption. We have shown how eDNA can be used to reveal the direct and indirect management effects through herbivore trophic interactions. We inferred community-level influences of the disturbances created by these management decisions and extracted conclusions on their effects on the ecosystem.

In the Oostvaardersplassen, the fenced nature of this reserve made it a unique opportunity to monitor the dietary shifts within the four herbivore species present because it limited their food availability, with only certain plant species at reach. We found the diet of Red deer was density-dependent. More specifically, we observed a reduction of niche overlap with increasing total herbivore population. The effects of culling could be traced in the diet of Red deer, which was the main target of the culling campaign, and revealed interesting dietary shifts. We suggest their diet changed in response to their own population size, reducing their niche overlap with increasing Red deer population. In this case, resource competition translated into shorter vegetation height, reducing resource availability and forcing herbivores to consume different plant taxon. We showed how eDNA can be a powerful tool to understand ecosystem functioning. Since culling focused on Red deer, the diet of the other species was not influenced by this factor. Yet, it provided interesting findings regarding their dietary shifts along the sampled years, which contributed to put the results of Red deer in perspective.
We consider our findings a great success for ecological research. We were able to link the dietary shifts to the large-scale culling undergone and this is a proof that eDNA can be used to investigate the effects of ecosystem management. Nevertheless, it also advocates for further investigation towards a more accurate dietary quantification from sampled scats. Certainly, one of the main difficulties for between species comparisons is the variability on how each degrades DNA while digesting the consumed plants. We explored how transforming the eDNA data influenced the ecological interpretation of the results. Using relative read abundance (RRA), frequency of occurrence (FOO) and presence absence (p/a). Each varies in terms of quantitative resolution and qualitative certainty, i.e. RRA gives the most detailed quantification of diet but at the risk of PCR added noise due to primer affinity (Wilcox et al., 2013) or overamplification (Shirazi et al., 2021) and p/a brings no quantitative information but is very reliable on a binary description of the plant species consumed. We advocate that, unless avoiding PCR amplification with capture probes, i.e. target DNA enrichment for shotgun sequencing, the choice of data approach to be used should account for the differences between the digestive strategy of the sampled species and balance the quantitative and qualitative drawbacks of each data transformation. Yet, a comparison between the multiple data transformations helps to visualise the variability between samples to better understand the overall ecological drivers.

In Bialowieza, despite the species sampled being similar to the Oostvaardersplassen, the ecological context was completely different. Instead of sampling several years to cover the dynamics in dietary changes, we sampled two species within one month during summer, when the plant diversity available peaks, to have a high-resolution snapshot of the trophic interactions between the European bison (hereafter bison) and the Red deer. Here, the goal was to compare how the two species change their dietary choices within the same ecosystem, which is modified by the management policies applied, which drive habitat quality and predation risk for these herbivores. However, the Bialowieza forest is not a fenced reserve, and the diversity of habitats and plant species within is much higher than in the Oostvaardersplassen. Moreover, we did not have any reference to what was the relative abundance of each plant species in the sampled parts of the forest. We therefore sampled for the range of environmental disturbances of interest, i.e., predation risk associated with habitat quality as a consequence of logging; and predation risk associated with the presence of wolf populations. The strict reserve has a logging ban, and the forest is of higher quality for the herbivores. The managed part of the forest is exploited for timber and the high proportion of spruce has led to lower habitat qualities for the two herbivore species. The presence of wolves was associated with areas far from human settlements and with low to no timber exploitation. Both were quantified by previous work (Bubnicki et al., 2019) and we used their classification of the forest in functional areas, from a multivariate grouping of the environmental conditions as explanatory variables for the dietary changes.
The results we obtained were very interesting for understanding the large herbivore feeding behaviour in the Bialowieza forest. We identified a trend between Red deer niche overlap and predation risk and between bison and habitat quality. Red deer is the main prey of wolves, and as such we detected a signal from their behavioural response within their diet. As bison undergo lesser risk, their feeding strategy showed a correlation with food quality instead. With this study, we highlighted the value of sampling more than one species, as they can be used as a reference to better understand the results from the other species. In this project, low predation risk for bison allowed us to put the red deer results in perspective.

For dietary studies, since the preservation of scats can be done very cost effectively, i.e. drying them with silica gel without need for freezing, we advocate that collecting more samples and across more species is always advisable in order to add perspective to the ongoing project or to have samples to work on potential projects in the future. Once in the sampling area, collecting more or less scats will have a minor impact on the overall budget. In this line, we consider that sporadic sampling strategies across large areas, such as the Alps, can help to develop powerful datasets to study more complex environmental disturbances. Herbivore scats are abundant, easy to identify and to preserve. A coordinated effort on this front could truly benefit the field of management research to integrate at larger and larger scale the dietary information as a response to environmental disturbances.

Biological communities as ecosystem indicators

In order to obtain a comprehensive picture of the environment of study, sampling for whole communities is an interesting approach that has revolutionised how we monitor ecosystems (Pawlowski et al., 2021). Compared to trophic interactions, which focus on the dietary composition of each individual, or to species detection, which aims to determine the presence of a species in the environment, eDNA can also be applied beyond the individual and target whole communities. This approach opens the technique to virtually any ecosystem in the world as long as we can collect biological material from it, regardless if it comes from soil, water, air or scats. Conceptually however, the limits of eDNA application for this purpose remain unclear, as the reliability of the community sampled using eDNA depends on many methodological and ecological aspects (Barnes & Turner, 2016; Mize et al., 2019).
In fact, the extent of ecological information within our eDNA sample is unknown until sequencing. For example, we might be missing the species of interest because we have not sampled at the right time or the sufficient amount, even with extensive *a priori* knowledge of the ecosystem of study. And even if our sampling is optimal, we may still encounter methodological limitations at the laboratory stage, linked to inhibition or overdominance of the DNA of certain species (Kelly et al., 2019; Uchii et al., 2019). This to illustrate the difficulties of inferring biological communities using only eDNA. For this reason, one of the goals of this thesis was to investigate the reliability of the technique by comparing it to traditional ones. In the fourth chapter, we compared the sampling of eDNA in water to traditional kick-net sampling of macroinvertebrates and in the fifth one, to traditional camera trap surveys of large vertebrates. Together they certified that the results obtained with eDNA are correlated to the ones using traditional techniques. This gave us the certainty that by using eDNA we would obtain an ecologically meaningful view of the sampled communities.

In the third and fourth chapters, we used eDNA to reveal spatial and temporal dynamics occurring within the ecosystems in the light of environmental drivers. We sampled the arthropod biological communities of vineyard soil and alpine river water using eDNA techniques and investigated the link between the observed community changes and the environmental disturbances under study. We were able to identify drivers of these communities thanks to the high resolution of eDNA data. However, we also identified several methodological limitations, which indicates that we need to improve not only how we sample biological communities through eDNA but also how we interpret the results from an ecological perspective. Although our findings showed clear benefits of this technique over traditional methods, further research is needed on the methodological aspects of eDNA if we intend to completely move on from traditional methods.

In the Swiss cantons of Vaud and Valais, we studied the soil arthropod community in the light of agricultural management and pesticide usage, i.e. conventional or organic management. We found a strong correlation linked to the soil type, which exceeded the correlation linked to the two management classification which was surprising as we were expecting the management practices to still be stronger drivers for the arthropod community than the type of soil of the vineyards. Only when converting the eDNA data into alpha diversity metrics did we find a clear signal of the management effect, with organic parcels having greater alpha diversity. Certainly, soil physicochemical properties drive the microorganisms within the soil,

particularly bacteria, and create unique communities in relation to it. However, our data showed that this linkage to the soil properties is stronger for arthropods than we had initially hypothesised. This highlighted the importance of having an in-depth good understanding of the drivers of community composition and its ecology. Luckily, our experimental setup was able to still provide interesting results regarding the management because we sampled extensively within each type of soil, which aligned with each Canton. Thanks to the extensive dataset of the management survey filled by the farmers, we were able to exploit the intricacies of the eDNA results in the light of the pesticide types and dosages. We identified which of these pesticides were significantly related to the community composition changes, and put these products in perspective of the environmental impacts they are known to have. As such, we have demonstrated how the use of eDNA can ultimately inform on the impact of products such as pesticides that could have been underestimated until now. Interestingly, the application of this methodology is not restricted to vineyards, and we consider that the standardisation of the methodology implemented in this approach can be of great use for agriculture at a global scale, integrating sampling strategies and management datasets to validate the pesticides identified and strengthen the conclusions reached.

In the Val Roseg (Graubünden, Switzerland), we investigated the seasonal dynamics of the river's macroinvertebrate community within the alpine floodplain. We investigated the potential role of tributary rivers as refuge within the floodplain for the arthropods. Furthermore, we studied the similarity between the eDNA methodology and traditional kick-net sampling, which has been the main technique for sampling macroinvertebrates in ecological studies for many years (Brua et al., 2011). We found a clear correlation between the two methodologies, and validated the use of eDNA in this extreme environment. Our results showed that the main factor affecting macroinvertebrate abundance was the river's sediment load, and not temperature, as previously thought, due to glacial melt, but that overall community composition appeared unaffected. Interestingly, we detected a clear shift at the start of the melting season, i.e. between February and April, within the tributaries in terms of community composition. Together, these findings showed how the two monitoring approaches can be complementary. The community compositions were overall similar, but the greater resolution of the eDNA method combined with the quantitative reliability of the kick-net sampling revealed to be very useful to describe the complex dynamics between the tributaries and the ecosystem as a whole. Nevertheless, our study also highlighted the limitations associated with each monitoring approach. While performing the kick-net sampling, we confirmed that one of the major limitations of this technique is the taxonomic expertise and time required to identify each individual. A solution to this would be to extract the DNA from the ethanol where the individuals are transferred to, in order to perform metabarcoding on it allowing for a quick taxonomical identification. This ethanol could serve as the source of eDNA to identify the sampled macroinvertebrates in a much more efficient way, similarly to studies using bulk communities (Gleason et al., 2021; Persaud et al., 2021), and bypassing the time-consuming individual identification. As such, the combination of the two techniques goes beyond what we had anticipated, and mixing the two could be better than just combining them to obtain an improved methodology for sampling river communities.

For eDNA, one of the main constraints was the clogging of the eDNA filters with sediments, obstructing the flow of water and compromising the detectability of species because of the reduced volume of water filtered. This issue encouraged us to search for solutions for future studies, and we consider that the clogging could actually be used as a potential solution. Sediments trapped in the filter are collected in sterile conditions and it is likely to contain eDNA attached to the sediment particles. We consider this to be a promising new approach to sample river communities, especially when the turbidity is high, as in glacier-fed streams. Alternatively, direct sampling of the river benthic sediments could also provide an interesting sampling methodology to monitor river macroinvertebrates. Unfortunately, we did not perform such studies in this thesis, but researchers should target this comparison to improve the sampling strategies in the future. In this line, it seems clear now that traditional techniques provide more resources and conceptual frameworks for eDNA research to use as the starting point to develop. Even if this method has arrived to replace traditional techniques, it could very well profit from the expertise collected along the previous years.

Following up on eDNA methodological considerations, we were surprised by the intricacy of adjusting the PCR conditions to maximise the DNA yield and the taxonomic coverage of our water samples. This was especially the case for water eDNA from Val Roseg and is one of the major challenges for eDNA standardisation, as it is very case-sensitive. In this sense, scats and soil, despite coming with known inhibition molecules, were less of a challenge to adjust the PCR conditions to find an optimal compromise between taxonomic resolution and overamplification. Even for chapter five, where we sampled water from remote body waters in desert areas to assess the similarity between eDNA and camera traps, it seems the DNA concentration of vertebrates was high enough to quickly find a compromise on the dilution and PCR conditions to optimise the DNA yield and taxonomic coverage without risk of overamplification. We were thus left with two potential explanations for the samples in Val Roseg: either the arthropod DNA concentration was too low or we were coextracting inhibitors that were compromising the PCR amplification. As such, we consider that what we encountered was a combination of the two. Moreover, the presence of sediments could be an important driver for an optimal amplification, but we did not observe any clear amplification biases linked to sediments, at least in terms of sequences retrieved. As mentioned in the previous part, the role of sediments is yet to be unravelled, and developing research in this direction will certainly bring interesting and useful results for alpine eDNA research.

Development in molecular tools has brought the possibility to emancipate from barcoding, and profit from PCR free sequencing thanks to capture probe enrichment, also known as hybridization capture, which binds to the target DNA to retain it while removing the non-target DNA. This increases the accuracy of DNA quantification of the results as it is free from potential overamplification or primer specificity issues. However, the DNA extraction process can alter the quantity of DNA of each taxa and again alter the quantification of the taxa present in the environmental sample. As such, research in the field will have to find a way to profit from all the knowledge gained through barcoding for improving capture-probe methodologies for ecological studies in the future.

Conclusions

In this thesis, we have explored the potential of eDNA as a tool for assessing the impact environmental disturbances caused by management practices in the overall ecosystem. We have tackled this complex task with the certainty that eDNA would be retrieved and quantified closely to the actual community composition sampled. However, this correlation is fascinatingly more complex than we had anticipated, and it has contributed to developing new approaches to sample communities more accurately. We have now advanced towards a better understanding of the dynamics of eDNA in the environment, and which factors could be biassing the reconstruction of biological communities in the process. However, more research is needed to increase the reliability of the obtained results. For this reason, we consider the best way to advance constructively is as we have done in this thesis, i.e. combining methodological questions with environmental concerns to design studies which will improve both the methodology and our understanding of the ecosystem. Despite this approach may seem overly ambitious or unfeasible, we have shown in this thesis that it is possible and constructive to do so. In this line, the advent of eDNA research is likely to take us much further, and the best way to do it is to maintain the bond between eDNA laboratory research with ecosystem dynamics and management applications. By doing so, eDNA techniques will expand its range of applications to unravel the pillars of its complex dynamics and its ecological implications.

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