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EFFECTS OF SOIL FAUNA ON EARLY-STAGE LITTER DECOMPOSITION ACROSS DIVERSE TROPICAL ECOSYSTEMS IN EAST MALAYSIA

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ABSTRACT

Litterbag studies from temperate zones have shown a significant effect of soil fauna on litter decomposition. However, understanding the decomposition dynamics in tropical regions remains limited compared to temperate regions. Here we investigated the impact of soil meso- and macrofauna on litter decomposition rates at three contrasting locations in tropical area of the Eastern part of Peninsular Malaysia (tropical forest near Tasik Kenyir, permaculture in Pahang and urban soils in Universiti Malaysia Terengganu campus). We conducted litterbag experiments with different mesh sizes and soil faunal sampling to investigate the effect of soil meso- and macrofauna on litter decomposition (Fig. 1). As decomposition is fast in the tropics, we expose litterbags for three months and collect them every month. Litter mass loss increased over time, with higher decomposition rates observed in tropical forests and permaculture compared to urban soils. Tropical forest soils host significantly more diverse communities of soil fauna than the other two sites. The principal component analyses (PCA) revealed divergence in the community structure of taxonomic and functional groups among different locations, with urban soils primarily comprising *Araneae*, *Protura*, and *Diplura*, while permaculture and tropical forests mainly consisted of *Acari* and *Collembola*. Size analyses revealed that soil macrofauna enhanced decomposition rates in permaculture, while mesofauna affected decomposition in urban soils. The C:N ratio of litter in litterbags increased after three months of incubation in permaculture and tropical forest without any significant differences among mesh sizes. Random forest analyses highlighted the importance of soil moisture and texture (content of sand, silt and clay) influencing soil biota associated with decomposition processes.

Keywords: decomposition rates; litterbags; litter quality; soil macrofauna; soil mesofauna

Introduction

Soil ecosystems host an immense diversity of organisms, ranging from microorganisms like bacteria, fungi and protists to larger soil animals such as termites and millipedes (Bardgett and van der Putten 2014). For instance, one single gram of soil harbors billions of microbial cells belonging to thousands of species (Fierer 2017). While soil microbes have received considerable attention during past decades (Fierer 2017; Mahé et al. 2017; Nilsson et al. 2018), soil animals have been often overlooked until recent years (Angst et al. 2024). Many studies have unveiled remarkable diversity and abundance of soil animals (Wall et al. 2008; Lavelle et al. 2022). For example, a single square meter of soil hosts hundreds of thousands of individuals of different groups of soil invertebrates, varying with soil type and microclimatic conditions (Petersen and Luxton 1982; Wu et al. 2011; Heděnec et al. 2022). According to body size, we can recognize microfauna (< 0.2 mm), mesofauna (> 0.2 mm), and macrofauna (> 2 mm) (Swift et al. 1979). Soil microfauna (nematodes, tardigrades, and rotifers) contribute indirectly to litter decomposition via their grazing effects on fungal and bacterial communities (Cesarz et al. 2013; Devetter et al. 2017). In contrast, macrofauna (isopods, millipedes) and mesofauna (springtails and mites) contribute directly to litter decomposition by litter fragmentation and modifying conditions favorable for bacterial and fungal decomposers (Joly et al. 2020; Mrnka et al. 2020; Coq et al. 2022).

Despite their small size, soil animals play crucial roles in ecosystem functioning, particularly in complex processes, such as litter decomposition and nutrient cycling (Filser et al. 2016; Nielsen 2019). Litter decomposition is a fundamental process in terrestrial ecosystems, influencing soil fertility, carbon sequestration, and nutrient

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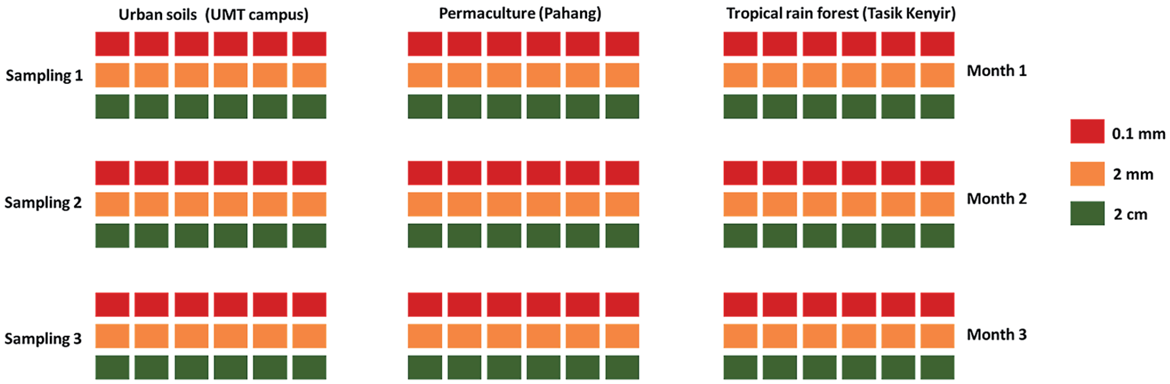

Fig. 1 Design of litterbag experiment.

cycling. In terrestrial ecosystems, more than 50% of the mean annual litterfall is returned to the soil via feeding activity of soil animals (Wall et al. 2008; García-Palacios et al. 2013; Heděnec et al. 2022). However, decomposition rates strongly vary between biomes, driven by multiple factors including climate, litter and substrate quality, and abundance and diversity of soil fauna (Wall et al. 2008; Heděnec et al. 2022). Soil macrofauna, such as isopods, millipedes, earthworms, and gastropods, are important drivers of leaf litter decomposition in temperate deciduous forests, while termites, millipedes, and ants are major decomposers in tropical regions (Petersen and Luxton 1982; Heděnec et al. 2022). However, the role of fauna in litter decomposition in tropical regions remains underexplored. For example, it remains unclear how land-use changes as well as differences in soil properties affect soil fauna communities and their role in litter decomposition. Land-use changes affect soil biodiversity in numerous ways (Jiangi and Purvis 2023), including pesticide use (Badani et al. 2023) and changes in soil properties (Oppong et al. 2023).

Many studies have revealed significant effects of soil fauna on litter decomposition rates (Barajas-Guzmán and Alvarez-Sánchez 2003; Wall et al. 2008; Zhang et al. 2008; García-Palacios et al. 2013; Heděnec et al. 2022). Soil macrofauna, such as isopods, millipedes, earthworms, gastropods, cockroaches and termites are responsible for litter fragmentation and colonization by microbial decomposers such as bacteria and fungi which enhance decomposition rates (Kheirallah 2004; Joly et al. 2018; Coq et al. 2022). Litterbags accessible or non-accessible for meso- and macrofauna provide a reliable tool to measure litter mass loss over time (Kampichler and Bruckner 2009; Frouz et al. 2015; Peguero et al. 2019; Peng et al. 2022b). Despite the increased number of litterbag studies (Kandeler et al. 1999; Kampichler and Bruckner 2009; Frouz et al. 2015; Peng et al. 2022b), most of these were performed in the temperate or subtropical zone while only a few studies were performed in tropical regions, especially in Peninsular Malaysia (Yamashita and Takeda 1998).

Most litterbag studies in temperate zones used relatively long incubation periods and infrequent litterbag sampling (Kandeler et al. 1999; Kampichler and Bruckner 2009; Peng et al. 2022b). If the same methodology were applied in tropical regions, there would be nothing left to study, as decomposition occurs more quickly than in temperate and subtropical regions. Significant mass loss occurs during the early period of incubation in tropical regions, which has been often overlooked in long-term studies (Yamashita and Takeda 1998; Frouz et al. 2015). Therefore, studying the early decomposition stages is crucial for capturing these rapid and distinct dynamics.

To fill this knowledge gap, we established a litterbag study to (i) assess the diversity and composition of soil meso and macrofauna in soils from various contrasting habitats with different land uses with different levels of anthropogenic pressure across peninsular Malaysia; (ii) to investigate the effect of soil meso and macrofauna on litter decomposition rates during three months of incubation and (iii) to investigate influencing factors shaping litter decomposition rates at various contrasting locations in peninsular Malaysia. We hypothesize that 1) soil in tropical rain forest will host the highest diversity and abundance of soil fauna via higher resource availability and stability. In contrast, permaculture and urban soils will host lower diversity and abundances of soil animals, due to lower availability of nutrients and higher anthropogenic disturbances; 2) we hypothesize that higher litter consumption in accessible litterbags incubated in tropical forest than in permaculture and urban soils due to stable microclimatic conditions and higher diversity of soil fauna 3) we expect that soil properties will shape litter decomposition via their effect on soil biota.

Material and Methods

Study site description

Three contrasting habitats (tropical rainforest, permaculture, and urban soil at the campus of the Universiti Malaysia Terengganu) with different land uses, vegetation covers, soil properties, and composition and diversity of soil fauna were selected in the Terengganu and Pahang states in Malaysia. The tropical rainforest is located near Kenyir Lake in the Hulu Terengganu district of

Terengganu State in Malaysia (5.145357° N, 102.760592° E). The area is characterized by dominant tree vegetation with a canopy cover of 80–90%. The dominant trees are *Dipterocarpus*, *Shorea*, *Lithocarpus*, *Pometia*, *Artocarpus*, and *Maniltoa*, while dominant understory vegetation is from the family of *Zingiberaceae* and *Annonaceae*. The permaculture is situated next to the Delimah Guest House of Taman Negara in Pahang State (4.383298° N, 102.40406° E). The permaculture area is approximately 150 m^2. The main crops are papayas, bananas and other vegetables such as okra and cucumbers. The permaculture is managed without any inorganic fertilizers and pesticides. The permaculture is fertilized by chicken manure every three months. The post-harvest residuals are left on the site as organic biofertilizers to support natural soil decomposers. The campus of the Universiti Malaysia Terengganu (UMT) is situated in the coastal area of Kuala Nerus district of the Terengganu State (5.407046° N, 103.092333° E). The campus has approximately 26 km^2 and its vegetation consists of different coastal tree species, such as *Swietenia macrophylla*, *Cyrtophyllum fragrans*, and *Casuarina equisetifolia*.

Experimental design

We used three types of litterbags with different mesh sizes – those accessible for soil mesofauna (nylon net with 2 mm mesh size), macrofauna (nylon net with 2 mm mesh size and with 2 cm holes), and litterbags made from fabric with 0.1 mm mesh size as a control to test the effect of soil animals on decomposition of leaf litter. Each bag was filled with freshly fallen litter of various tree species found on the UMT campus which are also common in tropical forests across Malaysia. The leaf litter from different trees collected in the botanical garden at the UMT campus (*Agathis*, *Artocarpus*, *Cyrtophyllum*, *Dipterocarpus*, *Mangifera*, *Melaleuca*, *Milettia*, and *Shorea*) was oven-dried at 65 °C for three days and equally mixed to include all dominant tree species characterized for both natural and anthropogenic habitats in tropical areas. The experiment was conducted at three sites, each containing three subplots. In each subplot, 6 litterbags containing 5 g of oven-dried leaf litter (dried at 60 °C for 48 hours) accessible to macrofauna, 6 accessible to mesofauna, and 6 control litterbags were deployed simultaneously. The litterbags were incubated and collected in three phases: after one month, 6 macrofauna-accessible, 6 mesofauna-accessible, and 6 control litterbags were retrieved from each subplot. After two months, another set of 6 litterbags from each category were collected, and finally, after three months, the remaining 6 litterbags of each type were retrieved. In total, 162 litterbags were retrieved (Supplementary file S1). The total mass of consumed litter was measured gravimetrically in the laboratory by subtraction of dry litter weights before and after incubation. The percentage of litter consumed, decomposition constant (*k* value), and effect size (based on the *k* value) of soil mesofauna and macrofauna were measured for each litterbag. Three litterbags of each mesh size category were randomly taken from each location to measure litter TOC, TN and C:N ratio after three months of incubation. The same number of replicates before incubation was measured as a control.

Soil fauna sampling and identification

Soil samples for mesofauna were collected using a shovel from a 10 × 10 cm square to a depth of 5 cm. A total of three compact soil blocks were taken from each location at each of the three months of incubation, resulting in a total of 27 samples. Soil mesofauna was extracted by dry extraction method using a modified Berlese-Tullgren extractor for three days (Pande and Berthet 1973). Soil mesofauna specimens were trapped in water with detergent and further stored in 70% ethanol for later identification (Peng et al. 2022a). Soil macrofauna was collected using the hand-sorting method. At each location, a soil block (25 × 25 cm and 5 cm in depth) was collected together with soil samples for mesofauna. In total, 27 monoliths were taken from all locations and sampling times. Soil monoliths were transferred to the lab and immediately processed using hand sorting of all soil fauna in size class larger than 2 mm (Swift et al. 1979; Ruiz et al. 2008). Soil animals were classified into 16 taxonomic groups at the family, order, subclass, or class level, including *Collembola*, *Acari*, *Diplura*, *Symphylla*, *Pauropoda*, *Protura*, *Lumbricidae*, *Diplopoda*, *Isoptera*, *Isopoda*, *Araneae*, *Chilopoda*, *Formicidae*, *Coleoptera*, Insect others, *Blattodea* (without *Isopotera*). Furthermore, soil fauna was classified into 7 functional groups, namely Predators, Omnivores, Saprophages: Macrofauna, Saprophages: Mesofauna, Macrofauna total, Mesofauna total and Total fauna (Swift et al. 1979; Artz et al. 2010; Filser et al. 2016; Orgiazzi et al. 2016).

Soil physico-chemical properties and litter chemistry

Soil samples were collected using a shovel from a 25 × 25 cm square to a depth of 5 cm. A total of three compact soil blocks were taken from each location. Soil moisture was measured gravimetrically, before and after drying at 65 °C for 72 hours using a drying oven at the Ecological laboratory of the Faculty of Science and Marine Environment (FSSM) at UMT. The pH was determined using a Horiba LAQUA PH210, obtained from the Plant Biotechnology Lab of FSSM, at a soil-to-water ratio of 1:5. Soil texture was determined as a percentage of sand, silt, and clay after sieving through the sieve of size 2 mm, 0.02 mm and 0.002 for sand, silt, and clay respectively. Available phosphorus (P) was analyzed using the Bray-1 extraction method with the Shimadzu UV Spectrophotometer, UV-1800 model. The percentage of organic matter (OM) in soil was measured by loss of ignition method using a muffle furnace at a temperature of 375 °C for 8 hours. Total Organic Carbon (TOC), Total Nitrogen (TN), and the Carbon-to-Nitrogen (C/N) ratio were determined for soil and selected litter samples using the Elementar UNICUBE® organic elemental analyzer at the Organic Chemistry Laboratory of the Faculty of Marine Engineering and Technology at UMT, with helium as a carrier gas.

Statistical analyses

We used logarithmic regression to fit the temporal trend of mass loss for each species and mesh size across three sampling locations. The decomposition rate (decomposition constant) (k value) was calculated according to the single exponential model for comparisons among different litterbags and sampling locations (Peng et al. 2022b, 2022c). Specifically, we calculated the k value (months^{-1}) using Eq. (1):

$$k = -\frac{1}{t}\ln\left(\frac{M_t}{M_0}\right) \quad (1)$$

where M_0 is the initial litter dry mass (g) and M_t is the dry mass at sampling time t (month). Alpha diversity indices (Species Richness (S), Shannon Index (H'), and Simpson's Index (D)) based on the number of taxonomic groups were calculated using the "vegan" R package (Oksanen 2015). Richness refers to the number of different species (or taxa) present in a given sample, site, or community, without considering their abundance. The principal component analyses (PCA) were employed to visualize distribution of population density of various taxonomic and functional groups of soil fauna and soil physico-chemical properties. Random forest analyses using "rfPermute" package were used to test the importance of different physico-chemical properties on litter mass loss and decomposition rate (Liu et al. 2020). The effects of litterbag mesh size, sampling locations, sampling times and their interactions on the percentage of mass loss were assessed using a linear regression model. The Analyses of Variance (ANOVA) were used to compare the mean percentage of mass loss, decomposition rate, alpha diversity indices and densities of taxonomic and functional groups of soil fauna between three different sampling locations, followed by post hoc test (Tukey's Honest Significant Difference) for observed means. To test how access of meso- and macrofauna modulates litter decomposition rate (k) as assessed using different litterbag mesh sizes, effect sizes were calculated as a proxy. The effect sizes for mesofauna, macrofauna, and all total soil fauna were calculated as the normalized effects using the natural log response ratio (lnRR) as follows:

$$lnRR_{mesofauna} = ln\left(\frac{k_{2\,mm}}{k_{0.2\,mm}}\right) \quad (2)$$

$$lnRR_{macrofauna} = ln\left(\frac{k_{2\,cm}}{k_{2\,mm}}\right) \quad (3)$$

$$lnRR_{total} = ln\left(\frac{k_{2\,cm}}{k_{0.2\,mm}}\right) \quad (4)$$

where $k_{0.2\text{mm}}$, $k_{2\text{mm}}$, and $k_{2\text{cm}}$ are the k values in the litterbags with mesh sizes of 0.1 mm, 2 mm, and 2 cm holes, respectively. The two-way ANOVA followed by Tukey's HSD test was run to test the effect of sampling location and incubation time on response ratio. The paired t-test was used to compare litter TOC, TN and C:N ratio in litterbags before and after three months of exposure in various sampling locations. Pearson correlation coefficient was used to test correlations of soil properties with litter mass loss and decomposition rates. All statistical analyses and graphical representations were conducted using the R-Studio program (R Core Team 2025) with the assistance of the "vegan" and "ggplot2" packages (Oksanen et al. 2012; Wickham 2014).

Table 1 Soil physico-chemical properties among various sampling locations.

	Sampling locations			
	Urban soil	**Permaculture**	**Tropical forest**	**ANOVA**
Moisture (%)	7.0±1.0c	16.9±1.9b	26.8±2.7a	***
pH	5.9±0.2b	6.1±0.2a	6.3±0.1a	***
Conductivity (uS cm^{-1})	36.0±8.7c	45.7±8.8b	55.4±8.9a	**
TOC (g kg^{-1})	13±2c	16±3b	19±5a	***
TN (g kg^{-1})	0.5±0.0c	0.6±0.0b	0.7±0.0a	***
C:N ratio	26±3b	27±4a	27±5a	***
Plant available P (mg kg $^{-1}$)	11.8±3.0a	8.9±1.9b	5.9±0.8c	***
C:P ratio	1100±980	1800±1100	3220±3200	ns
N:P ratio	42±10c	67±11b	118±28a	***
C:N:P ratio	2.3±1b	2.8±1.0b	4.4±0a	*
OM (%)	3.8±1.0c	5.5±1.4a	6.2±1.9a	***
Sand (%)	35.0±8.8c	45.0±11.3b	55.0±13.8a	***
Silt (%)	50.0±12.5a	37.5±9.4b	25.0±6.3c	***
Clay (%)	15.0±3.8c	17.5±4.4b	20.0±5.0a	***

One-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.001. Letters indicate statistical homogenous groups.

Results

Soil properties and soil fauna community composition

Soil physico-chemical properties differed significantly among the three sampling locations (Table 1). For instance, soil moisture, conductivity, TOC, TN, N:P, and percentage of organic matter were higher in the tropical forest, intermediate in permaculture, and lowest in urban soil. The percentage of clay particles and sand was highest in the soils of the tropical forest, intermediate in permaculture, and lowest in urban soil, while the highest proportion of silt particles was found in the urban soil. The concentration of phosphorus was highest in urban soils, intermediate in permaculture, and lowest in soils of the tropical forest. The soil C:P ratio did not show any significant differences.

Table 2 Alpha diversity of soil faunal community among various locations and sampling times.

		Richness (S)±SD	Shannon index (H')±SD	Simpson index (D)±SD
Locality	Urban soil	6±1b	1.48±0.2b	0.725±0.04b
	Permaculture	6±1b	1.30±0.1b	0.610±0.04b
	Tropical forest	8±0.4a	1.75±0.1a	0.792±0.02a
	ANOVA	*	*	**
Sampling time	Month 1	6±0.9b	1.52±0.2	0.718±0.04
	Month 2	5±0b	1.39±0.1	0.709±0.02
	Month 3	8±0a	1.62±0.1	0.700±0.06
	ANOVA	**	ns	ns

Alpha diversity indices are based on the number of taxonomic groups. Two-way ANOVA; *P < 0.05, **P < 0.01. Letters indicate statistical homogenous groups.

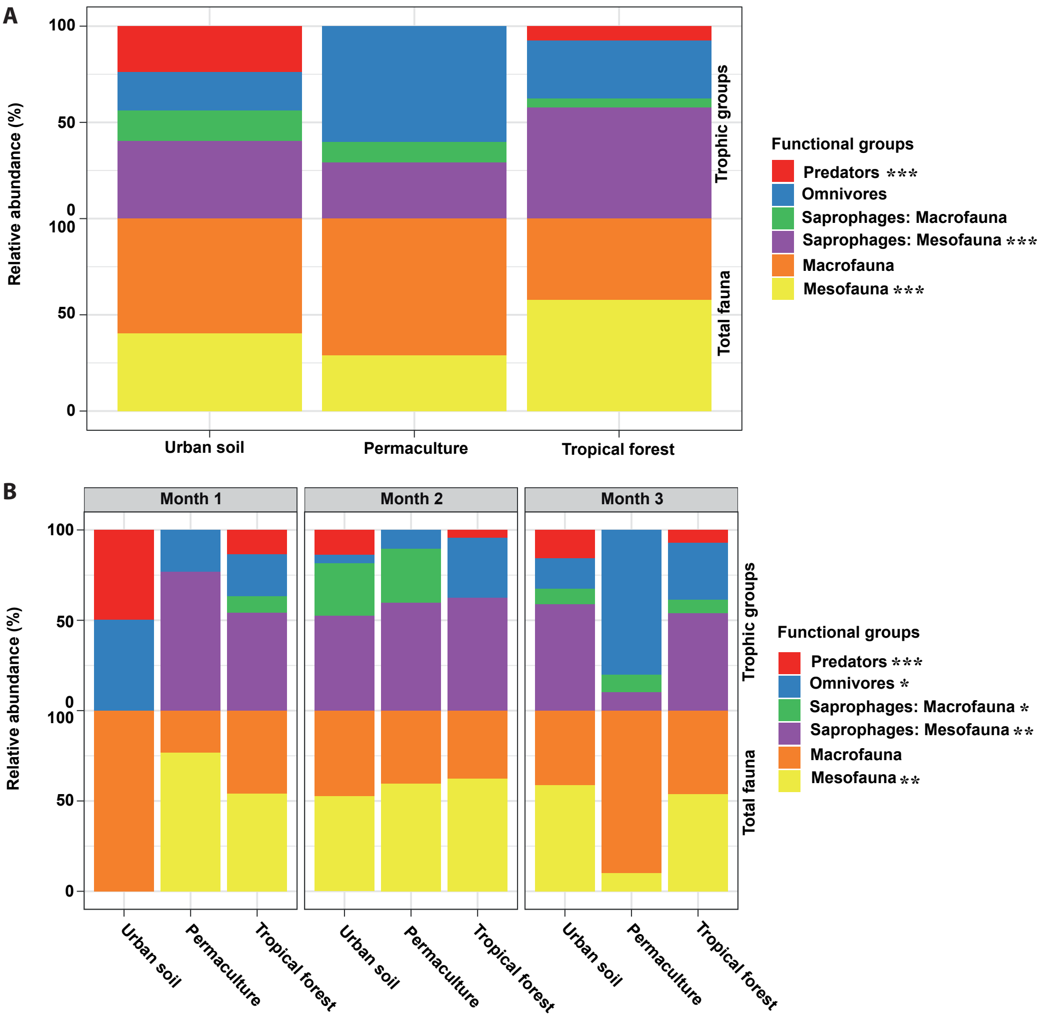

Fig. 2 Relative abundance of functional groups of soil fauna among different locations (A) and sampling times (B). Two-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.001.

Table 3 Abundance of taxonomic and functional groups of soil fauna (individuals per m2) among various locations and sampling times.

Group		Locations				Sampling times			
		Urban soil	Permaculture	Tropical rain forest	ANOVA	Month 1	Month 2	Month 3	ANOVA
Collembola		33±10b	17±1.8c	84±15a	***	20±6c	74±18.9a	40±8b	***
Acari		20±6c	46±4b	74±15a	***	27±9c	70±13a	44±9b	**
Diplura		0±0b	0±0b	20±10a	*	20±10a	0±0b	0±0b	*
Symphylla		0±0b	3±2a	0±0b	*	3±2a	0±0b	0±0b	*
Pauropoda		0±0b	4±2a	0±0b	*	4±2a	0±0b	0±0b	*
Protura		7±4a	0±0b	0±0b	*	0±0b	0±0b	7±4a	*
Lumbricidae		20±10a	19±5a	0±0b	*	0±0c	30±9a	9±5b	***
Diplopoda		4±2	0±0	3±2		3±2	0±0	4±2	
Isoptera		0±0c	4±2b	11±3a	***	4±2b	0±0c	11±3a	***
Isopoda		0±0b	3±2a	0±0b	*	0±0b	0±0b	3±2a	*
Araneae		29±8a	0±0c	19±3b	***	30±9a	12±3b	6±2b	***
Chilopoda		7±2a	0±0c	3±2b	***	0±0b	3±2a	7±2a	***
Formicidae		20±8	124±60	50±15		30±6	40±18	124±60	
Coleoptera		3±2b	0±0b	20±6a	***	3±2	7±3	13±7	
Insect others		3±2c	13±2b	20±5a	***	7.6±2b	6.4±2b	22±4a	***
Blattodea		4±2	8±2	3±2		7±2a	0±0b	8±2a	**
Predators		36±6a	0±0c	23±2b	***	30±9a	16±4b	13±3b	***
Omnivores		30±8	145±63	93±12		48±4b	53±22b	168±58a	*
Sapro-phages	Macrofauna	24±9	26±7	14±4		7±3b	30±9a	27±6a	*
	Mesofauna	60.7±16b	70±8b	177±21a	***	74±19b	144±30a	91±15b	**
Total	Macrofauna	90±10	171±68	130±8		84±14	100±17	208±60	
	Mesofauna	61±16b	70±8b	177±21a	***	74±19b	144±30a	91±15b	**
Total fauna		150±15b	242±63a	307±29a	*	158±16b	243±47a	298±53a	*

Two-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.001. Letters indicate statistical homogenous groups.

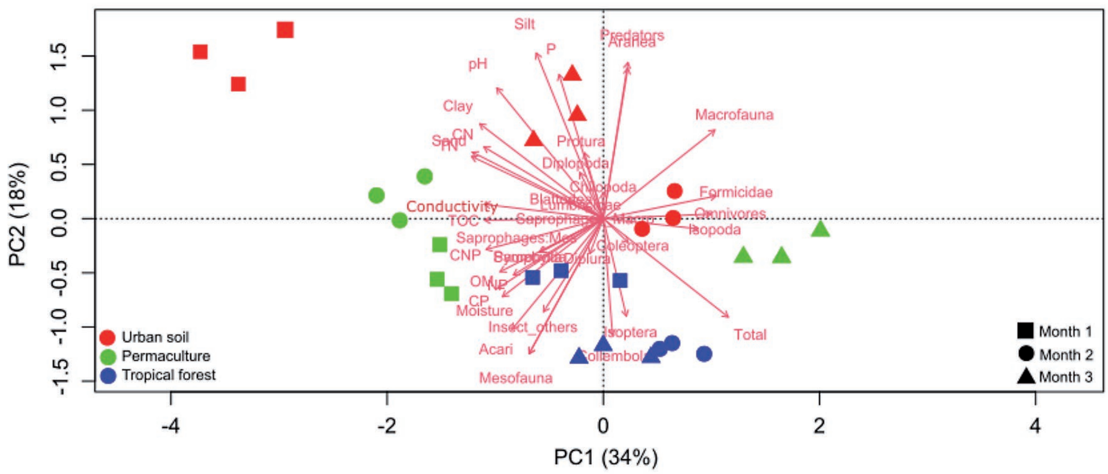

Fig. 3 Principal component analyses (PCA) of community structure of taxonomic and functional groups of soil fauna and soil physico-chemical properties among various sites and sampling times.

The alpha diversity of soil faunal groups, based on the number of taxonomic groups, differed among various locations and sampling times (Table 2). Soils in tropical forests hosted significantly higher taxonomic richness, Shannon index, and Simpson index than those in permaculture and urban areas. Taxonomic richness, Shannon index, and Simpson index did not differ between urban soils and permaculture. Furthermore, the taxonomic richness of soil fauna differed significantly among sampling times, showing an increasing trend over time. In contrast, the Shannon index and Simpson index did not show any significant differences among sampling times.

The abundance of various taxonomic and functional groups differed among locations and sampling times (Table 3; Fig. 2). For example, *Acari* exhibited the highest abundance in tropical soils, intermediate abundance in permaculture, and the lowest abundance in urban soils. Furthermore, the abundance of *Acari* varied among sampling times. The total abundance of soil fauna was higher in tropical soils and in permaculture than in urban soils. Both soil mesofauna and saprophagous mesofauna showed significantly higher densities in tropical forests compared to permaculture and urban soil. In contrast, soil macrofauna and saprophagous macrofauna did not

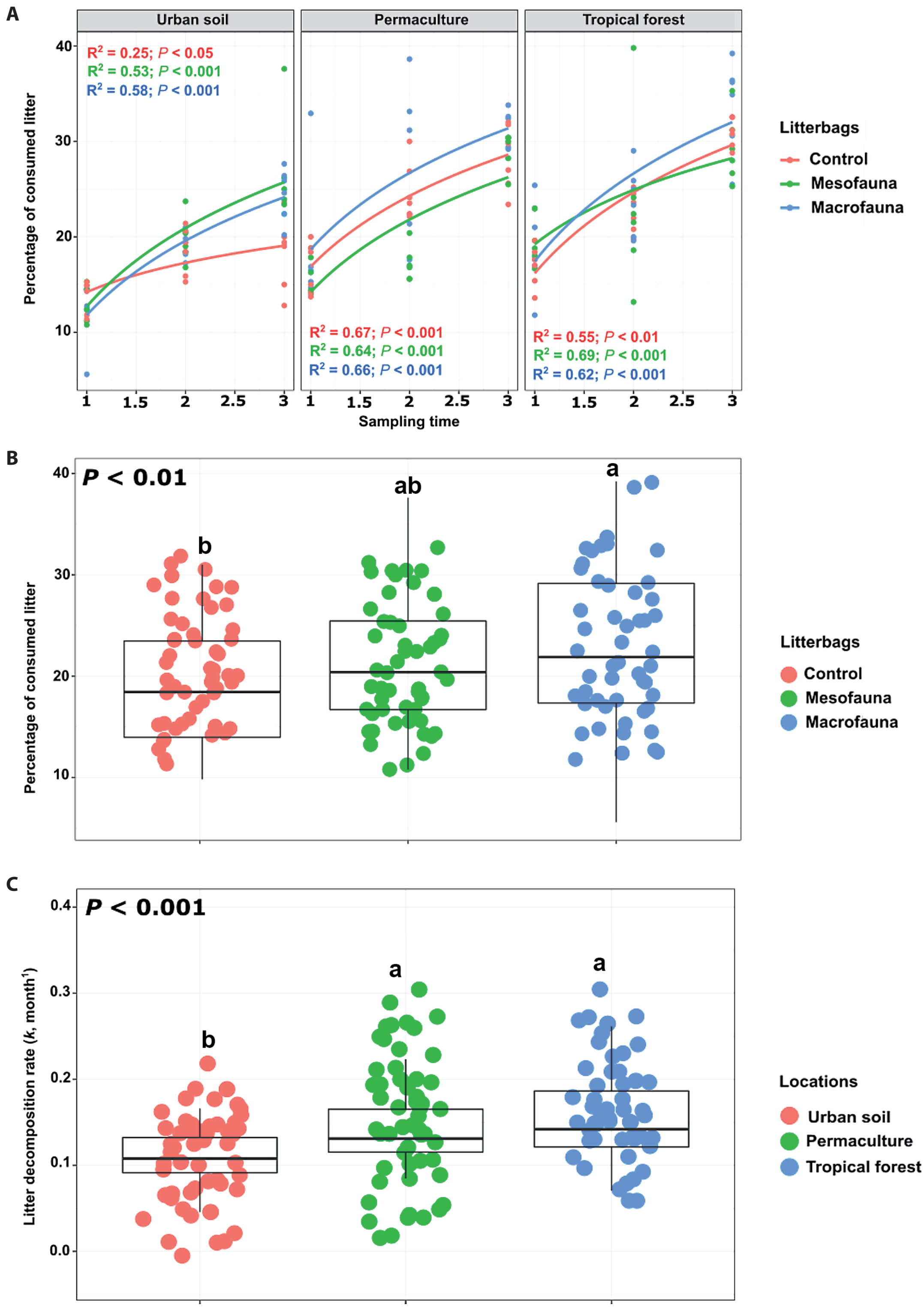

Fig. 4 Percentage of litter mass loss among sampling times (A) and different mesh sizes (B) and decomposition rates among different sampling sites (C). Letters indicate statistical homogenous groups.

show any significant differences among sampling sites. However, both soil macrofauna and saprophagous macrofauna exhibited differences among samplingtimes.

The PCA diagram revealed divergence in the community structure of taxonomic and functional groups among different locations and sampling times (Fig. 3). In urban soils, the community of soil fauna primarily comprised *Aranea*, *Protura*, and *Diplura*, and was associated with high pH, silt content, and P content. Conversely, the structure of soil fauna in permaculture and tropical forests mainly consisted of Acari and Collembola, with their community composition associated with a high C:N:P ratio, organic matter content, and soil moisture.

Effect of soil fauna on litter decomposition

Our results revealed an increase in litter mass loss in all types of litterbags over the course of increased incubation time (Fig. 4A). The litter mass loss was lowest after one month of incubation and highest after three months. The percentage of litter mass loss differed among litterbags with varying accessibility to soil meso- and macrofauna (Fig. 4B). Litterbags accessible to macrofauna exhibited a significant difference from control litterbags but did not show any significant differences compared to those accessible to mesofauna. Litterbags accessible to mesofauna did not show any significant difference in litter mass loss compared to control litterbags. Litterbags accessible to macrofauna in tropical forest and permaculture exhibited a higher percentage of mass loss than those in urban soils. Litter decomposition rates (*k* values) differed significantly among litterbags incubated at different locations (Fig. 4C). Litterbags in tropical forests and permaculture exhibited higher decomposition rates than those in urban soils. The litter chemistry in litterbags incubated for three months in different locations differed significantly among sampling locations but was not affected by mesh size. The TOC and CN ratio of litter in litterbags incubated in tropical forest and permaculture increased while urban soils showed no significant changes (Figs 5A–B). In addition, TN of litter incubated in all locations decreased significantly after three months of incubation (Fig. 5C).

Effect size measurement revealed that accessibility to soil mesofauna significantly increased decomposition

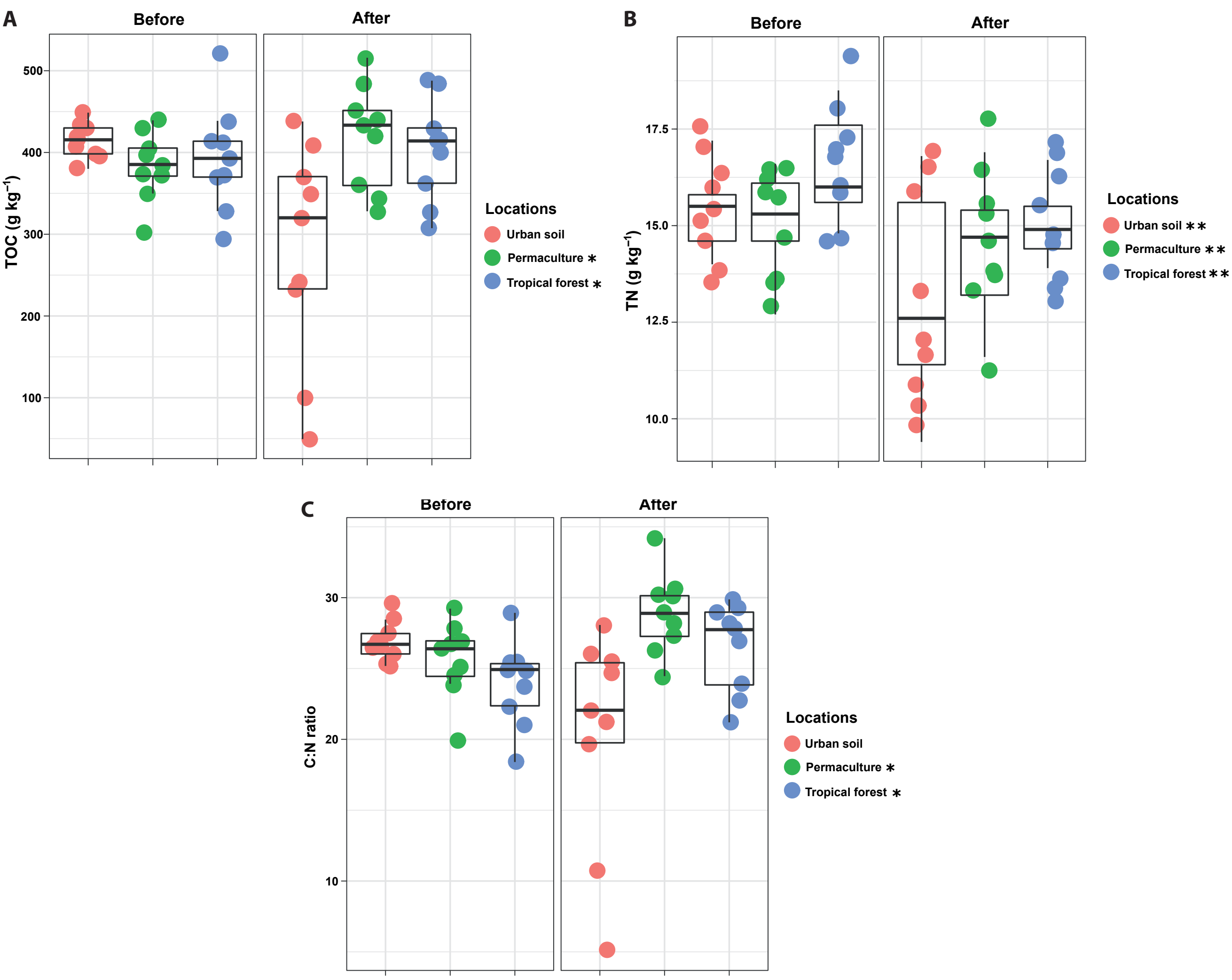

Fig. 5 Total Organic Carbon (A), total Nitrogen (B) and C:N ratio (C) of litter in litterbags incubated in different sampling locations. Asterisks indicate statistically significant differences (Paired t-test; *P < 0.05, **P < 0.01).

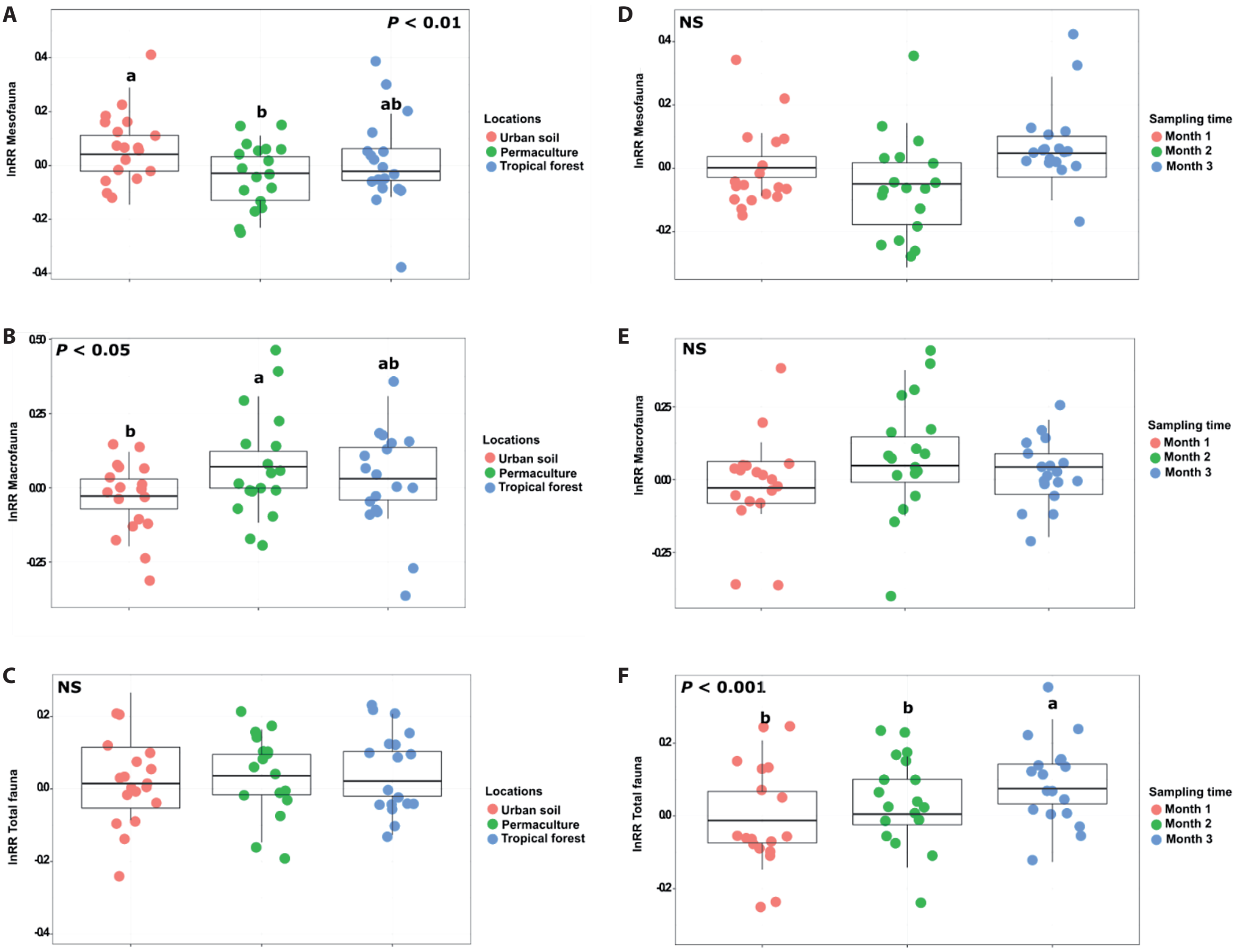

Fig. 6 Effect of soil mesofauna, macrofauna and total fauna on litter decomposition rates among locations (A–C) and sampling times (D–F). Letters indicate statistical homogenous groups.

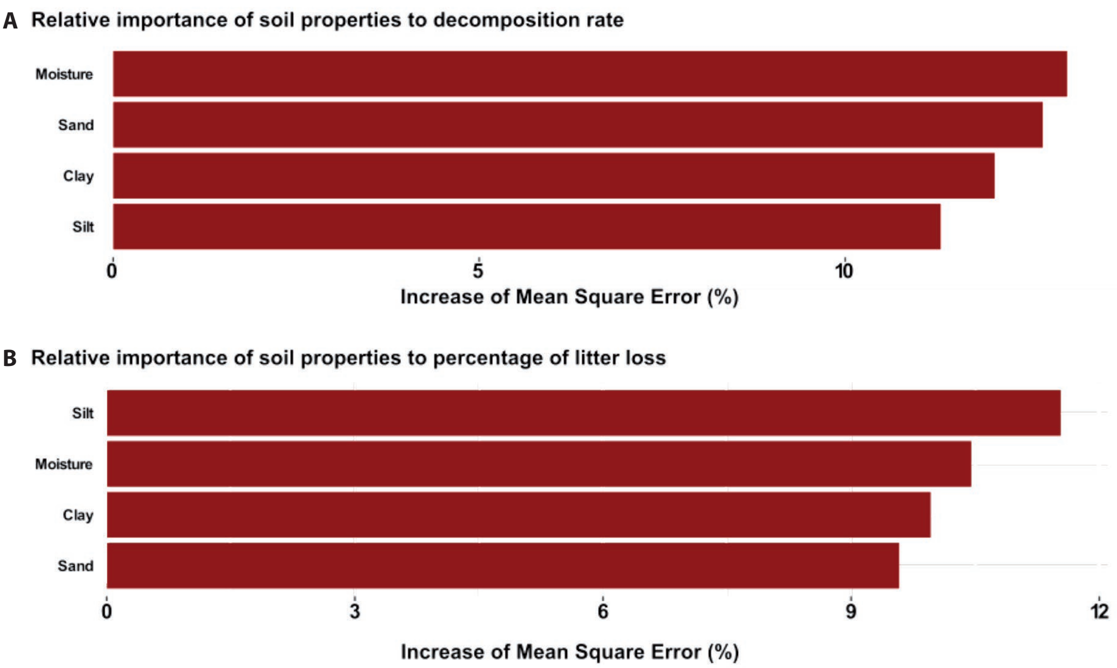

Fig. 7 Relative importance of soil physico-chemical properties to decomposition rates (A) and percentage of litter mass loss (B).

Table 4 Pearson correlation coefficients of soil physico-chemical properties and litter decomposition expressed as percentage of litter mass loss and decomposition rate.

	Percentage	Decomposition rate (k, months)
Moisture	0.33*	0.34*
pH	0.11	0.14
Conductivity	0.06	0.12
TOC	0.07	0.10
TN	0.25	0.27
CN	−0.04	−0.01
P	−0.17	−0.16
CP	0.20	0.20
NP	0.27	0.27
CNP	0.16	0.16
OM	0.13	0.16
Clay	0.35*	0.37*
Silt	−0.35*	−0.36*
Sand	0.35*	0.37*

Asterisks indicate statistically significant differences, *P < 0.05, Bonferonni corrections were used to adjust multiple correlations.

rates in litterbags incubated in urban soils more than in permaculture without any other significant differences (Fig. 6A). Soil macrofauna increased litter decomposition rates in permaculture compared to urban soil but did not show any differences between urban soils and tropical forest or tropical forest and permaculture (Fig. 6B). Accessibility to both macrofauna and mesofauna together did not show any effect on decomposition rates (Fig. 6C). The effect of accessibility to mesofauna or macrofauna on litter decomposition did not differ among different sampling times, however, accessibility to both mesofauna and macrofauna together had a significantly larger effect on litter decomposition three months after incubation (Figs 6D–F).

Random forest analyses revealed the importance of soil moisture, sand content, clay content, and silt content, respectively, for decomposition rates (Fig. 7A). Similarly, silt content, soil moisture, clay content, and sand content, respectively, significantly contributed to litter mass loss (Fig. 7B). Pearson correlation coefficient revealed that litter decomposition rates and the percentage of mass loss increased significantly with soil moisture and with the content of clay and sand, while they decreased with silt content (Table 4).

Discussion

Soil fauna variation across locations

Our results revealed more diverse communities of soil fauna in tropical rainforest than in permaculture and urban soils. We hypothesize that variations in the diversity of soil fauna are mediated by abundant and diverse resources in tropical forest, stable environmental conditions, low levels of disturbance, and complex ecological interactions. For example, the structural complexity of rainforests creates numerous microhabitats, from the mineral soils to the forest floor, which supports different soil fauna species adapted to specific niches and thus increasing overall soil biodiversity (Korboulewsky et al. 2016). In agreement with Korboulewski et al. (2016), we suggest that soil fauna is directly affected by the physical characteristics (microhabitats) and chemical composition (resource quality) of the litter specific to each tree species. In addition, soil communities are also affected by humus characteristics which are strongly linked with the litter chemistry of aboveground vegetation and often vary at different site locations (Korboulewsky et al. 2016; Schelfhout et al. 2017).

The relative abundances of various taxonomic and functional groups differed among locations and sampling times. We expect that abundances of various taxonomic and functional groups are linked to resource availability. We suggest that high nutrient content related to rapid decomposition processes in tropical habitats further supports growth and reproduction of soil organisms (Wall et al. 2008; Zhang et al. 2008; Peguero et al. 2019; Schaefer et al. 2009). Our results indicate changes in the composition of various taxonomic and functional groups of soil fauna with significantly higher densities of soil mesofauna and saprophagous mesofauna in the tropical forest compared to permaculture and urban soil. We suggest that changes in the composition of various groups of soil fauna are driven directly by aboveground vegetation via the feeding preferences of different animals. For example, a study by Gerlach et al. (2014) revealed different feeding preferences of isopods for various native and introduced plants. Moreover, a laboratory study by Heděnec et al. (2023) showed a higher consumption rate of high-quality leaf litter consumed by soil macrofauna. Similarly, a field study by Peng et al. (2022a) from the temperate zone revealed a shift in community structure of soil meso- and macrofauna in soils beneath different tree species which also differed in litter chemistry.

Our study revealed that the community structure of soil fauna in urban soils was associated with soil pH, silt content, and P content while the structure of soil fauna in permaculture and tropical forests mainly was primarily shaped by C:N:P ratio, organic matter content, and soil moisture. In addition, community structure diverged between different sampling locations. The sampling locations showed differences in soil physico-chemical properties, as well as by vegetation cover. We expect that soil physico-chemical properties are shaped by parent material and dominant vegetation (Birkhofer et al. 2012; De Schrijver et al. 2012). We expect that soil physico-chemical properties shape various groups of soil fauna indirectly via their direct effect on soil microbiota. For example, soil pH or moisture can stimulate bacterial or fungal growth and thus can stimulate the abundance of bacterial or fungal feeders (Rousk and Bååth 2011; Hedĕnec et al. 2024).

Soil fauna accessibility enhances litter decomposition

Our results revealed an increase in litter mass loss for three months of incubation. We hypothesize that soil fauna would affect litter decomposition even at the early decomposition stage within three months of litterbag exposure. Increased decomposition over time can be attributed to the progressive colonization and activity of soil animals, which intensify decomposition during time. We suggest that warm and humid tropical climate supports litter decomposition in comparison with drier and colder biomes (Wall et al. 2008; Frouz et al. 2015; Hedĕnec et al. 2022). We also expect that stable moisture and temperature in tropical climates can accelerate the physiological activity of soil animals as well as soil microbiota, which can potentially result in high decomposition rates (Powers et al. 2009; Peguero et al. 2019).

Our study showed that litterbags accessible to macrofauna exhibited a significantly higher litter mass loss than control litterbags but did not show any significant differences compared to those accessible to mesofauna. This indicates the crucial role of macrofauna in breaking down litter, likely due to their ability to fragment larger pieces of organic matter, thereby increasing the surface area available for microbial decomposition (García-Palacios et al. 2013; Frouz 2018; Fujii et al. 2018). Our results partly corroborate with similar litterbag studies across different habitat types with various dominant vegetation (Peguero et al. 2019; Peng et al. 2022b), however, our results showed significant litter mass loss only in litterbags accessible for macrofauna. We hypothesize that body size can affect litter decomposition rate via a higher consumption rate per body weight. For instance, a study by Ardestani et al. (2019) showed that big-size animals consumed more leaves per unit of body weight than small-sized animals.

Interestingly, litterbags in tropical forests and permaculture did not differ significantly in decomposition rates, suggesting that both environments provide favorable conditions for decomposers, possibly due to higher resource availability, moisture levels, and more stable microclimatic conditions compared to urban soils. However, our results based on effect size showed that soil macrofauna showed the highest effect on litter mass loss in permaculture. This suggests that in highly biodiverse tropical forests, other factors such as microbial activity and environmental conditions might play a more dominant role in driving decomposition (Wall et al. 2008; García-Palacios et al. 2013). In contrast, in permaculture systems, where biodiversity is managed and possibly less diverse than in natural forests, macrofauna might have a more pronounced effect.

Our effect size comparison showed that mesofauna exhibited the highest effect on litter mass loss in urban soils. This could be explained by the fact that urban soils typically have lower organic matter and nutrient levels compared to permaculture and tropical forests as shown by our data. Therefore, the presence and activity of mesofauna can have a disproportionately large impact on litter decomposition, as these organisms become critical drivers of the process (Peguero et al. 2019). Moreover, reduced biodiversity in urban soils means that mesofauna, which might otherwise be one of many decomposer groups, plays a more central role in the decomposition process in urban soils.

Our results indicated an increase of TOC and C:N ratio of litter in litterbags after three months of incubation while TN of litter in litterbags decreased significantly after three months of incubation. This suggests that soil fauna preferred the most palatable litter with a low CN ratio and left in litterbags only litter with a high C:N ratio. In addition, the passing of consumed leaf litter through the gut system also reduces the chemistry of feces which may affect the total C:N ratio of remaining litter in litterbags.

Soil properties shape fauna-mediated litter decomposition

The random forest analyses underscored the complex interplay of soil properties in shaping decomposition processes, revealing how different soil texture components, such as clay, sand, and silt, can significantly influence the rates and efficiency of litter decomposition. High clay content, for example, has been found to enhance moisture retention in soils, which is beneficial for the activity of decomposers such as microbes and soil fauna that require moist conditions to thrive (Cortez 1998; Butterly et al. 2010). Moisture is critical for enzymatic reactions involved in the breakdown of organic matter, and adequate moisture levels can facilitate the growth and activity of decomposing organisms, thereby accelerating decomposition processes (Brockett et al. 2012).

In contrast, the presence of clay in excessive amounts can also impede soil aeration, creating anaerobic conditions that are less favorable for many decomposers. In poorly aerated soils, the activity of these microorganisms is restricted, slowing down the decomposition process (Wang et al. 2021; Qian et al. 2022). Furthermore, anaerobic conditions can lead to the production of toxic substances such as methane and hydrogen sulfide, which can inhibit the activity of soil fauna and further inhibit microbial decomposition (van Agtmaal et al. 2015). Therefore, while clay content is beneficial for moisture retention, it must be balanced to avoid negative impacts on soil aeration. Furthermore, clay soils, with their fine particles, tend to be dense and compact, which can impede the movement and penetration of larger soil organisms like macrofauna (Swift et al. 1979; Frouz et al. 2006; Ruiz et al. 2008). These conditions are limiting factors for such fauna to create and maintain their own tunnels, potentially limiting their ability to forage and thrive.

Sand, on the other hand, contributes to better aeration and drainage due to its larger particle size and greater pore spaces between particles (Lavelle et al. 2020). Soils with higher sand content tend to be well-aerated, which supports the activity of aerobic decomposers and facilitates rapid organic matter breakdown (Coleman et al. 2004; Wang et al. 2021). However, sandy soils can suffer from poor moisture retention, especially in dry conditions, which can limit the availability of water to decomposers and slow down decomposition (Coleman et al. 2004; de Vries et al. 2012; Alster et al. 2013). Thus, the positive effects of sand on soil aeration need to be balanced with sufficient moisture retention to support continuous decomposition activity. Sandy soils, with larger particles and greater pore spaces, offer less resistance to movement and are more easily penetrated by both macrofauna and mesofauna (Swift et al. 1979; Wall et al. 2008; Xin et al. 2012). These conditions facilitate the creation of tunnels and burrows, enabling soil organisms to navigate and exploit their environment effectively.

Silt particles alter moisture retention and aeration (Coleman et al. 2004). Silt improves soil structure and thus the availability of nutrients to soil organisms. However, too much silt can lead to compaction and reduce soil pore spaces, negatively impacting both aeration and water infiltration (Hartmann et al. 2014). The optimal decomposition rates are achieved when soil texture maintains a balance between these components – clay for moisture retention, sand for aeration, and silt for nutrient availability and structure (Callesen et al. 2003; Coleman et al. 2004; Velasquez et al. 2007). This balance ensures that soil conditions remain conducive to the activity of decomposers, supporting efficient organic matter breakdown and nutrient cycling in various soil environments.

Implications, limitations and future perspective

Our study revealed that soil fauna has a large impact on litter decomposition. Efficient litter decomposition by soil fauna enhances nutrient cycling, thereby improving soil fertility and crop yields (De Vries et al. 2013; Edlinger et al. 2023). The implications of efficient litter decomposition extend beyond immediate soil fertility. The presence of a robust soil fauna community can also enhance crop resilience against pests and diseases, reducing reliance on chemical pesticides (Griffiths et al. 2000; Teixeira et al. 2019; Vannier et al. 2019; Delgado-Baquerizo et al. 2020). Furthermore, our findings emphasize the importance of understanding and conserving soil fauna diversity in urban and permaculture settings, as these ecosystems are often subject to anthropogenic disturbances which can impact soil fauna communities and, consequently, litter decomposition processes (Peng et al. 2022b). By recognizing the critical role of soil fauna in litter decomposition and ecosystem functioning, efforts can be made to integrate conservation and management practices that support soil fauna biodiversity and abundance.

We suggest that our study also includes several limitations but the methods used in our study make our results comparable to other studies. Firstly, decomposition was measured solely through mass loss from the litterbags, which does not fully capture the underlying mechanisms driving these processes. Mass loss can result from various factors, including microbial mineralization, leaching, and the fragmentation and washing out of small organic matter particles, making it difficult to distinguish the specific contributions of these processes (Kampichler and Bruckner 2009; Frouz 2018). Additionally, the role of soil fauna in litter decomposition is complex; fauna can both accelerate and slow down microbial activity through their consumption and transformation of litter (Frouz 2018; Angst et al. 2024). Furthermore, the use of litterbags with different mesh sizes may not perfectly replicate natural conditions where litter is freely accessible to all decomposers. We suggest that our study focused on specific sites and may not capture the full range of soil fauna diversity and dynamics in tropical ecosystems. Additionally, the short-term nature of the study may not fully represent long-term trends in litter decomposition dynamics. Despite the above-mentioned limitation, our study provides new insights into the initial dynamics of decomposition that are often neglected in long-term studies. We suggest that future research should employ more comprehensive methods to highlight the intricate mechanisms of litter decomposition and to fully understand the ecological roles of soil fauna. For example, combining litterbag experiments with laboratory cutting-edge methods, such as the stable isotope method, amplicon sequencing method or shotgun metagenomic method is promising to investigate specific groups of soil biota involved in specific decomposition processes (Baldrian 2017; Sultana et al. 2019; Zhang et al. 2020).

Conclusion

Our study revealed the complex interplay between soil properties, soil fauna composition, and environmental factors in shaping litter decomposition processes. For example, the significant variation observed in soil physico-chemical properties among different sampling locations highlights the diverse environmental conditions supporting various groups of soil fauna that litter decomposition. Furthermore, our results showed the impact of soil fauna on litter decomposition, with macrofauna playing a particularly significant role. Understanding the interactions between soil fauna and environmental factors, such as soil moisture and nutrient content, can provide valuable insights for ecosystem management and conservation strategies aimed at enhancing litter decomposition and nutrient cycling in terrestrial ecosystems.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT – OpenAI version 3.2 in order to grammar check and edit English language since author(s) are not native English speakers. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Supplementary file S1

	Treatment	Locality	Time	Before	After	Percentage	k
A1	2 cm	Tropical_rain_forest	1	5.0	3.73	25.4	0.29
A2	2 cm	Tropical_rain_forest	1	5.0	4.41	11.8	0.13
A3	2 cm	Tropical_rain_forest	1	5.0	3.95	21.0	0.24
A4	2 cm	Tropical_rain_forest	1	5.1	4.20	17.6	0.19
A5	2 cm	Tropical_rain_forest	1	5.0	4.15	17.0	0.19
A6	2 cm	Tropical_rain_forest	1	5.1	4.18	18.0	0.20
A7	2 mm	Tropical_rain_forest	1	5.1	4.10	19.6	0.22
A8	2 mm	Tropical_rain_forest	1	5.1	3.93	22.9	0.26
A9	2 mm	Tropical_rain_forest	1	5.0	3.85	23.0	0.26
A10	2 mm	Tropical_rain_forest	1	5.0	4.06	18.8	0.21
A11	2 mm	Tropical_rain_forest	1	5.0	4.10	18.0	0.20
A12	2 mm	Tropical_rain_forest	1	5.1	4.25	16.7	0.18
A13	0.2 mm	Tropical_rain_forest	1	5.0	4.08	18.4	0.20
A14	0.2 mm	Tropical_rain_forest	1	5.0	4.23	15.4	0.17
A15	0.2 mm	Tropical_rain_forest	1	5.0	4.32	13.6	0.15
A16	0.2 mm	Tropical_rain_forest	1	5.0	4.02	19.6	0.22
A17	0.2 mm	Tropical_rain_forest	1	5.0	4.15	17.0	0.19
A18	0.2 mm	Tropical_rain_forest	1	5.0	4.12	17.6	0.19
B1	2 cm	Urban_soil	1	5.0	4.38	12.4	0.13
B2	2 cm	Urban_soil	1	5.1	4.36	14.5	0.16
B3	2 cm	Urban_soil	1	5.1	4.37	14.3	0.15
B4	2 cm	Urban_soil	1	5.1	4.45	12.7	0.14
B5	2 cm	Urban_soil	1	5.0	4.72	5.6	0.06
B6	2 cm	Urban_soil	1	5.1	4.46	12.5	0.13
B7	2 mm	Urban_soil	1	5.1	4.47	12.4	0.13
B8	2 mm	Urban_soil	1	5.0	4.44	11.2	0.12
B9	2 mm	Urban_soil	1	5.1	4.36	14.5	0.16
B10	2 mm	Urban_soil	1	5.1	4.32	15.3	0.17
B11	2 mm	Urban_soil	1	5.1	4.55	10.8	0.11
B12	2 mm	Urban_soil	1	5.1	4.36	14.5	0.16
B13	0.2 mm	Urban_soil	1	5.1	4.34	14.9	0.16
B14	0.2 mm	Urban_soil	1	5.1	4.32	15.3	0.17
B15	0.2 mm	Urban_soil	1	5.0	4.41	11.8	0.13
B16	0.2 mm	Urban_soil	1	5.1	4.37	14.3	0.15
B17	0.2 mm	Urban_soil	1	5.1	4.52	11.4	0.12
B18	0.2 mm	Urban_soil	1	5.1	4.34	14.9	0.16
C1	2 cm	Permaculture	1	5.1	4.35	14.7	0.16
C2	2 cm	Permaculture	1	5.1	4.37	14.3	0.15
C3	2 cm	Permaculture	1	5.1	4.32	15.3	0.17
C4	2 cm	Permaculture	1	5.1	4.24	16.9	0.18
C5	2 cm	Permaculture	1	5.1	3.42	32.9	0.40
C6	2 cm	Permaculture	1	5.1	4.26	16.5	0.18
C7	2 mm	Permaculture	1	5.1	4.14	18.8	0.21

	Treatment	Locality	Time	Before	After	Percentage	k
C8	2 mm	Permaculture	1	5.1	4.19	17.8	0.20
C9	2 mm	Permaculture	1	5.1	4.27	16.3	0.18
C10	2 mm	Permaculture	1	5.0	4.30	14.0	0.15
C11	2 mm	Permaculture	1	5.0	4.28	14.4	0.16
C12	2 mm	Permaculture	1	5.0	4.29	14.2	0.15
C13	0.2 mm	Permaculture	1	5.0	4.00	20.0	0.22
C14	0.2 mm	Permaculture	1	5.1	4.38	14.1	0.15
C15	0.2 mm	Permaculture	1	5.0	4.25	15.0	0.16
C16	0.2 mm	Permaculture	1	5.0	4.08	18.4	0.20
C17	0.2 mm	Permaculture	1	5.1	4.14	18.8	0.21
C18	0.2 mm	Permaculture	1	5.1	4.4	13.7	0.15
D1	2 cm	Urban_soil	2	5.0	4.03	19.4	0.11
D2	2 cm	Urban_soil	2	5.1	4.03	21.0	0.12
D3	2 cm	Urban_soil	2	5.1	4.09	19.8	0.11
D4	2 cm	Urban_soil	2	5.0	4.09	18.2	0.10
D5	2 cm	Urban_soil	2	5.1	4.16	18.4	0.10
D6	2 cm	Urban_soil	2	5.1	4.22	17.3	0.09
D7	2 mm	Urban_soil	2	5.1	4.06	20.4	0.11
D8	2 mm	Urban_soil	2	5.1	4.16	18.4	0.10
D9	2 mm	Urban_soil	2	5.1	4.05	20.6	0.12
D10	2 mm	Urban_soil	2	5.0	4.16	16.8	0.09
D11	2 mm	Urban_soil	2	5.1	3.89	23.7	0.14
D12	2 mm	Urban_soil	2	5.1	4.13	19.0	0.11
D13	0.2 mm	Urban_soil	2	5.0	3.93	21.4	0.12
D14	0.2 mm	Urban_soil	2	5.0	3.97	20.6	0.12
D15	0.2 mm	Urban_soil	2	5.1	4.32	15.3	0.08
D16	0.2 mm	Urban_soil	2	5.1	4.29	15.9	0.09
D17	0.2 mm	Urban_soil	2	5.1	4.16	18.4	0.10
D18	0.2 mm	Urban_soil	2	5.1	4.11	19.4	0.11
E1	0.2 mm	Tropical_rain_forest	2	5.0	4.01	19.8	0.11
E2	0.2 mm	Tropical_rain_forest	2	5.0	3.74	25.2	0.15
E3	0.2 mm	Tropical_rain_forest	2	5.0	3.82	23.6	0.13
E4	0.2 mm	Tropical_rain_forest	2	5.1	3.98	22.0	0.12
E5	0.2 mm	Tropical_rain_forest	2	5.1	3.85	24.5	0.14
E6	0.2 mm	Tropical_rain_forest	2	5.0	3.96	20.8	0.12
E7	2 cm	Tropical_rain_forest	2	5.0	4.00	20.0	0.11
E8	2 cm	Tropical_rain_forest	2	5.0	3.75	25.0	0.14
E9	2 cm	Tropical_rain_forest	2	5.0	4.02	19.6	0.11
E10	2 cm	Tropical_rain_forest	2	5.1	3.62	29.0	0.17
E11	2 cm	Tropical_rain_forest	2	5.1	3.91	23.3	0.13
E12	2 cm	Tropical_rain_forest	2	5.1	3.78	25.9	0.15
E13	2 mm	Tropical_rain_forest	2	4.9	2.95	39.8	0.25
E14	2 mm	Tropical_rain_forest	2	5.16	4.48	13.2	0.07
E15	2 mm	Tropical_rain_forest	2	5.0	4.07	18.6	0.10
E16	2 mm	Tropical_rain_forest	2	5.02	3.94	21.5	0.12

	Treatment	Locality	Time	Before	After	Percentage	k
E17	2 mm	Tropical_rain_forest	2	5.0	3.88	22.4	0.13
E18	2 mm	Tropical_rain_forest	2	5.1	3.87	24.1	0.14
F1	2 cm	Permaculture	2	5.1	3.51	31.2	0.19
F2	2 cm	Permaculture	2	5.1	4.01	21.4	0.12
F3	2 cm	Permaculture	2	5.0	3.88	22.4	0.13
F4	2 cm	Permaculture	2	5.0	4.12	17.6	0.10
F5	2 cm	Permaculture	2	5.1	3.41	33.1	0.20
F6	2 cm	Permaculture	2	5.1	3.13	38.6	0.24
F7	0.2 mm	Permaculture	2	5.1	3.73	26.9	0.16
F8	0.2 mm	Permaculture	2	5.0	3.88	22.4	0.13
F9	0.2 mm	Permaculture	2	5.1	3.97	22.2	0.13
F10	0.2 mm	Permaculture	2	5.1	3.90	23.5	0.13
F11	0.2 mm	Permaculture	2	5.1	3.87	24.1	0.14
F12	0.2 mm	Permaculture	2	5.0	3.50	30.0	0.18
F13	2 mm	Permaculture	2	5.0	4.16	16.8	0.09
F14	2 mm	Permaculture	2	5.1	4.19	17.8	0.10
F15	2 mm	Permaculture	2	5.0	4.15	17.0	0.09
F16	2 mm	Permaculture	2	5.0	4.22	15.6	0.08
F17	2 mm	Permaculture	2	5.0	4.22	15.6	0.08
F18	2 mm	Permaculture	2	5.0	3.98	20.4	0.11
G1	0.2 mm	Urban_soil	3	5.1	4.13	19.0	0.07
G2	0.2 mm	Urban_soil	3	5.1	4.11	19.4	0.07
G3	0.2 mm	Urban_soil	3	5.0	4.00	20.0	0.07
G4	0.2 mm	Urban_soil	3	5.0	4.36	12.8	0.05
G5	0.2 mm	Urban_soil	3	5.0	3.82	23.6	0.09
G6	0.2 mm	Urban_soil	3	5.0	4.25	15.0	0.05
G7	2 mm	Urban_soil	3	5.1	3.88	23.9	0.09
G8	2 mm	Urban_soil	3	5.0	3.88	22.4	0.08
G9	2 mm	Urban_soil	3	5.0	3.75	25.0	0.10
G10	2 mm	Urban_soil	3	5.0	3.83	23.4	0.09
G11	2 mm	Urban_soil	3	5.0	3.69	26.2	0.10
G12	2 mm	Urban_soil	3	5.0	3.12	37.6	0.16
G13	2 cm	Urban_soil	3	5.0	3.68	26.4	0.10
G14	2 cm	Urban_soil	3	5.1	3.69	27.6	0.11
G15	2 cm	Urban_soil	3	5.0	3.88	22.4	0.08
G16	2 cm	Urban_soil	3	5.1	4.07	20.2	0.08
G17	2 cm	Urban_soil	3	5.0	3.77	24.6	0.09
G18	2 cm	Urban_soil	3	5.1	3.78	25.9	0.10
H1	2 cm	Tropical_rain_forest	3	5.0	3.47	30.6	0.12
H2	2 cm	Tropical_rain_forest	3	5.0	3.18	36.4	0.15
H3	2 cm	Tropical_rain_forest	3	5.1	3.10	39.2	0.17
H4	2 cm	Tropical_rain_forest	3	5.0	3.19	36.2	0.15
H5	2 cm	Tropical_rain_forest	3	5.1	3.32	34.9	0.14
H6	2 cm	Tropical_rain_forest	3	5.1	3.80	25.5	0.10
H7	2 mm	Tropical_rain_forest	3	5.1	3.30	35.3	0.15

	Treatment	Locality	Time	Before	After	Percentage	k
H8	2 mm	Tropical_rain_forest	3	5.0	3.60	28.0	0.11
H9	2 mm	Tropical_rain_forest	3	5.1	3.74	26.7	0.10
H10	2 mm	Tropical_rain_forest	3	5.1	3.81	25.3	0.10
H11	2 mm	Tropical_rain_forest	3	5.1	3.61	29.2	0.12
H12	2 mm	Tropical_rain_forest	3	5.0	3.44	31.2	0.12
H13	0.2 mm	Tropical_rain_forest	3	5.0	3.56	28.8	0.11
H14	0.2 mm	Tropical_rain_forest	3	5.1	3.51	31.2	0.12
H15	0.2 mm	Tropical_rain_forest	3	5.1	3.59	29.6	0.12
H16	0.2 mm	Tropical_rain_forest	3	5.1	3.53	30.8	0.12
H17	0.2 mm	Tropical_rain_forest	3	5.1	3.44	32.5	0.13
H18	0.2 mm	Tropical_rain_forest	3	5.1	3.44	32.5	0.13
I1	2 cm	Permaculture	3	5.0	3.53	29.4	0.12
I2	2 cm	Permaculture	3	5.0	3.54	29.2	0.12
I3	2 cm	Permaculture	3	5.0	3.38	32.4	0.13
I4	2 cm	Permaculture	3	5.0	3.37	32.6	0.13
I5	2 cm	Permaculture	3	5.1	3.66	28.2	0.11
I6	2 cm	Permaculture	3	5.0	3.31	33.8	0.14
I7	0.2 mm	Permaculture	3	5.0	3.83	23.4	0.09
I8	0.2 mm	Permaculture	3	5.0	3.72	25.6	0.10
I9	0.2 mm	Permaculture	3	5.1	3.58	29.8	0.12
I10	0.2 mm	Permaculture	3	5.0	3.65	27.0	0.10
I11	0.2 mm	Permaculture	3	5.1	3.48	31.8	0.13
I12	0.2 mm	Permaculture	3	5.0	3.40	32.0	0.13
I13	2 mm	Permaculture	3	5.1	3.80	25.5	0.10
I14	2 mm	Permaculture	3	5.1	3.57	30.0	0.12
I15	2 mm	Permaculture	3	5.1	3.66	28.2	0.11
I16	2 mm	Permaculture	3	5.1	3.55	30.4	0.12
I17	2 mm	Permaculture	3	5.1	3.55	30.4	0.12
I18	2 mm	Permaculture	3	5.1	3.55	30.4	0.12

THE SUPPRESSIVE EFFECT OF WOOD ANTS ON BARK BEETLE COLONIZATION

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ABSTRACT

Climate change supports bark beetle outbreaks in European forests, posing a significant threat to the economically important Norway spruce (*Picea abies*). While chemical control methods in pest control are increasingly restricted, biological control using natural enemies, such as wood ants (*Formica* spp.), offers a sustainable alternative. Despite their known role as generalist predators of various forest pests, the direct impact of wood ants on bark beetle colonization remains underexplored. This study investigated the suppressive effect of *Formica rufa* ants on six-toothed spruce bark beetle (*Pityogenes chalcographus*) colonization. Branch traps were installed near 34 wood ant nests across five localities in Czechia. At each nest, one trap was placed 1 meter from the nest (treatment) and another 40 meters away (control). In total, traps near ant nests showed a mean reduction of approximately 40% in beetle colonization. Results confirmed similar suppressive metrics, consistent across all study localities. Our findings demonstrate that ants significantly suppress bark beetle colonization. This confirms the crucial role of wood ants as biological control agents against bark beetle damage and supports their integration into sustainable forest management strategies.

Keywords: biological control; *Formica rufa*; forest pests; Norway spruce; non-consumptive effects; *Pityogenes chalcographus*

Introduction

Climate change is disrupting the health of economically important tree species across Europe (Jaime et al. 2019, 2024). Trees weakened by drought are particularly susceptible to pest infestations (Potterf et al. 2025; Véle and Neudertová Hellebrandová 2025). Norway spruce (*Picea abies*), a key economic tree of European forestry, is widely cultivated, often in plantations, which are ecologically less stable (Spiecker 2000). Among the most dangerous threats are bark beetles, which are responsible for large-scale diebacks of secondary spruce forests in Central Europe (Grégoire and Evans 2007; Brázdil et al. 2022). In response, there is a growing trend towards converting spruce plantations into more ecologically resilient mixed forests (Löf et al. 2023). Nevertheless, Norway spruce remains the most widely planted and economically significant tree species (MZe 2023). While chemical control methods are available for bark beetle management, their use is increasingly restricted due to negative impacts on non-target species and potential health risks (Leroy 2025).

An alternative to chemical control is the use of natural enemies, which aligns with the principles of integrated forest protection (Matyjaszczyk and Skrzecz 2020). Entomopathogenic fungi, parasitoids, and predators represent key biotic factors with strong potential for application in biological control programs (Lipták et al. 2013). Among bark beetle predators, species such as the ant beetle *Thanasimus formicarius* and woodpeckers are notable (Wegensteiner et al. 2015). In contrast, wood ants are not typically classified as significant bark beetle predators, although wood ants (*Formica* spp.) are recognized as significant natural enemies of various forest insect pests (Adlung 1966; Wegensteiner et al. 2015). Ants' advanced social structure and cooperative foraging behaviour make them highly effective generalist predators (Traniello 1989). Their pest control ability is enhanced by a positive response to volatiles emitted by infested plants (Schettino et al. 2017).

Wood ants use honeydew from aphids as their main source of food, the rest is protein food (Domisch et al. 2009). As omnivorous generalists, wood ants adapt their diet to available food resources (Richter and Economo 2023). During pest outbreaks, they may shift their foraging strategy, favouring insect predation over honeydew collection. In such instances, insects can constitute over 90% of their diet (Domisch et al. 2009). Wood ants actively prey on insects across all developmental stages: eggs, larvae, pupae, and adults. In European forests, *Formica* species prey on several defoliating pest species, which can contribute to reduced herbivore damage to trees (Adlung 1966; Skinner and Whittaker 1981; Warrington and Whittaker 1985). For example, a single *Formica rufa* colony with 500,000 workers can collect between 1,000 and 100,000 larvae of the spruce sawfly (*Pristiphora abietina*) per day (Bruns 1954). Their protective influence can extend up to tens of meters from the nest (Laine and Niemelä 1980).

Indirect evidence of wood ants' influence on bark beetle populations was provided by Trigos-Peral et al. (2021), who observed a lower incidence of bark beetle-infested trees around ant nests. However, this descriptive study did not clarify whether the observed differences could

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be attributed to other factors, such as variations in soil quality near the nests leading to increased tree vitality (White 1985). Furthermore, it is known that bark beetle outbreaks can occur even in the vicinity of ant nests (Véle and Frouz 2023). Driven by the need for empirical evidence, we designed and executed an experimental study to further elucidate the role of wood ants in mitigating bark beetle attacks on trees. Specifically, we examined whether there is a difference in the number of galleries and maternal holes on branch traps placed within the territory of ant nests compared to those on control sites.

Methods

This study was conducted across five localities in the Czech Republic near Březí nad Oslavou (49.5058447N, 15.9550525E), Račín (49.6344272N, 15.8346483E), Horní Řasnice (50.9808175N, 15.1750342E), Chuchelna (50.6165419N, 15.2904494E), and Bítouchov (50.6222733N, 15.3334508E). The five localities were selected to represent a gradient of bark beetle outbreak intensity, from severely affected (Březí nad Oslavou, Račín) to moderately affected (Horní Řasnice) and minimally affected areas (Chuchelna, Bítouchov), as documented by Lubojacký et al. (2023, 2024). All localities were situated in pure even-aged managed Norway spruce stands at least 50 years of age, at elevations ranging from 450 to 610 m a. s. l. (Březí nad Oslavou: 550 m, Račín: 610 m, Horní Řasnice: 450 m, Chuchelna: 450 m, Bítouchov: 470 m).

For our investigation, we selected isolated nests of *Formica rufa*, which were sparse and widely spaced across the study areas. The nest mounds were of medium size, characterized by a mean diameter of 147 ± 31 cm and a height of 64 ± 15 cm. At each nest, we installed two branch traps. One trap was placed 1 meter from the nest, within the ant's territory, and the second was positioned 40 meters away, outside the territorial range. The traps were constructed from bark beetle-uninfested trees that were felled approximately 14 days before installation. Each trap consisted of two branches, each 7 cm in diameter and 1.1 meters in length. Traps were placed on tree trunks at a height of approximately 4 meters (Fig. 1) and oriented toward the south to increase the probability of bark beetle colonization (Wermelinger 2004). Traps were not, however, exposed to intense direct sunlight due to canopy shading. A total of 68 traps were installed in the second half of March 2024, before the start of bark beetle swarming, which typically occurs at the turn of April and May. The traps were collected in early June, after the first swarming period (Holuša and Fiala 2024). Following collection, the bark was removed from the traps, and the numbers of galleries and maternal holes were counted. A *Pityogenes chalcographus* male first creates a short nuptial chamber and then attracts the female. Each female then makes her own maternal chamber (Holuša and Fiala 2024). Thus, one counted gallery indicates the presence of one male, while the number of maternal holes corresponds to the number of females.

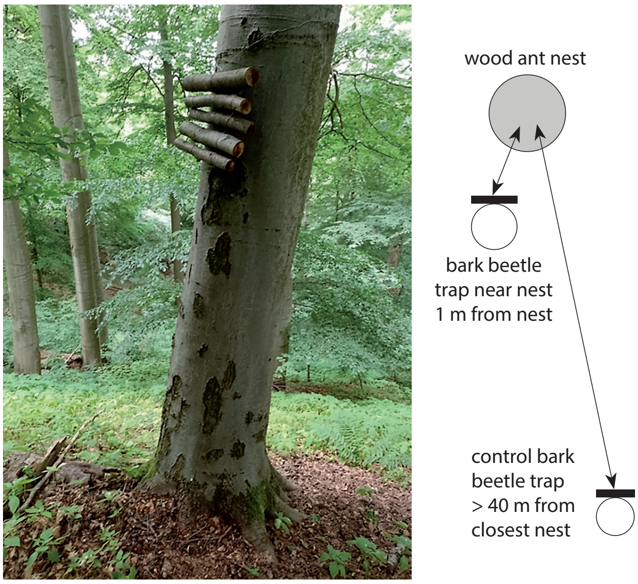

Fig. 1 Picture of bark beetle trap on the tree and schematic of the location of bark beetle traps in relation to wood ant nest.

Due to their diameter, the traps were primarily designed to capture *P. chalcographus*. This is a smaller species (1.6–2.9 mm) that often accompanies the larger spruce bark beetle (*Ips typographus*, 4.2–5.5 mm) on older trees. Both species have similar bionomics and often occur together on the same tree (Pfeffer 1955), leading us to hypothesize that the impact of ants on both species would be similar.

To evaluate the effect of ant nest proximity on bark beetle colonization, we performed a two-way Analysis of Variance (ANOVA). The independent variables were "locality" (representing the different study sites) and "ant nest" (a binary factor representing traps placed 1 meter from the nest vs. 40 meters from the nest). The dependent variables were the number of males, females, and the total count of beetles. A post hoc LSD test was used to identify statistically homogeneous groups and to determine significant differences between the nest and control traps. All statistical analyses were performed in Statistica 10.0.

Results

Our results demonstrated that the presence of *Formica rufa* nests significantly suppressed colonization by *P. chalcographus* on experimental traps. The mean number of galleries as well as maternal holes was significantly lower on traps placed 1 meter from an ant nest compared to control traps placed outside nest territory (Fig. 2). Specifically, traps located near nests recorded an average of 10.03 ± 11.77 males and 40.10 ± 57.16 females, while

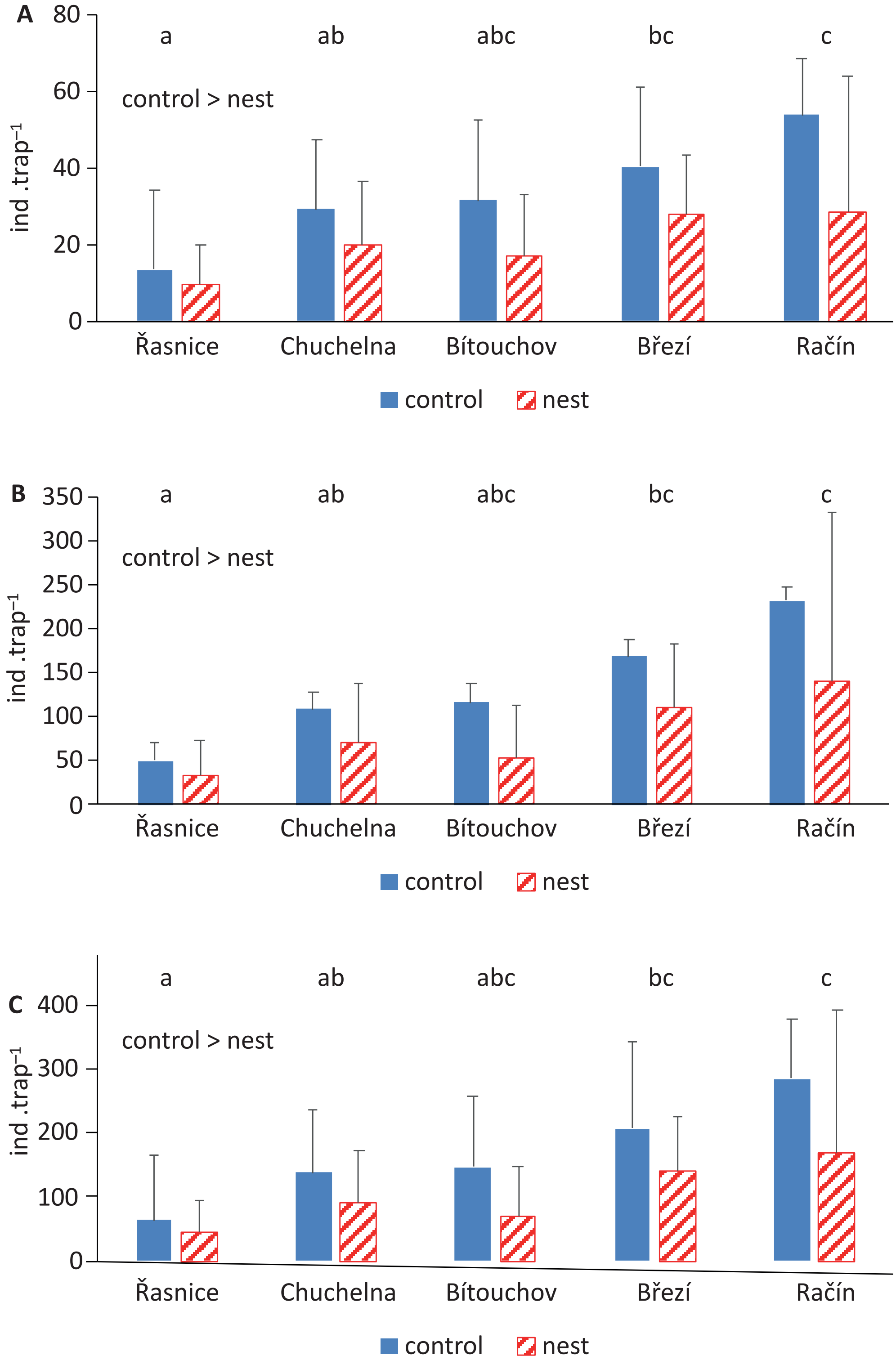

Fig. 2 Number of bark beetle individuals trapped in traps located wood ant nests and in control nests away from the nest, shown for males (A), females (B) and total (C). Means and SD are shown, statistically homogeneous groups are labelled by the same letters (LSD post hoc test $p<0.05$), significant differences between nest and control traps are indicated, for p values of two-way ANOVA see Table 1.

control traps showed 16.44 ± 12.95 and 66.13 ± 59.5 individuals, representing a 39% and 39.4% reduction, respectively.

A two-way ANOVA confirmed that the distance from the ant nest had a statistically significant effect on total colonization, as well as on male and female counts individually. The effect of locality was also significant, reflecting differences in beetle abundance across study locations. However, no significant interaction was found between locality and nest distance, indicating that the suppressive effect of ants was consistent across all areas, regardless of local beetle population density (Table 1).

Table 1 The effect of locality and distance from wood ant nest on bark beetle infestation, p-values of two-way ANOVA are given.

Effect of	Males	Females	Total
Locality	0.0071	0.0051	0.0053
Ant nest	0.0157	0.0388	0.0328
Interactions	ns	ns	ns

Discussion

Our study demonstrates a clear suppressive effect of red wood ant (*Formica rufa*) presence on bark beetle *P. chalcographus* colonization. We found that trees located near ant nests experienced approximately 40% fewer beetle infestations compared to control trees in areas without ants. This suppressive effect remained consistent across all study localities, regardless of the overall intensity of bark beetle infestation in the region.

These findings align with previous research of Trigos-Peral et al. (2021), who described a significant correlation between an increasing number of *Formica polyctena* nests and a reduction in the number of bark beetle-infested trees. Similarly, other studies have reported comparable levels of pest abundance reduction. Total sawfly *Pristiphora abietina* larvae populations were reduced by more than 50% within 29 meters from wood ant nests (Wellenstein 1957). Lower pest abundances near wood ant nest have been recorded in many species, for example: *Diprion pini*, *Neodiprion sertifer*, *Coleophora laricella*, *Tortrix viridana*, *Operophtera brumata*, *Panolis flammea*, *Lymantria monacha* (Adlung 1966).

Several mechanisms likely contribute to the observed reduction in beetle colonization near ant nests. The primary mechanism is that, just like bark beetles, ants move along trees to find most of their food, which they acquire through hunting and collecting honeydew (Sudd and Lodhi 1981; Lenoir 2003; Domisch et al. 2009). The most well-known mechanism is predation. Wood ants prey on many insect species, but they are generally considered primarily predators of herbivorous insect larvae, which are often found in places accessible to worker ants (Adlung 1966). However, hunting the developmental stages of bark beetles is impossible, as the entry holes of small beetles like *P. chalcographus* are too narrow for wood ants to pass through. Therefore, only adult beetles can be preyed upon. This aligns with observations that beetles constitute over 10% of the wood ants' diet, with adult *Curculionidae* (weevils) being numerically well-represented, while the presence of their larvae in the food spectrum is low (Domisch et al. 2009).

Beyond direct predation, non-consumptive effects (NCEs) are another crucial way ants might regulate bark beetle populations. NCEs play a significant role in predator-herbivore interactions by modifying prey and predator behaviour. These modifications can include altered foraging strategies, changes in habitat selection, shifts in life history traits, and heightened anti-predator responses. Such effects are well-documented across many animal species, including ants (Wills and Landis 2018; Batabyal 2023).

A typical example of these interactions, which often occur indirectly, is the relationship between ants and aphids on plants, because honeydew is a predominant part of the ants' diet (Rico-Gray and Oliveira 2007; Domisch et al. 2009). Ants also use aphids as a supplementary food source to alleviate the effects of acute fungal infections (Rissanen et al. 2023). For this reason, ants tend to protect aphids from their natural enemies (Novgorodova and Gavrilyuk 2012). For instance, when *Formica rufa* ants encountered adult ladybugs *Hippodamia variegata* (which is a predator of aphids) near aphid colonies, they frequently exhibited aggressive behaviour, leading to a significant reduction in beetle egg-laying (Mir et al. 2024). Ants may behave similarly towards other animals they encounter on trees. Animals may also be deterred by volatile compounds produced by ants, as demonstrated with spiders and ticks (Mestre et al. 2014; Gooding et al. 2024). Even large beetles, like carabids, actively try to avoid individual contact with moving ants (Dorosheva and Reznikova 2006). Seedlings of conifer species artificially made attractive to ants suffered less feeding damage by the large pine weevil *Hylobius abietis*, suggesting that ant presence deters this pest, likely through tactile cues but possibly also via volatile chemicals and visual signals (Maňák et al. 2013, 2016). Furthermore, the influence of ant-bird competition has been observed. Birds visited trees without ants more frequently and foraged there for longer periods than trees with ant activity (Haemig 1994). This suggests that ants can indirectly affect other organisms by altering their foraging behaviour.

There is a complex balance between the bark beetle infestation density required to successfully overcome a tree's defences, which depends on tree vitality (Mulock and Christiansen 1986; Schroeder and Lindelöw 2002). Because of this, we cannot definitively say whether the observed reduction in the number of infesting beetles guarantees a tree's survival. However, it's well-known that a "mass attack" is necessary to overcome a healthy tree's defences (Lehmanski et al. 2023). In such cases, we can expect that healthy trees will be protected by ants. This could explain observations of bark beetle-infested trees near ant nests (Véle and Frouz 2023), as that study was conducted after a period when trees were weakened by severe drought (Brázdil et al. 2022). Despite their proven suppressive effect, the use of wood ants as a biological control agent has significant limitations. Their protective effect is inherently local, extending only tens of meters from the nest (Adlung 1966; Laine and Niemelä 1980), and cannot be scaled up to the landscape level without a sustained density of nests throughout the managed area. Their presence also has a negative impact on beneficial insect populations (Véle and Dobrosavljević 2021). Furthermore, the mutualism between wood ants and aphids represents a trade-off, as ant colonies actively protect aphid populations, which can themselves negatively affect tree vitality (Kilpeläinen et al. 2009). These limitations should be taken into account when integrating wood ants into forest conservation strategies. Nevertheless, wood ants should be considered an integral part of pest management strategies, as their presence supports the protection of valuable trees and the prevention of bark beetle infestations.

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ON CANARY ISLANDS POLYPORES: NEWS AND PROBLEMS

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ABSTRACT

A total of 20 selected species of polypores recorded on the Canary Islands are characterized and commented. New biogeography and ecology data of polypores of Macaronesia are presented. Four species: *Antrodiella fissiliformis*, *Diplomitoporus flavescens*, *Haploporus odorus* and *Skeletocutis albocremea* are proposed for exclusion from the Canary Islands mycobiota. Several species are confirmed for the first time: *Ceriporiopsis consobrina*, *Ceriporiopsis pseudogilvescens*, *Gloeophyllum* cf. *abietinum*, *Gloeophyllum trabeum*, *Perenniporia meridionalis*, *Porodaedalea* sp., *Sidera vulgaris*. Two new genera of polypores, *Gloeophyllum* and *Porodaedalea*, are recorded as new for Canary Islands. The results are based on the review of herbarium specimens and field research.

Keywords: biogeography; Macaronesia; Polyporales; taxonomy; wood-inhabiting fungi

Introduction

The Canary Islands are a group of volcanic islands near the western coast of the North Africa; the nearest of them is situated only about 100 km from Africa. Due to the historical isolation of all archipelagos, a high degree of endemism has been documented in different groups of organisms (Arechavaleta et al. 2010; Beierkuhnlein et al. 2021). For example, 33% of plants are endemic species (Welss and Lindacher 1994). Biodiversity research has a long tradition here (e.g. Webb and Berthelot 1836–50; Wollaston 1864).

The species biodiversity in Fungi (including lichens) on the Canary Islands is the highest of all the Macaronesia islands and it reaches over 3000 species, which exceeds the number of plant species here (Beltrán-Tejera 2011). The greatest biodiversity of fungi is found on the western islands La Gomera, La Palma, and Tenerife, with more extensive cloud laurel forests (del Arco Aguilar et al. 2010). Maybe that mycologist have paid less attention to more eastern islands with specific dry biotopes, but there may be hidden diversity as was shown in *Euphorbia* scrubs on Tenerife (Kout et al. 2017; Quijada and Baral 2017).

The research of Canary Islands fungi started with Wolfredo Wildpret de la Torre (Wildpret et al. 1969) from the local university on the island of Tenerife, who has been however focused mainly on gilled fungi. The Aphyllophoroid group was explored L. Ryvarden (1972, 1976) and the Canary Islands mycologists have followed him with many articles and books (e.g. Rodríguez-Armas and Beltrán-Tejera 1995). The last formal checklist of the species from the Canary Island mentions 89 polypores (in the sense of Ryvarden and Melo 2014) with high prevalence of white rot species (Arechavaleta et al. 2010), and some of them are critically reviewed in this article.

Material and Methods

The selection of the presented species is based on studied specimens from mycological herbarium on Tenerife (TFC Mic.) and on specimens collected during field research on Tenerife between 29.11.–20.12.2013 and 15.10.–29.11.2014.

The polypores were determined as described in monographic books (e.g. Ryvarden and Melo 2014). The micromorphological features were evaluated in Melzer's reagent and Cotton blue using an Olympus BX 51 light microscope. The species names of the examined specimens are kept in the original designation of herbarium specimens (except *Obba* cf. *rivulosa*).

The checked specimens were compared with material collected in Europe (H, O, PRM, UPL) and they have been deposited in University of La Laguna, Tenerife (TFC Mic.) and duplicates (dupl.) in Mycological herbarium of Department of Biology, Geoscience and Environmental Education, University of West Bohemia (abbreviated UPL). For the abbreviations of herbaria, see Thiers (2023) (continuously updated). All the authors' names are abbreviated according to Index Fungorum (http://www.indexfungorum.org/Names/AuthorsOfFungalNames.asp).

Some specimens' determinations were confirmed by DNA sequences, which were deposited into GenBank (https://www.ncbi.nlm.nih.gov/genbank/). DNA work was based on previous publications (e.g. Vlasák and Kout 2011), the ITS region was amplified and sequenced with ITS5 and ITS4 primers (White et al. 1990).

Results and Discussion

Twenty species of the polypores were reviewed from the Canary Islands and new distribution data are pre-

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sented. Four species: *Antrodiella fissiliformis* (Pilát) Gilb. & Ryvarden, *Diplomitoporus flavescens* (Bres.) Domański, *Haploporus odorus* (Sommerf.) Bondartsev & Singer and *Skeletocutis albocremea* A. David are proposed for exclusion from the Canary Islands checklist. All of them belong to the more northern species (Ryvarden and Melo 2014).

Seven species are confirmed for the first time: *Ceriporiopsis consobrina* (Bres.) Ryvarden, *Ceriporiopsis pseudogilvescens* (Pilát) Niemelä & Kinnunen, *Gloeophyllum* cf. *abietinum* (Bull.) P. Karst., *Gloeophyllum trabeum* (Pers.) Murrill, *Perenniporia meridionalis* Decock & Stalpers, *Porodaedalea* Murrill sp., and *Sidera vulgaris* (Fr.) Miettinen. *Gloeophyllum* P. Karst. and *Porodaedalea* are reported as new genera for the Canary Islands.

Boletopsis Fayod sp. and *Obba rivulosa* (Berk. & M.A. Curtis) Miettinen & Rajchenb are discussed in terms of morphological variability and the presence of possible cryptic species.

The rest of the species were recorded from new localities (islands) and biotopes, in some cases, significant taxonomic remarks are added. All species are commented below.

Antrodia tenerifensis Kout & Vlasák

Specimen – **Spain**, **Canary Islands, La Gomera**: Garajonay National Park, near Vivero de Meriga, on wood of *Eucalyptus*, 850 m a. s. l., 28 Apr. 2001, Beltrán-Tejera et al. (TFC Mic. 13064, dupl. UPL).

Fig. 1 *Antrodia tenerifensis* Kout & Vlasák. Tenerife, Güímar, 22 Nov. 2014 [Kout, UPL (isotype)].

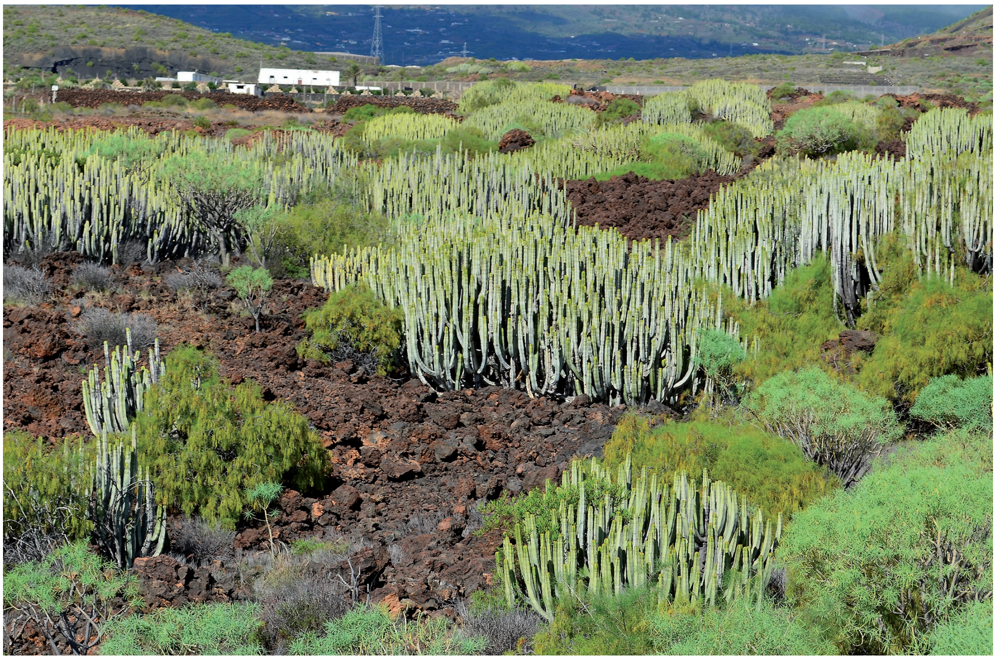

Fig. 2 *Euphorbia* scrubs. Habitat of type locality of *Antrodia tenerifensis* Kout & Vlasák in Güímar (Tenerife).

Small, resupinate polypore of sordid whitish colour, which was recently described as a new species from *Euphorbia* scrubs on Tenerife (Kout et al. 2017, Figs 1, 2). It has not been found anywhere else yet, so it could be considered an endemic species of the Canary Islands. Some sterile specimens of *Antrodia* sp. in the TFC Mic. herbarium with similar morphology indicate however wider distribution within the Canary Islands. TFC Mic. 13064 (dupl. UPL) from La Gomera (Garajonay National Park) with fayal brezal biotope expands the distribution area of *A. tenerifensis* to next island and new biotope, in my opinion. *Antrodia tenerifensis* has been confirmed as a core species of the genus *Antrodia* in current phylogeny of the brown-rot fungi (Liu et al. 2022).

Antrodiella fissiliformis (Pilát) Gilb. & Ryvarden

Specimen – **Spain, Canary Islands, La Palma**: Caldera de Taburiente National Park, slope of Pico Bejenado, on the decayed wood of *Pinus canariensis*, 1325 m a. s. l., 06 Nov. 1999, Beltrán-Tejera et al. (TFC Mic. 9280, dupl. UPL).

Additional specimen examined – Antrodiella mentschulensis (Pilát ex Pilát) Ryvarden. **Czech Republic, Plzeň Region**: Chynínské buky Nature Reservation, on dead lying hardwood, approx. 760 m a. s. l., 05 Oct. 2014, J. Kout (TFC Mic. 24915).

TFC Mic. 9280 is a semipileate thin polypore with a whitish, hirsute pileus surface, not much reminiscent of more massive fruitbodies of *A. fissiliformis*. The record from *Pinus* [Beltrán-Tejera (ed.) 2004] seems to be improbable, too, because *A. fissiliformis* is a hardwood species (Ryvarden and Melo 2014).

The microscopical revision of TFC Mic. 9280 confirmed it as *Skeletocutis* sp., because of typical, tiny, allantoid spores and specific incrustations on hyphae. Most likely, it is *Skeletocutis amorpha* (Fr.) Kotl. & Pouzar collected recently also in Teide National Park (Beltrán-Tejera et al. 2019). Accordingly, *Antrodiella fissiliformis* is proposed to be removed from the Canary Islands mycobiota.

Boletopsis Fayod sp.

Specimen – **Spain, Canary Islands, Tenerife**: above La Esperanza, Candelaria, Lomo Colorado, on the ground in humid pine woodland (*Pinus canariensis*), 1431 m a. s. l., 31 Oct. 2014, J. Kout et al. (UPL).

Additional specimen examined – **Slovakia,** Žilina Region: Liptovský Mikuláš Dist., under hill Hybica, on the ground under *Picea abies*, about 960 m a. s. l., 02 Oct. 2016, L. Hejl (UPL).

The species of *Boletopsis* Fayod has been mentioned from the Canary Islands as *Boletopsis subsquamosa* (L.) Kotl. & Pouzar for a long time. This name, however, is considered a synonym of *Boletopsis leucomellaena* Donk, and so was the species included in formal checklist (del Arco Aguilar et al. 2010). *Boletopsis leucomellaena* is known as a species from spruce forests (Niemelä and Saarenoksa 1989), but all records from the Canary Islands are from the pine forests (Ryvarden 1976; González Luis and Beltrán-Tejera 1987; Bañares Baudet 1988). Nevertheless, European pine forest species *Boletopsis grisea* (Peck) Bondartsev & Singer (Ryvarden and Gilbertson 1993) differs in several principal features from the checked Canary specimen, which does not correspond

Fig. 3 *Ceriporia bresadolae* (Bourdot & Galzin) Donk. Tenerife, Chio, 24 Oct. 2014 [Kout, UPL]. Comparison of the same part of the fruitbody in fresh (top) and after several years in the herbarium (bottom).

to any known species (Zhou et al. 2022). The occurrence under an endemic tree *P. canariensis* may indicate an undescribed cryptic species of *Boletopsis,* and the work on this question is in progress.

Ceriporia bresadolae (Bourdot & Galzin) Donk

Specimens – **Spain, Canary Islands, La Palma**: Caldera de Taburiente National Park, Barranco Verduras, on the bark, 920 m a. s. l., 30 Apr. 2000, Beltrán-Tejera et al. (TFC Mic. 10324, dupl. UPL); ibid., Lomo de Tacote, on pine wood without bark, 1059 m a. s. l., 26 Jan. 2001, Muñoz & Rebolé (TFC Mic. 12345, dupl. UPL). **Canary Islands, Tenerife**: Teide National Park, base of Pico Cabras, on wood of *Pinus canariensis* without bark, 1688 m a. s. l., 04 Apr. 2009 Beltrán-Tejera et al. (TFC Mic. 21922, dupl. UPL); Chio, on the fallen twigs of *Pinus canariensis* with and without bark, approx. 620 m a. s. l., 24 Oct. 2014, J. Kout et al. (UPL, Figs 3, 4).

Ceriporia bresadolae occurs mainly in the northern hemisphere on conifer substrates. It belongs to the *Ceriporia purpurea* (Fr.) Donk group and it has been not distinguished from *C. purpurea* for long time. The main distinguishing features are the larger pores of *C. bresadolae* against *C. purpurea* and its conifer ecology (Spirin et al. 2016). The whitish margin of fruitbody in *C. bresadolae* is also considered an important feature, which, however, becomes reddish in some specimens in herbarium. The older Canary Islands collections of *Ceriporia purpurea* from pines (Beltrán-Tejera et al. 2019) probably represent *C. bresadolae*. *Meruliopsis taxicola* (Pers.) Bondartsev is also similar, but can be well distinguished by careful microscopical examination (Ryvarden and Melo 2014).

C. bresadolae has been confirmed at Gran Canaria and the records from the Canary Islands are the southernmost in species distribution (Spirin et al. 2016).

Ceriporia spissa (Schwein. ex Fr.) Rajchenb.

This species has been regularly reported from laurel forests on the Canary Islands (e.g. Rodríguez-Armas and Beltrán-Tejera 1995; Rodríguez-Armas et al. 2003; Beltrán-Tejera et al. 2006). However, it was proven that *C. spissa* is North American species and bright orange *Ceriporia* Donk from laurel forests on the Canary Islands is a newly described taxon *Ceriporia triumphalis* Spirin & Kout (Spirin et al. 2016, Figs 5, 6). Its distribution clearly follows biotope of humid evergreen laurel forest where the species inhabits dead wood of hardwoods (Rodríguez-Armas and Beltrán-Tejera 1995).

Additional specimens examined – **Spain, Canary Islands, La Gomera**: Garajonay National Park, 900 m a. s. l., 13 Feb. 2000, Beltrán-Tejera & Rodríguez-Armas (TFC Mic. 9406, dupl. UPL); ibid., 790 m a. s. l., 11 Nov. 2000, Beltrán-Tejera & González Martín (TFC Mic. 12791, dupl. UPL); ibid., 800 m a. s. l., 12 Feb. 2001, Beltrán-Tejera et al. (TFC Mic. 12698, dupl. UPL); ibid., 960 m a. s. l., 14 Feb. 2001, Beltrán-Tejera et al. (TFC Mic. 13280, dupl. UPL); ibid., 925 m a. s. l., 29 Nov. 2002, Beltrán-Tejera et al. (TFC Mic. 14019, dupl. UPL).

Ceriporiopsis consobrina (Bres.) Ryvarden

Specimens – **Spain, Canary Islands, La Gomera**: Garajonay National Park (eastern border), 745 m a. s. l., 31 Feb. 2002, Beltrán-Tejera et al. (TFC Mic. 11833, dupl. UPL, Fig. 7); ibid., 950 m a. s. l., 09 Dec. 2000, Beltrán-Tejera et al. (TFC Mic. 12863, dupl. UPL).

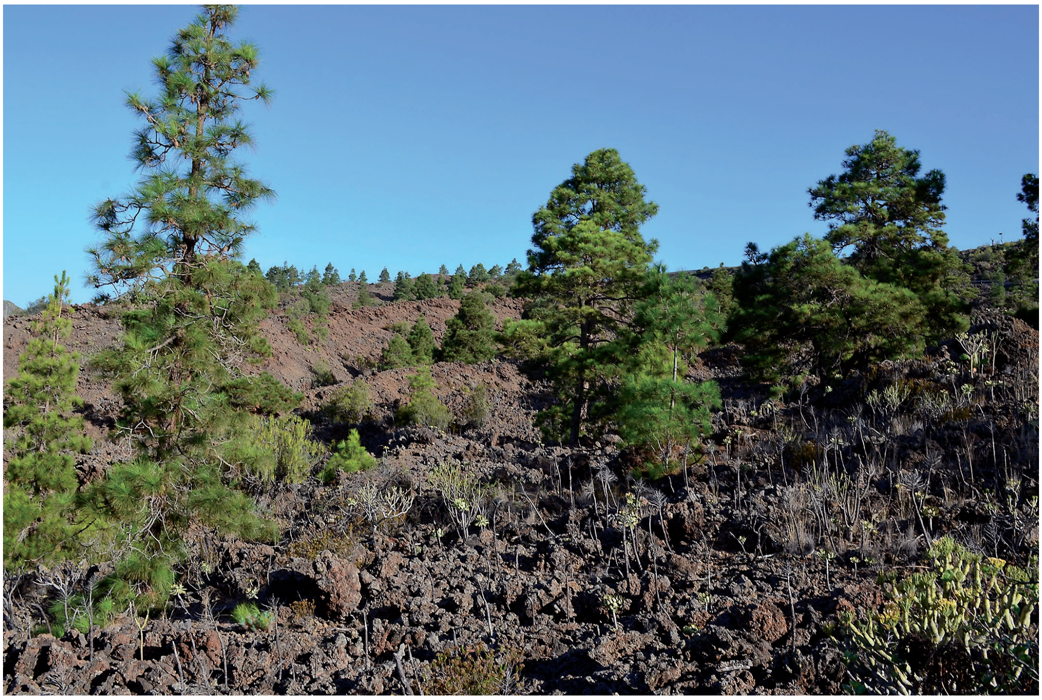

Fig. 4 Biotop of *Ceriporia bresadolae* (Bourdot & Galzin) Donk with *Pinus canariensis* in Chio (Tenerife).

Fig. 5 *Ceriporia triumphalis* Spirin & Kout, young fruitbody in situ. Tenerife, Anaga Mts., near Pico del Inglés, 16 Nov. 2014 [Kout, UPL].

Fig. 6 *Ceriporia triumphalis* Spirin & Kout, herbarium specimen. Tenerife, Anaga Mts., 14 Dec. 2013 [Kout, UPL (isotype)].

Additional specimen examined – **Czech Republic, Plzeň Region**: Plzeň-City Dist., Chotíkovský forest, on branch of shrubby willow, 27 Nov. 2016, J. Kout (PRM 958938).

A resupinate species with whitish poroid to irpicoid hymenophore that is known mainly from *Salix* wood in Europe (Ryvarden and Melo 2014). Generally, the European records are scattered, and *C. consobrina* is considered a rare species, which may be due to unremarkable external appearance. It seems to be rare also in humid laurisilva on the Canary Islands, and it is for the first time recorded there. *C. consobrina* collections have to be inspected under microscope because there are more species that may have a torn hymenophore, e.g. *Irpex lacteus* (Fr.) Fr. or *Schizopora paradoxa* (Schrad.) Donk reported from the Canary Islands (Arechavaleta et al. 2010).

Ceriporiopsis pseudogilvescens **(Pilát) Niemelä & Kinnunen**

Specimen – **Spain, Canary Islands, Tenerife**: Teide National Park, near Centro de visitantes de El Portillo, on dead wood of *Spartocytisus supranubius*, 05 Dec. 2013, J. Kout et al. (TFC Mic. 24868 – newly assigned number, dupl. UPL, GenBank OQ311334).

Beltrán-Tejera et al. (2019) mentioned this specimen as *Ceriporiopsis resinascens* (Romell) Domański. The distinction between *C. pseudogilvescens* and similar *C. resi-*

Fig. 7 *Ceriporiopsis consobrina* (Bres.) Ryvarden. La Gomera, Garajonay National Park, Feb. 2002 [Beltrán-Tejera & al., TFC Mic. 11833].

nascens is not easy without sequencing (Tomšovský et al. 2010). Despite of the considerable similarity both mentioned species, their separated species identity has been repeatedly confirmed by molecular analysis (most recently in Chen et al. 2021). Therefore, this specimen from Teide National Park has been confirmed by ITS sequence with more than 99% identity with *C. pseudogilvescens* from China in GenBank (KU509523, MZ637069). The occurrence of *C. resinascens* on the Canary Islands is thus unclear.

***Diplomitoporus flavescens* (Bres.) Domański**

Specimen – **Spain, Canary Islands, La Palma**: Fuencaliente, by Montaña de los Faros, on burnt wood of *Pinus canariensis*, 1300 m a. s. l., 14 Feb. 1994, Beltrán-Tejera et al. (TFC Mic. 6816, dupl. UPL).

Additional specimens examined – **Czech Republic, Plzeň Region**: Petrovka Nature Reserve, on the wood of *Pinus sylvestris*, approx. 360 m a. s. l., 01 Dec. 2014, J. Kout (TFC Mic. 24922, 24923).

TFC Mic. 6816 is a resupinate specimen on burnt wood (Beltrán-Tejera et al. 2003) with sordid whitish colour mixed up by brownish spots. The unburnt wood is decayed by brown rot. TFC Mic. 6816 looks differently from yellowish polypore *D. flavescens*, which grows often on recently dead pines often covered by bark yet (Kout and Vlasák 2011). There are probably no records of *D. flavescens* from burnt wood (Vampola and Charvátová 2021) and *Diplomitoporus* Domański creates white rot (Domański 1970). The microscopical revision identified TFC Mic. 6816 as *Antrodia sinuosa* (Fr.) P. Karst., a common species in pine forests on the Canary Islands, often recorded there on burnt wood [Beltrán-Tejera (ed.) 2004]. *Diplomitoporus flavescens* remains unknown on the Canary Islands archipelagos and probably is not occurring there, because its distribution area is limited to Europe (Ryvarden and Gilbertson 1993).

***Gloeophyllum* cf. *abietinum* (Bull.) P. Karst.**

Specimens – **Spain, Canary Islands, La Gomera**: Garajonay National Park, Las Carboneras, 702 m a. s. l., 31 Jan. 2002, Beltrán-Tejera et al. (TFC Mic. 11933, TFC Mic. 11934, both dupl. UPL).

Both checked specimens of *Gloeophyllum* aff. *abietinum* show lamellate hymenophore and dark brown fruitbodies. Nevertheless, no spores were detected and characters of cystidia do not correspond to *Gloeophyllum abietinum*. The observed cystidia were dark brown, thick-walled, occasionally with bulbous apical part. Tropical *Gloeophyllum striatum* (Fr.) Murrill, the other lamellate species, known from the close Africa (Ryvarden et al. 2022), disagrees in microscopical features, too. Exact species identification is still open. In any case, the genus *Gloeophyllum* has not been known at Canary Islands to date. There is, however, a record of *G. abietinum* from Azores on non-native *Cryptomeria* within Macaronesia (Ryvarden and Spooner 2004).

***Gloeophyllum trabeum* (Pers.) Murrill**

Specimens – **Spain, Canary Islands, La Palma**: Caldera de Taburiente National Park, Roque del Huso, 900 m a. s. l., 29 Apr. 2000, Beltrán-Tejera et al. (TFC Mic. 10173, dupl. UPL). **Canary Islands, Tenerife**: San Miguel, man-made wood of *Pinus radiata*, Dec. 2014, Beltrán-Tejera (UPL, Fig. 8, dupl. TFC Mic., GenBank OQ311335).

Gloeophyllum trabeum has never been recorded from the Canary Islands to date. The checked specimens are ochre brown with poroid hymenophore and species

Fig. 8 *Gloeophyllum trabeum* (Pers.) Murrill. Tenerife, San Miguel, Dec. 2014 [Beltrán Tejera, UPL].

identity of specimen from Tenerife was confirmed by sequencing.

***Hapalopilus rutilans* (Pers.) Murrill**

Common European polypore *Hapalopilus rutilans* was mentioned on La Palma and Tenerife (Arechavaleta et al. 2010). It seems to me that *Hapalopilus* P. Karst. is very rare on the Canary Islands, as I collected it only once in several months there, and the collection has been sequenced as very similar *Hapalopilus eupatorii* (P. Karst.) Spirin & Miettinen (Zíbarová et al. 2021). Then the spreading of *H. rutilans* on the Canary Islands is uncertain.

***Haploporus odorus* (Sommerf.) Bondartsev & Singer**

Specimen – **Spain, Canary Islands, La Palma**: Caldera de Taburiente National Park, on burnt wood of *Pinus canariensis*, 1325 m a. s. l., 06 Nov. 1999, Beltrán-Tejera et al. (TFC Mic. 9285, dupl. UPL).

Additional specimen examined – **Finland, North Savo**: Pieksämäki, Jäppilä, on *Salix caprea*, 100 m a. s. l., 18. Jun 2004, V. Spirin (H 6006910, dupl. UPL).

Haploporus odorus in Macaronesia on *Pinus* (Beltrán-Tejera [ed.] 2004) is a biogeographical and ecological oddity, as this species is known from boreal area and mainly on willow. The collection was compared with the true *H. odorus* from Finland (H 6006910).

TFC Mic. 9285 is sterile, resupinate, spongy specimen with hyphae reminiscent of *Dichomitus* D.A. Reid. Thick-walled skeletal hyphae are abundant, richly branched with branches tapering to the end, whereas rarely branched skeletal hyphae in *H. odorus* have no tapering ends. This clear difference excludes *H. odorus,* and the species should be removed from the Canary Islands checklist. Because of pine substrate and character of skeletal hyphae, it would be possible to assign TFC Mic. 9285 to *Dichomitus squalens* (P. Karst.) D.A. Reid, I believe.

***Obba* cf. *rivulosa* (Berk. & M.A. Curtis) Miettinen & Rajchenb.**

Specimen – **Spain, Canary Islands, La Palma**: Fuencaliente, Montaña de los Faros, on burnt wood of *Pinus canariensis*, 1300 m a. s. l., 14 Feb. 1994, Beltrán-Tejera et al. (TFC Mic. 6821, dupl. UPL, Figs 9, 10).

Additional specimen examined – **Italy, Emilia-Romagna**: Forlì-Cesena, Sasso Fratino Natural Reserve, *Abies*, 850 m a. s. l., 25 Sept. 2007, Bernicchia (8381, O).

Obba rivulosa (Berk. & M.A. Curtis) Miettinen & Rajchenb, previously as *Physisporinus rivulosus* (Berk. & M.A. Curtis) Ryvarden, belongs to the rare species (Ryvarden and Melo 2014). Checked specimen TFC Mic. 6821 corresponds to *P. rivulosus* by subglobose spores, pointed cystidiols and resinous layer in fruitbody. There are slight differences in dimensions of pores (2–4/mm), basidia (25–30 × 7–9 μm) and spores (5.5–7 × 4.5–5 μm). All of them are bigger than those of *P. rivulosus* (Kotiranta 1985). Later descriptions (Ren et al. 2017) also do not exactly correspond to this Canary Island specimen. It may be another cryptic species, and more collections are needed to be sure about its identity.

***Perenniporia fulviseda* (Bres.) Dhanda**

Specimen – **Spain, Canary Islands, Tenerife**: Anaga, between Pico Inglés and Ermita Cruz del Carmen, north slope, in the hole after the coup of *Erica*, approx. 970 m a. s. l., 16 Nov. 2014, J. Kout (UPL).

Remarkable species with rhizomorphs and small spores, regularly under 5 μm in length, which is less than in most European species of *Perenniporia* Murrill (De-

Fig. 9 *Obba* cf. *rivulosa* (Berk. & M.A. Curtis) Miettinen & Rajchenb. La Palma, Fuencaliente, 14 Feb. 1994 [Beltrán-Tejera & al., UPL].

Fig. 10 Vertical section through fruitbody of *Obba* cf. *rivulosa* (Berk. & M.A. Curtis) Miettinen & Rajchenb. with visible resinous layer [Beltrán-Tejera & al., UPL].

cock and Stalpers 2006). *Perenniporia fulviseda* is missing in the checklist from 2010 (Arechavaleta et al. 2010), but it has been mentioned from Tenerife, Anaga Mountains, by Decock and Stalpers (2006). It is sparsely distributed there as shows only one record from my field work and no records from herbarium of the University of La Laguna, Tenerife. Not mentioned also in complex study of aphyllophoroid fungi from laurel forests on the Canary Islands (Rodríguez-Armas and Beltrán-Tejera 1995).

Perenniporia meridionalis Decock & Stalpers

Specimens – **Spain, Canary Islands, Fuerteventura**: Jandía Natural Rark, on *Asteriscus sericeus*, 770 m a. s. l., 05 Dec. 2007, Beltrán Tejera et al. (TFC Mic. 18733). **Canary Islands, La Palma**: Caldera de Taburiente National Park, 1200 m a. s. l., 25 Nov. 2000, Beltrán-Tejera et al. (TFC Mic); ibid., on *Ficus carica*, 1150 m a. s. l., 20 Oct. 2001, Beltrán-Tejera et al. (TFC Mic. 11214). **Canary Islands, Tenerife**: Chio, on dead wood with bark, 24 Oct. 2014, J. Kout et al. (UPL).

Specimens of *Perenniporia meridionalis* may be hidden under *Perenniporia medulla-panis* (Jacq.) Donk or *Perenniporia tenuis* (Schwein.) Ryvarden in herbaria because the species has been separated and described as new only in 2006 (Decock and Stalpers 2006). *Perenniporia meridionalis* distribution is limited to warmer parts of Europe where it seems to be not rare on various deciduous trees, shrubs and even conifers (Vampola and Charvátová 2021).

Porodaedalea sp.

Specimen – **Spain, Canary Islands, La Palma**: Caldera de Taburiente National Park, Refugio de la Punta de Los Roques, on living pine, 2069 m a. s. l., 31 Mar. 2001, Gonzáles Martín et al. (TFC Mic. 12393, dupl. UPL, Fig. 11).

Porodaedalea Murrill is a genus of hymenochaetoid polypores separated from *Phellinus* Quél. that includes species with daedaleoid-poroid hymenophore and growing on conifers (Murrill 1905). Important features for the species resolution are number of pores per mm and host tree (Wu et al. 2019).

Canary Islands specimen of *Porodaedalea* was collected on pine; however, the morphological features do not correspond to the common *Porodaedalea pini* (Brot.) Murrill. The specimen is small, and it looks like *Porodaedalea cedrina* Pilát ex Tomšovský & Kout (Bernicchia and Gorjón 2020). Moreover, the altitude of the *Porodaedalea* collection on the Canary Islands is untypical for *P. pini*, which is a species from lower elevations (Tomšovský 2002).

Fig. 11 *Porodaedalea* Murrill sp. La Palma, Caldera de Taburiente National Park, 31 Mar. 2001 [Gonzáles Martín & al., UPL].

Unfortunately, the sequencing was not successful. It can be concluded that *Porodaedalea* is recorded on the Canary Islands for the first time. Exact species identification needs specimens in a fresh condition for the isolation of DNA.

***Postia caesia* (Schrad.) P. Karst. s.l.**

Specimen – **Spain, Canary Islands, Tenerife**: Anaga, Cuadra de don Benito, approx. 870 m a. s. l., 14 Dec. 2013, J. Kout et al. (UPL).

Well-known bluish polypore *P. caesia* s.l. creates a complex of about 25 closely related species that are difficult to distinguish (Miettinen et al. 2018). There are more specimens from this species complex in the TFC herbarium (often under genus *Oligoporus* Bref.), but their detailed revision has not been done yet. New findings are expected, if only due to ecological irregularities of some specimens, e.g. *Oligoporus subcaesius* (A. David) Ryvarden & Gilb. from *Pinus canariensis* (Rodríguez-Armas et al. 2003).

***Sidera vulgaris* (Fr.) Miettinen**

Specimens – **Spain, Canary Islands, La Gomera**: Garajonay National Park, near Cruce de los Roques, 1150 m a. s. l., 12 Feb. 2000, Beltrán-Tejera et al. (TFC Mic. 101015, dupl. UPL); ibid., near the border of national park, on the bark of *Myrica faya*, 1000 m a. s. l., 09 Dec. 2000, Beltrán-Tejera et al. (TFC Mic. 13030, dupl. UPL); ibid., nearby Los Llanos de Crispín, on the wood of pine, 1060 m a. s. l., 19 Jan. 2001, Beltrán-Tejera et al. (TFC Mic. 13450, dupl. UPL); ibid., near Pico Garajonay, on wood of *Erica* without bark, 1090 m a. s. l., 14 Feb. 2001, Beltrán-Tejera et al. (TFC Mic. 13734, dupl. UPL). **Canary Islands, Tenerife**: Anaga, M. Chamuscada, approx. 800 m a. s. l., 30 Nov. 2013, J. Kout et al. (UPL); ibid., Casa Forestal, on bark and wood of *Myrica faya*, approx. 930 m a. s. l., 02 Nov. 2014, J. Kout et al. (UPL, Fig. 12); ibid., Pico Inglés, on decayed wood without bark, approx. 960 m a. s. l., 07 Nov. 2014, J. Kout (UPL).

White resupinate polypore with lunate basidiospores and stellate clusters of crystals on hyphae (Niemelä and Dai 1997) that has not yet been reported from the Canary Islands. It was hidden under the name of related species *Skeletocutis lenis* (P. Karst.) Niemelä in literature and herbaria (Rodríguez-Armas and Beltrán-Tejera 1995). However, specimens of *Sidera* Miettinen & K.H. Larss. can also be found under other names as *Schizopora* Velen. or *Skeletocutis*.

Sidera vulgaris seems to be a common species in laurisilva and fayal brezal biotope, contrary to *Sidera lenis* (P. Karst.) Miettinen (syn. *Skeletocutis lenis*), whose occurrence in Macaronesia is uncertain, as it is considered a boreal species. *S. vulgaris* was also recorded on the Azores islands in Macaronesia yet (Ryvarden and Spooner 2004).

***Skeletocutis albocremea* A. David**

Specimen – **Spain, Canary Islands, Tenerife**: Pinar de La Esperanza, on *Pinus canariensis*, 14 Apr. 1997, J. Mosquera (TFC Mic. 8045, dupl. UPL).

This resupinate specimen is up to 4 mm thick which is hardly corresponding to the description where "less than 1mm thick" is stressed (Spirin 2005). The dimensions of spores do not agree too, and all features fit better to the *Skeletocutis stellae* (Pilát) Jean Keller. There is probably no other record of *S. albocremea* from the Canary Islands and so it should be removed from the checklist of fungi of the Canary Islands.

Fig. 12 *Sidera vulgaris* (Fr.) Miettinen. Tenerife, Casa Forestal, 2 Nov. 2014 [J. Kout & al., UPL].

Skeletocutis nivea **(Jungh.) R. Keller**

Specimens – **Spain, Canary Islands, La Gomera**: Garajonay National Park, 12 Feb. 2001, E. Beltrán-Tejera et al. (TFC Mic. 12714); ibid. 14 Feb. 2001, E. Beltrán-Tejera et al. (TFC Mic. 13603). **Canary Islands, Tenerife**: Vueltas de Taganana, 28 Dec. 1971, E. Beltrán (TFC Mic. 57); Anaga, Roque Negro, 17 Dec. 2013, J. Kout et al. (TFC Mic. 24893).

Regularly occurring polypore in laurel forests. Specimens are semipileate and correspond to how this species has been interpreted in the European area. However, molecular revision is needed. A recent treatment of *S. nivea* complex concluded that *S. nivea* is a species occurring in Asia – New Zealand region and in Europe, there are four other species, which are unidentifiable from morphological and ecological features (Korhonen et al. 2018).

Polypore species of the Canary Islands are mostly the same species as those occurring in the Central Europe, but there are some surprising differences. The most interesting is the absence of some common European species there, as the above discussed *H. rutilans* or only once recorded *Fomitopsis pinicola* (Sw.) P. Karst. (TFC Mic. 6178 from burned stump of *Pinus canariensis* with uncertain identification because there are no spores in specimen. Similarly, *Fomes fomentarius* (L.) Fr. is mentioned only from Tenerife, *Daedaleopsis confragosa* (Bolton) J. Schröt. from La Palma (Arechavaleta et al. 2010), and I have never seen these species there during my fieldwork.

Unique polypores of the Canary Islands mostly come from the biotopes that are unknown or limited in Europe as laurel forest, fayal brezal or specific dry habitats (del Arco Aguilar et al. 2010). Specific and endemic plant species play also undoubtedly a role. Still, endemic species of polypores (*Antrodia tenerifensis*) are rare and their presence is not as remarkable as absence of wide-spread polypores of Europe.

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ENZYMATIC DIGESTION COMBINED WITH µFT-IR IMAGING FOR RECOVERY AND CHARACTERISATION OF POLYMER PARTICLES FROM *MYTILUS GALLOPROVINCIALIS* TISSUE

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ABSTRACT

Microplastic contamination in marine organisms requires analytical approaches capable of efficiently removing biological matrices while preserving polymer integrity. Building upon previous work identifying enzymatic digestion as a suitable treatment method, the present study applies a pancreatic enzyme protocol (Kreon®25000) to recover polymer particles from *Mytilus galloprovincialis* tissue and to evaluate particle characteristics by µFT-IR imaging. Frozen mussel tissue (2 g) was artificially spiked with reference particles of PVC, HDPE, PA, and PET and subjected to enzymatic digestion under controlled conditions. Digestion removed 99.8% of the biological matrix, allowing subsequent filtration, microscopic inspection, and spectroscopic identification. A total of 2334 particles were detected. Recovery varied among polymers, with HDPE showing the highest numerical recovery and PET the lowest. Particle size distributions differed markedly: HDPE, PA, and PVC were dominated by particles <50 µm, whereas PET particles were predominantly larger. Morphological analysis revealed irregular fragment-like shapes across all polymer types. µFT-IR imaging enabled polymer identification and spatial mapping, revealing heterogeneous particle distribution and localized clustering patterns on filter surfaces. Comparison of ATR-FTIR reference spectra with µFT-IR spectra obtained after digestion confirmed preservation of diagnostic polymer bands, indicating that enzymatic treatment did not alter polymer chemical structure. The results demonstrate that enzymatic digestion combined with µFT-IR imaging provides a reliable and polymer-preserving workflow for microplastic analysis in marine biological matrices. The findings highlight the influence of particle size and spatial distribution on recovery and detection, underscoring the importance of standardized imaging strategies for accurate quantification.

Keywords: contamination control; microplastic analysis; marine biota; size classification; spatial distribution; spectroscopic identification

Introduction

Microplastics (MPs) are synthetic polymer particles introduced into the marine environment through long-term accumulation and subsequent fragmentation of plastic materials (Barnes et al. 2009; Kalogerakis et al. 2017; Yang et al. 2021). They are typically defined as particles smaller than 5 mm and occur in various morphological forms, including fragments, fibers, films, and spheres. Their physicochemical properties vary depending on polymer type, degree of weathering, and environmental conditions (Tirkey and Upadhyay 2021; Golmohammadi et al. 2023; U.S. Environmental Protection Agency 2024). Based on their origin, microplastics are classified as primary particles manufactured at microscopic size for specific applications and secondary particles formed through mechanical, photochemical, and thermal degradation of larger plastic debris (Duis and Coors 2016; Cverenkárová et al. 2021; Song et al. 2024). MPs have been detected throughout the marine environment, including the water column, sediments, and marine biota (Van Cauwenberghe et al. 2015; Sunny et al. 2025).

Due to their filter-feeding behavior, bivalve mollusks, including *Mytilus galloprovincialis*, efficiently retain suspended particles from the surrounding environment, including microplastic fragments and fibers (Pizzurro et al. 2022, 2023; Kovačić et al. 2024). This species is widely distributed in coastal zones and exhibits limited mobility, making it a suitable bioindicator for assessing local pollution levels (Pizzurro et al. 2023; Mihailov et al. 2025). The accumulation of microplastic particles in *M. galloprovincialis* tissues provides valuable insight into the exposure of coastal ecosystems to synthetic polymers.

The analysis of microplastics in biological tissues is associated with methodological limitations, particularly at the stage of organic matter removal. Frequently used acidic and alkaline treatments may affect the surface structure and chemical composition of certain polymers, creating conditions for partial degradation or analytical losses (Pfeiffer and Fischer 2020; Di Fiore et al. 2024; Tuuri et al. 2024). The lack of harmonized protocols further limits the possibility for direct comparability between different studies (Hermsen et al. 2018; Al-Azzawi et al. 2020).

Turmanova, S., Hristov, Y., Kiryakova, D., Ivanova, E., Atanasova, P., Kolchakova, G., Ilieva, A., Mollova, E.: Enzymatic digestion combined with µFT-IR imaging for recovery and characterisation of polymer particles from *Mytilus galloprovincialis* tissue
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Enzymatic digestion has emerged as a promising alternative, allowing selective removal of biological material under controlled conditions with minimal impact on polymer structure (Mo et al. 2018; von Friesen et al. 2019; Di Fiore et al. 2024; Khan and Zaidi 2025). This approach facilitates subsequent spectroscopic identification and morphological characterization of particles (Chen et al. 2020; Morgado et al. 2021).

Previous research systematically evaluated enzymatic, acidic, and alkaline digestion protocols for the removal of biological matrices in *Mytilus galloprovincialis*, identifying enzymatic digestion as the most reliable approach for preserving polymer integrity and ensuring high recovery efficiency (Turmanova et al. 2026). Building upon these findings, the present study applies the previously validated enzymatic protocol to mussel tissue artificially spiked with reference polymer particles and performs detailed μFT-IR imaging analysis. The study focuses on polymer identification, particle morphology, size distribution, and spatial distribution on filters, thereby advancing methodological reliability in microplastic analysis of marine biota. The aim of this study was to evaluate the recovery, characterization, and spatial distribution of selected polymer particles from Mytilus galloprovincialis tissue following enzymatic digestion using μFT-IR imaging.

Materials and Methods

Materials

Black Sea mussels, *Mytilus galloprovincialis*, collected from the Burgas Bay (Black Sea, Bulgaria), were used as model biological material in the present study. Mussels were stored frozen at −20 °C until analysis. Reference polymer particles were used to simulate microplastic contamination: polyvinyl chloride (PVC, 100–200 μm), high-density polyethylene (HDPE, 100–500 μm), polyamide (PA, 100–200 μm), and polyethylene terephthalate (PET, 100–250 μm). Enzymatic digestion was performed using Kreon®25000 (Abbott Laboratories GmbH, Germany), a pancreatic enzyme containing lipase (25,000 Ph. Eur units), amylase (18,000 Ph. Eur units), and protease (1,000 Ph. Eur units).

Sample preparation and polymer spiking

Frozen *Mytilus galloprovincialis* tissue (2.0 g) was used for the experiment. Samples were artificially spiked with known masses of microplastic particles representing four polymer types: polyvinyl chloride (PVC), high-density polyethylene (HDPE), polyamide (PA), and polyethylene terephthalate (PET). Particle size ranges were 100–200 μm for PVC and PA, 100–500 μm for HDPE, and 100–250 μm for PET. Separate samples were prepared for each polymer type, with only one polymer introduced per sample to allow polymer-specific recovery assessment and to prevent spectral overlap. A procedural control without added polymers was processed in parallel to verify background cleanliness. All handling procedures were conducted under contamination-controlled conditions, and samples were covered when not in use.

Enzymatic digestion using Kreon®25000

Enzymatic digestion was performed using Kreon® 25000 (Abbott Laboratories GmbH, Germany). A total of 0.125 g of Kreon®25000 was dissolved in 20 mL of 1 M Tris-HCl buffer solution (pH 8.0), corresponding to the optimal activity range of pancreatic enzymes (Berdutina et al. 2000; von Friesen et al. 2019). The spiked mussel samples were added to the enzyme solution and incubated at 37.8 °C for 2 hours under constant stirring (300 rpm). These conditions were selected based on a previously optimized enzymatic protocol validated for *Mytilus galloprovincialis,* which demonstrated efficient biological matrix removal (99.8%) (Turmanova et al. 2026).

Filtration

Following enzymatic digestion, the resulting solutions were vacuum-filtered through metal filters with a pore size of 5 μm. Filters were rinsed with ultrapure water to remove residual reagents, dried at 40 °C, and stored in sealed containers until further analysis.

Microscopic inspection of filters

Filters were examined using a BSCOPE BS.1153-EPLH microscope (Euromex, Netherlands) to document representative polymer particles retained on the filters after digestion. Micrographs were captured at 40× magnification to illustrate particle morphology and surface features. Optical microscopy was used for particle visualization, while polymer identification was confirmed by μFT-IR analysis.

Identification of polymer particles by μFT-IR imaging

Prior to μFT-IR analysis, particles retained on the metal filters were carefully transferred onto Anodisc membrane filters (0.2 μm) to improve infrared transmission and enhance spectral quality during imaging. Polymer particles were analyzed using a μFT-IR imaging microscope (LUMOS II, Bruker Optik GmbH, Germany). The instrument was equipped with a focal plane array (FPA) detector (32 × 32 pixels) providing a spatial resolution of 5 μm. Spectral imaging was performed in the range of 1000–4000 cm^{-1} with a spectral resolution of 5 cm^{-1}. Measurements were conducted in reflection mode, and background spectra were collected prior to each analysis. The effective spectral region used for polymer identification ranged approximately from 1200 to 3500 cm^{-1}.

μFT-IR data were processed using Purency Microplastics Finder software for automated particle detection and polymer identification. Particles were classified as polymer particles when the spectral match met the acceptance criteria defined in the reference spectral libraries. Only particles with an acceptable spectral match score above 70% were considered confirmed microplastics. The spatial coordinates of identified particles were recorded during imaging and used for subsequent analysis of particle morphology and spatial distribution on the filters. The μFT-IR imaging system also enabled visualization of the overall filter surface, allowing assessment of residual biological material and particle retention after digestion.

ATR-FTIR Spectroscopy of polymer particles

Reference polymer particles (HDPE, PA, PVC, and PET) were analyzed using an ALPHA II FT-IR spectrometer (Bruker Optik GmbH, Germany) equipped with a Platinum ATR module and a diamond crystal. Spectra were recorded in the range of 400–4000 cm^{-1} at a spectral resolution of 4 cm^{-1} and averaged over 32 scans to improve the signal-to-noise ratio. The resulting spectra served as reference profiles for comparison with μFT-IR spectra obtained from filter-retained particles after enzymatic digestion. Each polymer type was analyzed separately prior to use as reference material and re-analyzed after enzymatic digestion under the same ATR-FTIR conditions to verify spectral consistency and confirm that the digestion procedure did not alter polymer chemical structure.

Particle characterization and data analysis

Particle characterization was performed based on μFT-IR imaging data. Particle size was determined as the equivalent circular diameter calculated from the projected particle area. Particles were grouped into size classes (< 50 μm, 50–100 μm, 100–300 μm, and > 300 μm) to evaluate size distribution.

Particle morphology was assessed using shape descriptors derived from imaging data. Particles were classified as irregular fragments based on aspect ratio and particle outline. Only particles larger than 5 μm were included in the analysis.

The spatial coordinates obtained from μFT-IR imaging were used to generate spatial distribution and density maps of polymer particles on the filters. Descriptive statistical analysis (particle counts, size distribution, and percentage distribution by size class) and graphical representations were performed using Microsoft Excel (Microsoft Corp., USA).

Quality assurance and contamination control

All glassware and tools were thoroughly rinsed with filtered ultrapure water prior to use. Sample preparation

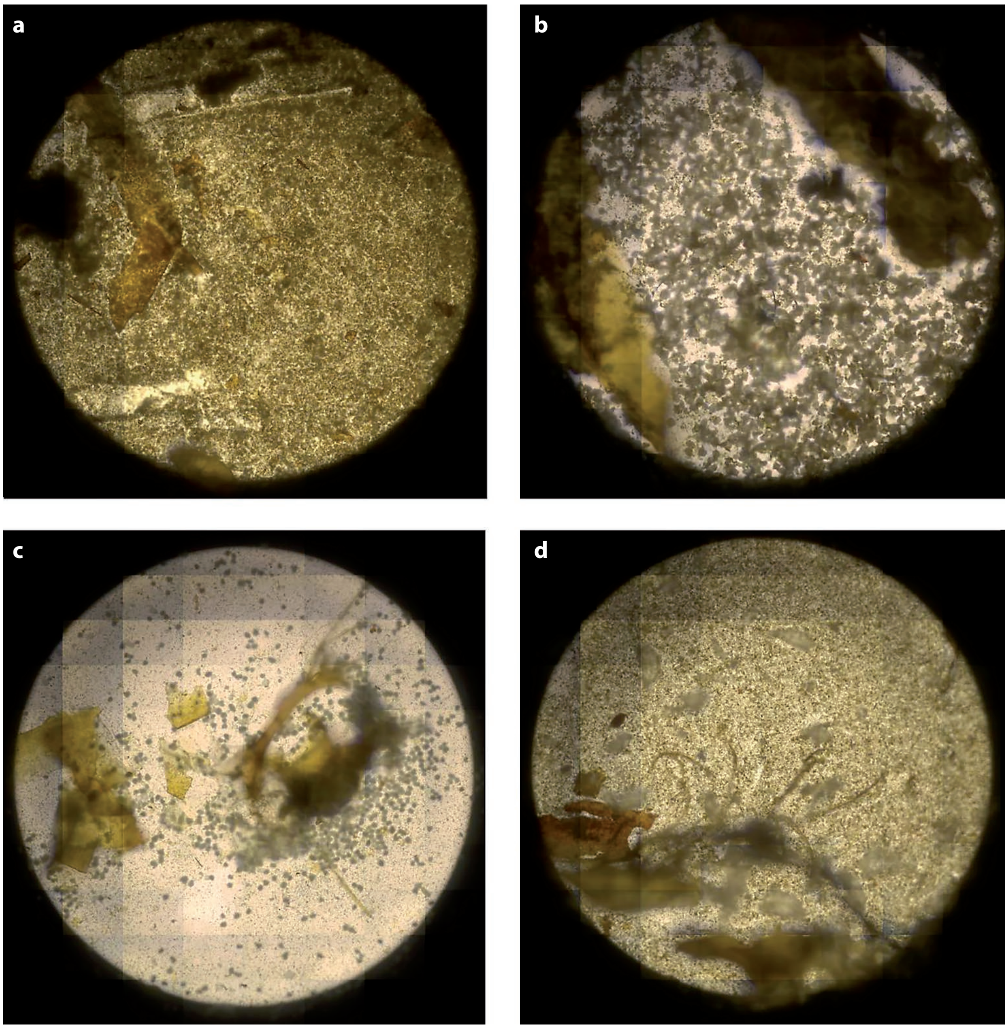

Fig. 1 Representative filter images after enzymatic digestion showing (a) HDPE, (b) PA, (c) PVC and (d) PET.

Table 1 Recovery and size characteristics of detected polymers.

Polymer	Added mass (mg)	Detected particles (n)	Particles per mg	Median size (μm)	Size range (μm)
HDPE	12.38	1107	89	15.4	0.9–64.8
PA	15.47	827	53	27.0	1.6–110.4
PVC	17.47	350	20	26.9	2.4–146.6
PET	8.55	50	5	70.7	8.1–275.5

and filtration were conducted in a clean working environment, and samples were covered whenever possible to minimize contamination.

Results and Discussion

Recovery and detection of polymer particles

Polymer particles representing HDPE, PA, PVC, and PET were successfully detected, and identified from *Mytilus galloprovincialis* mussel tissue following enzymatic digestion using the previously validated Kreon®25000 protocol. The digestion effectively removed the biological matrix while preserving polymer integrity, enabling subsequent microscopic and μFT-IR identification. μFT-IR imaging of the filters confirmed clear retention of polymer particles on the filter surface, with only minimal residual biological material remaining (Fig. 1).

A total of 2334 particles were detected across all polymer types (Table 1). Recovery efficiency differed among polymers, reflecting differences in particle size distribution and material properties. HDPE exhibited the highest number of detected particles (1107), followed by PA (827) and PVC (350), while PET showed substantially lower recovery (50). When normalized to the added polymer mass, recovery ranged from 5 to 89 particles per mg, with HDPE showing the highest recovery efficiency and PET the lowest. The lower numerical recovery of PET is consistent with its larger particle size distribution and higher density, relative to polyolefins (Faszczewska et al. 2026), which can influence particle retention and detectability during filtration and imaging.

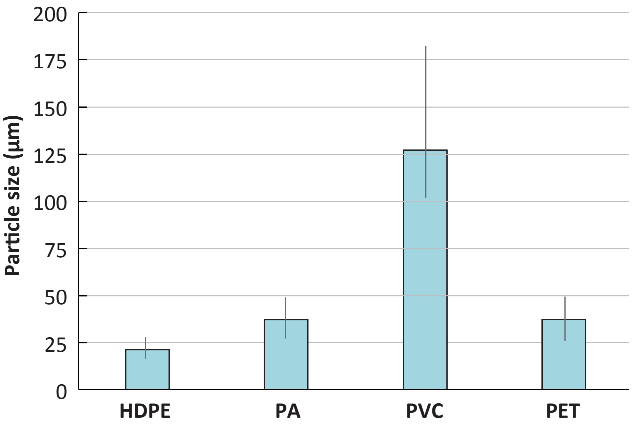

Fig. 2 Particle size distribution of HDPE, PA, PVC and PET particles detected after enzymatic digestion.

High recovery rates observed for HDPE and PA support the suitability of enzymatic digestion for efficient removal of organic tissue while preserving polymer particles. This observation is consistent with previous studies reporting that enzymatic digestion effectively removes biological material and minimizes polymer alteration compared with strong chemical treatments (Karami et al. 2017; Prata et al. 2019). The polymer-dependent differences in recovery are consistent with previous studies indicating that particle size, density, and polymer properties influence microplastic detection and recovery in biological matrices (Cole et al. 2014; Dehaut et al. 2016).

Particle size distribution

Particle size analysis revealed pronounced differences among polymer types (Table 1 and Fig. 2). Median particle sizes ranged from 15.4 μm for HDPE to 70.7 μm for PET, indicating substantial variability in particle dimensions after digestion and filtration.

HDPE particles were predominantly small, with 96% measuring < 50 μm, while only 4% fell within the 50–100 μm size class. Similarly, PVC and PA particles were largely within the smallest size fraction, with 69% and 71% < 50 μm, respectively. In contrast, PET exhibited a markedly different size distribution, with only 14% < 50 μm, while 46% and 40% of particles occurred in the 50–100 μm and 100–300 μm ranges, respectively (Table 2).

The predominance of smaller particles among HDPE, PA, and PVC reflects both the initial particle size characteristics and possible size reduction during sample handling and processing. Smaller particles are also more readily retained on filters and detected by μFT-IR imaging, contributing to their higher representation in the dataset.

Conversely, the larger size distribution observed for PET is consistent with its higher mechanical rigidity and resistance to fragmentation reported for polyester polymers (Andrady 2011). The lower proportion of small PET particles may also contribute to its reduced recovery ef-

Table 2 Percentage distribution of detected particles by size class.

Size class (μm)	HDPE	PA	PVC	PET
< 50	96%	71%	69%	14%
50–100	4%	23%	21%	46%
100–300	0%	6%	10%	40%
> 300	0%	0%	0%	0%

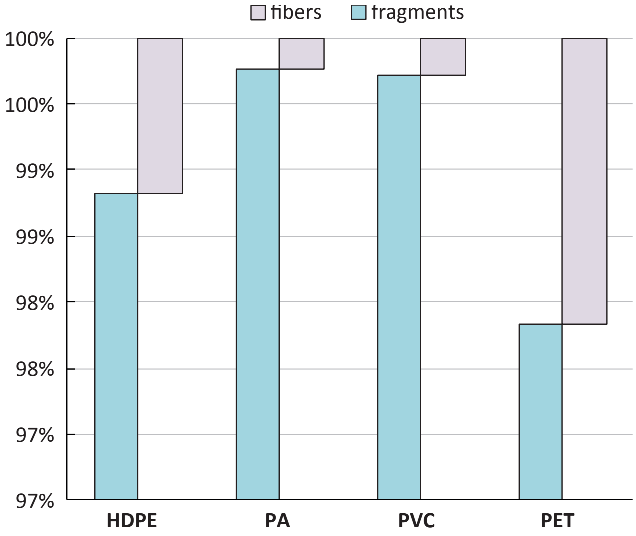

Fig. 3 Morphological distribution of HDPE, PA, PVC and PET particles detected after enzymatic digestion.

ficiency, as larger particles are more prone to loss during transfer and handling steps. The dominance of particles < 50 μm is consistent with previous microplastic recovery studies in biological matrices, where small particles represent the dominant fraction detected after digestion and filtration (Cole et al. 2014; Li et al. 2015). This size range is of particular ecological relevance, as smaller microplastics exhibit higher bioavailability and increased potential for trophic transfer.

Overall, these findings demonstrate that the enzymatic digestion protocol enables recovery and detection of polymer particles across a broad size spectrum while maintaining size characteristics necessary for reliable quantitative analysis.

Morphological characteristics of recovered polymer particles

Microscopic examination revealed that the recovered polymer particles exhibited irregular shapes and heterogeneous surface features characteristic of fragment-like microplastics. Most particles were classified as irregular fragments rather than fibers or films. Morphological differences were observed among polymer types. HDPE particles appeared predominantly as angular fragments, while PA particles showed more compact shapes with smoother edges. PVC particles exhibited irregular geometry with varied opacity, whereas PET particles were generally larger and more compact, often displaying

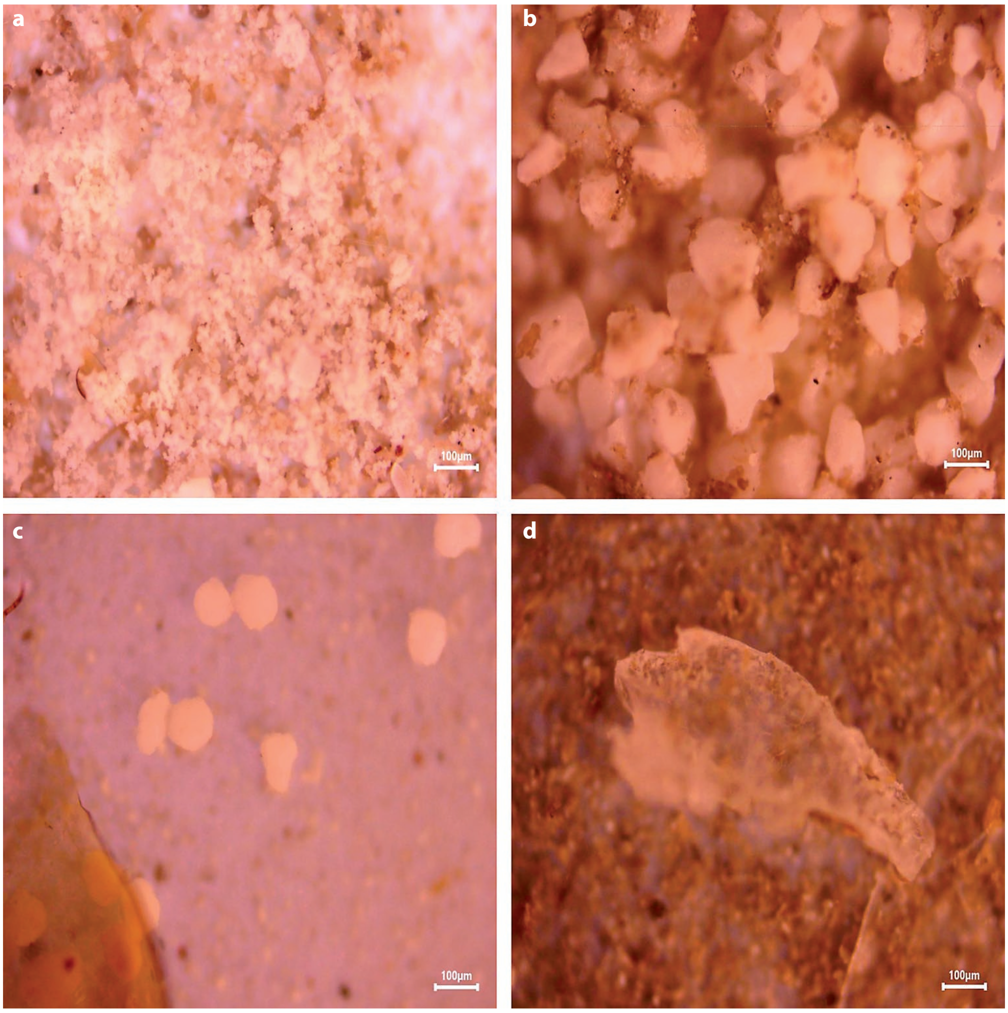

Fig. 4 Representative optical micrographs of polymer particles retained on filters after enzymatic digestion showing (a) HDPE, (b) PA, (c) PVC and (d) PET.

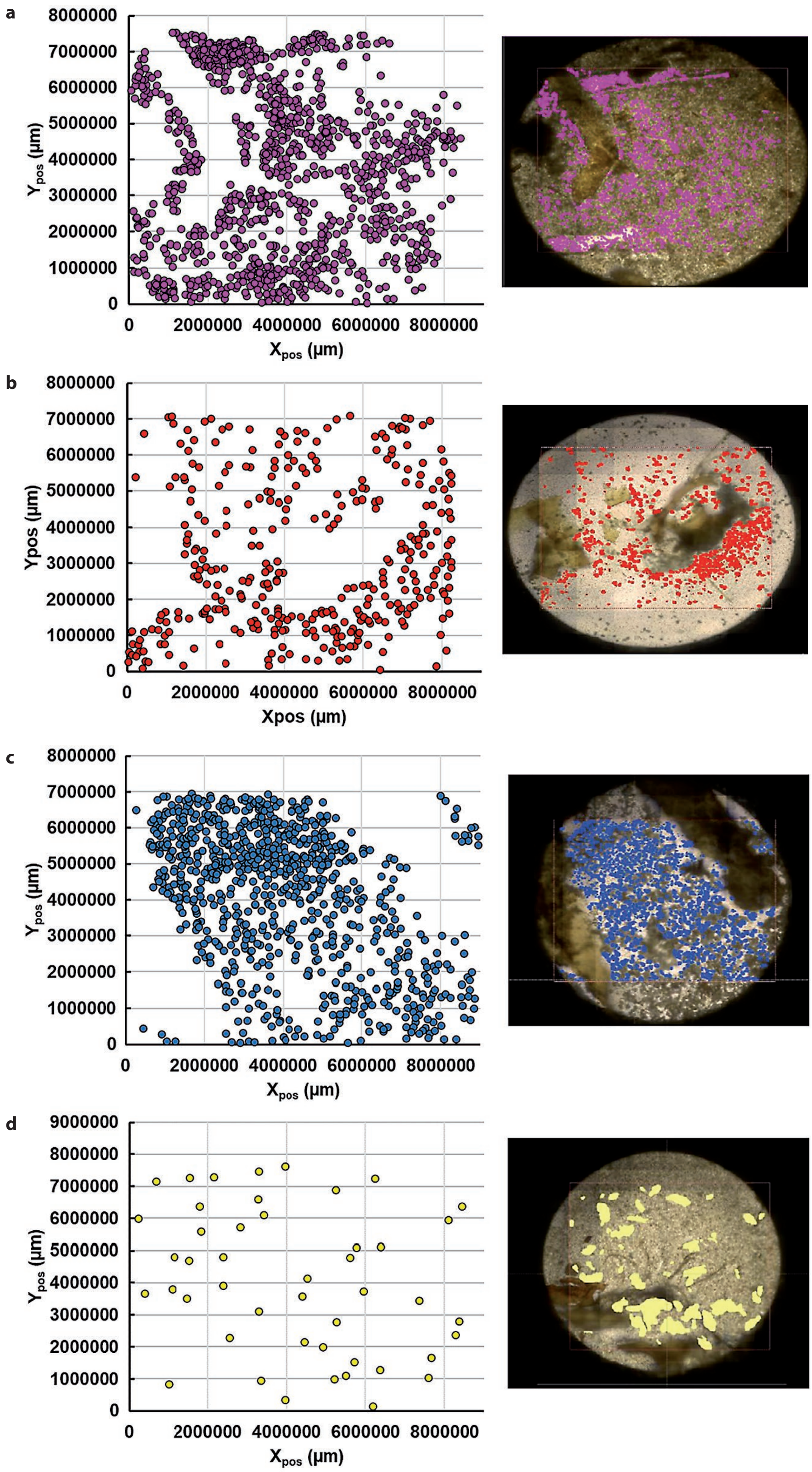

Fig. 5 µFT-IR imaging maps showing the spatial distribution and polymer identification of particles on filter surfaces: (a) HDPE, (b) PA, (c) PVC and (d) PET.

smoother surfaces and well-defined edges. Quantitative analysis of particle morphology confirmed the predominance of irregular fragments across all polymer types (Fig. 3). Shape descriptors derived from µFT-IR imaging data indicated that most particles had aspect ratios characteristic of fragment-like particles rather than elongated structures.

Representative optical micrographs illustrating the morphology and surface characteristics of the recovered particles are presented in Fig. 4a–d, highlighting differences in transparency, surface features, and edge definition among polymer types.

The predominance of irregular fragment-like shapes is consistent with morphologies commonly reported for environmental secondary microplastics (Cole et al. 2011; Hidalgo-Ruz et al. 2012). No visible surface degradation, melting, or structural alteration was observed following enzymatic digestion, indicating that the digestion protocol preserved polymer morphology. The observed variability in particle morphology and size suggests that particle shape may influence settling behavior and spatial distribution on the filters, which is further examined in the following section.

Spatial distribution and clustering patterns of polymer particles

Spatial analysis based on µFT-IR imaging coordinates revealed heterogeneous distribution patterns of polymer particles across the filter surfaces (Fig. 5 and Fig. 6). Scatter maps showed that particles were not uniformly dispersed but instead formed localized clusters and density gradients. HDPE particles, characterized by their small size and high abundance, appeared relatively evenly distributed across the filter surface. In contrast, PA and PVC particles showed moderate clustering, with localized accumulations observed in specific regions. PET particles, which were generally larger and less numerous, appeared in discrete areas rather than evenly distributed.

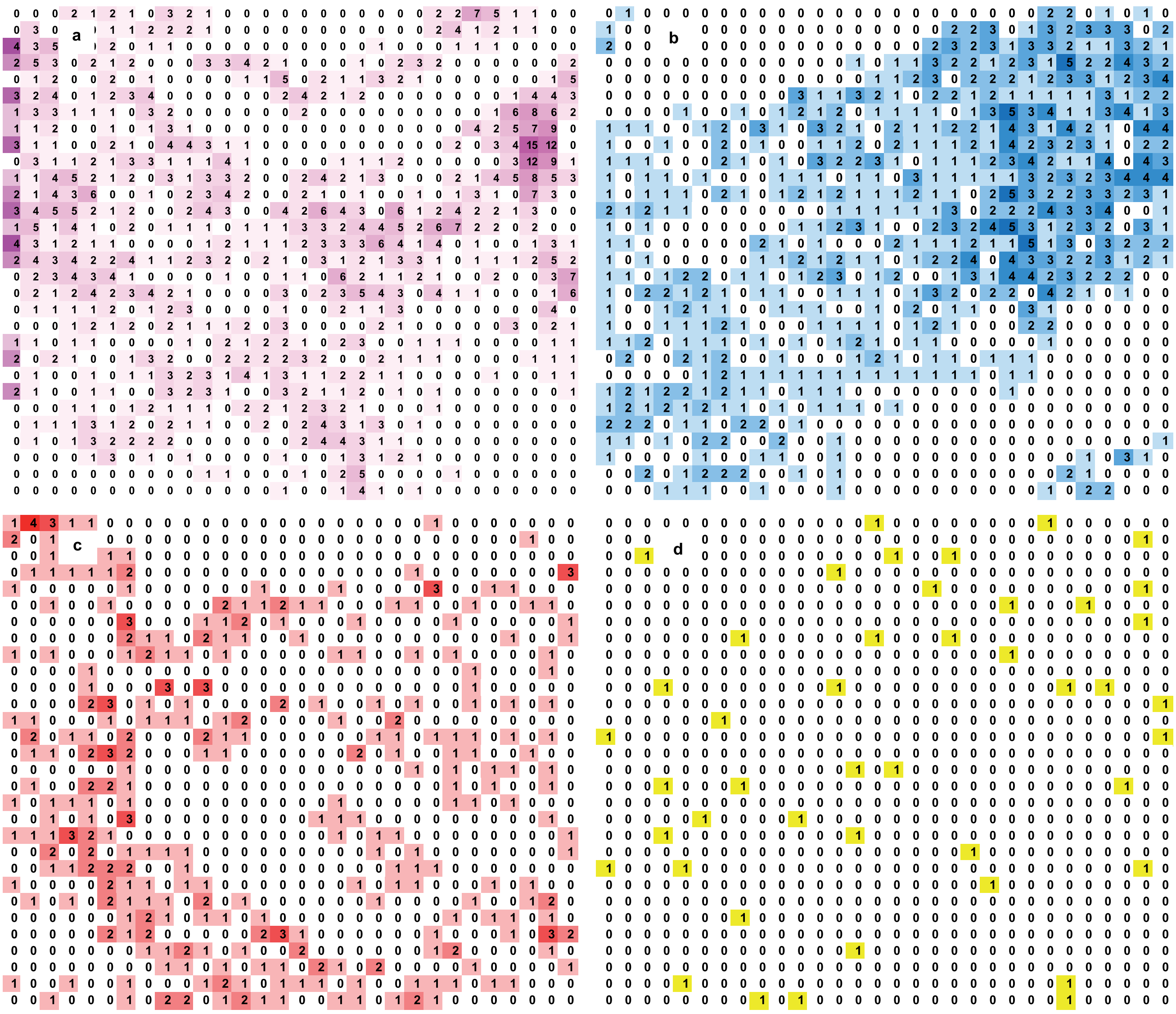

Fig. 6 Density maps showing spatial clustering and concentration patterns of polymer particles on filters after enzymatic digestion: (a) HDPE, (b) PA, (c) PVC and (d) PET.

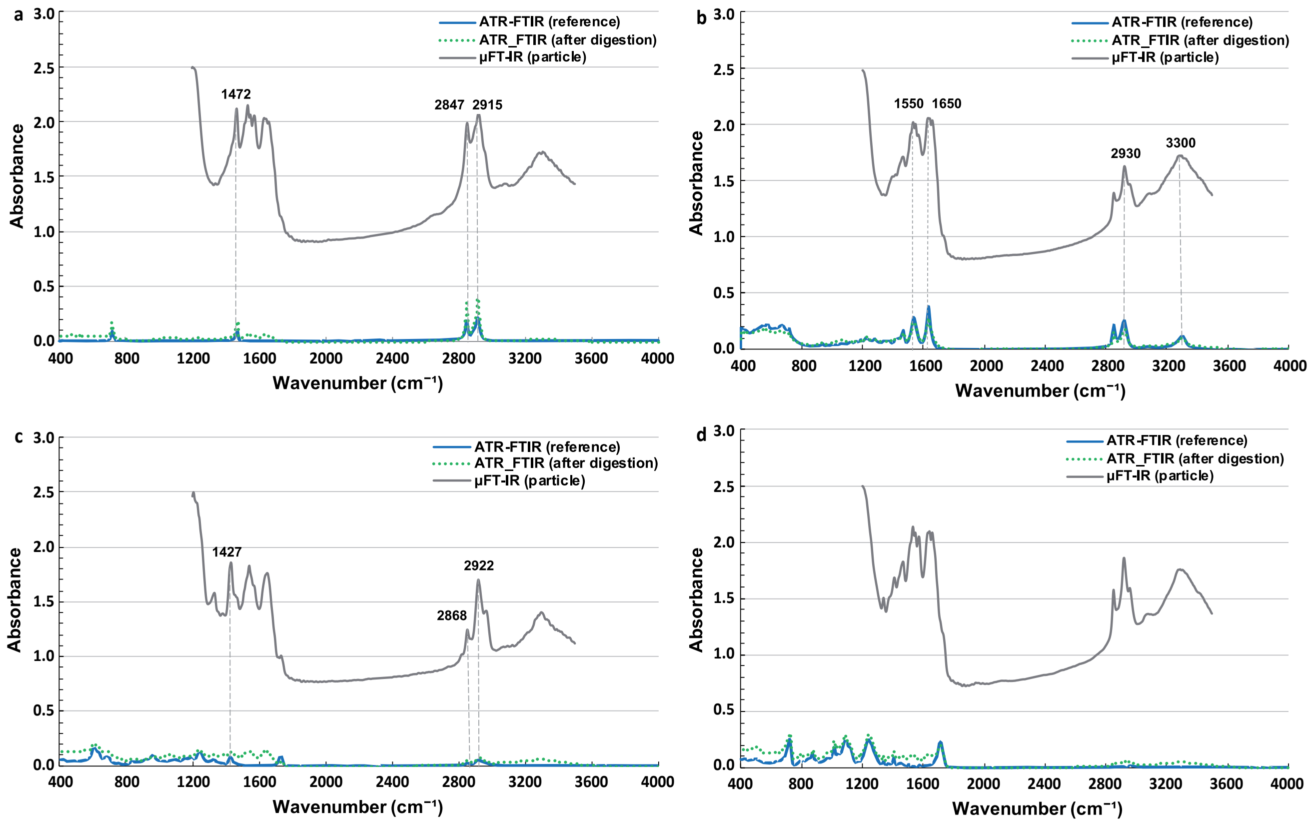

Fig. 7 Comparative ATR-FTIR reference spectra recorded before and after digestion and corresponding μFT-IR spectra of HDPE (a), PA (b), PVC (c) and PET (d) particles recovered following enzymatic digestion.

Density maps further highlighted these spatial trends, revealing zones of increased particle concentration (Fig. 6). Such heterogeneity likely reflects hydrodynamic processes during vacuum filtration, particle-particle interactions, and differences in settling behavior influenced by particle size and morphology. Non-uniform particle deposition on filter membranes has been reported in previous microplastic studies and represents an important methodological consideration, particularly when only subareas of a filter are analyzed, as this may affect quantitative estimates (Löder et al. 2017; Mintenig et al. 2019). Spatial clustering may influence quantitative estimates and should therefore be considered when designing imaging strategies. Overall, spatial mapping indicates that particle size and morphology may influence distribution behavior during filtration, reinforcing the importance of whole-filter imaging or standardized scanning protocols in microplastic analysis.

Spectroscopic identification and polymer integrity

Polymer identity was confirmed through comparison of μFT-IR spectra obtained from recovered particles with ATR-FTIR reference spectra recorded prior to enzymatic digestion (Fig. 7a–d). All four polymers exhibited characteristic absorption bands consistent with their known chemical structures. For HDPE, strong C–H stretching vibrations were observed in the region of 2848–2915 cm^{-1} (Fig. 7a), along with characteristic bending vibrations near 1470 and 720 cm^{-1} (Campanale et al. 2023; Circelli et al. 2024). PA particles displayed prominent amide I and amide II bands around 1650 and 1550 cm^{-1}, respectively (Fig. 7b), as well as N–H stretching vibrations near 3300 cm^{-1} (Narayanan and Janardhanan 2024; Schwab et al. 2024). PVC spectra (Fig. 7c) were characterized by C–Cl related absorptions in the fingerprint region between 600–700 cm^{-1} (Fernández-Sanmartín et al. 2024), while PET showed a distinct ester carbonyl band near 1715–1730 cm^{-1} (Fig. 7d) and aromatic ring vibrations in the 1400–1600 cm^{-1} range (Lujic et al. 2025).

The preservation of diagnostic absorption bands indicates that the enzymatic digestion procedure did not induce detectable chemical alteration of the polymers. Enzymatic digestion selectively degrades biological proteins, lipids, and carbohydrates (Mo et al. 2018; von Friesen et al. 2019; Di Fiore et al. 2024; Khan and Zaidi 2025) while leaving synthetic polymers structurally intact, in contrast to strong oxidative or acidic treatments that may cause surface oxidation or chain scission (Karami et al. 2017; Prata et al. 2019). Minor differences between ATR-FTIR and μFT-IR spectra may arise from differences in measurement geometry (ATR contact mode versus reflection imaging), particle thickness, surface roughness, and scattering effects associated with filter-based measurements, as discussed in vibrational microspectroscopy studies (Käppler et al. 2016). Such differences do not indicate chemical degradation but rather methodological variability inherent to spectroscopic techniques. The strong spectral agreement between reference and recovered particles demonstrates the suitability of the combined enzymatic digestion and µFT-IR imaging approach for accurate polymer identification in *Mytilus galloprovincialis* and similar marine biological matrices.

Methodological implications and study limitations

The present study demonstrates that enzymatic digestion using Kreon®25000 combined with µFT-IR imaging provides a reliable workflow for the recovery, identification, and characterization of polymer particles from *Mytilus galloprovincialis* mussel tissue. The protocol effectively removed biological material while preserving particle morphology and chemical integrity, enabling robust spectroscopic confirmation and spatial analysis.

Several methodological insights emerge from the results. First, particle size strongly influences detectability and numerical recovery. Polymers dominated by smaller size fractions (e.g., HDPE) yielded higher particle counts per unit mass, whereas larger particles (e.g., PET) resulted in lower numerical abundance despite comparable mass addition. This highlights the importance of considering both particle number and size distribution when evaluating recovery efficiency. Second, spatial clustering observed on filters indicates that particle deposition during vacuum filtration is not entirely uniform. Such heterogeneity may introduce variability if only partial areas of the filter are analyzed. Whole-filter imaging or standardized scanning strategies are recommended to minimize sampling bias, as emphasized in methodological studies on representative microplastic analysis (Löder et al. 2017). Third, the preservation of diagnostic FT-IR bands confirms that enzymatic digestion is polymer-compatible and avoids the chemical alterations sometimes associated with strong oxidative or acidic digestion protocols (Karami et al. 2017; Prata et al. 2019).

Despite these strengths, certain limitations should be acknowledged. The present study was conducted using controlled spiking experiments, with one analyzed filter per polymer type. While this design allows clear evaluation of polymer recovery and integrity, it does not address environmental variability or replicate-based statistical uncertainty. Future studies should incorporate multiple replicates and environmentally aged particles to further validate the robustness of the method under complex real-world conditions. Additionally, detection efficiency decreases for particles approaching the spatial resolution limit of µFT-IR imaging, potentially leading to underestimation of the smallest microplastic fraction (Käppler et al. 2016).

Overall, the findings support enzymatic digestion combined with µFT-IR imaging as a robust and polymer-preserving approach for microplastic analysis in biological matrices, while highlighting the importance of standardized imaging strategies and replication in future investigations.

Conclusion

This study demonstrates that enzymatic digestion using Kreon®25000 provides an effective and polymer-preserving approach for the recovery and identification of microplastic particles from *Mytilus galloprovincialis* tissue. The protocol achieved efficient removal of the biological matrix while maintaining particle morphology and chemical integrity, enabling reliable microscopic and spectroscopic analysis. Recovery efficiency varied among polymers and was strongly influenced by particle size and material properties. HDPE and PA exhibited higher numerical recovery, whereas PET showed lower particle counts, likely due to larger particle size and higher density. Size distribution analysis revealed dominance of particles <50 µm for HDPE, PA, and PVC, while PET particles were predominantly larger. µFT-IR imaging enabled accurate polymer identification and spatial mapping, revealing heterogeneous particle deposition and localized clustering patterns on filter surfaces. Morphological analysis confirmed the predominance of irregular fragment-like particles. Comparison of ATR-FTIR reference spectra with µFT-IR spectra obtained after digestion confirmed preservation of diagnostic polymer bands, demonstrating that enzymatic treatment did not induce detectable chemical changes. Overall, the combined use of enzymatic digestion and µFT-IR imaging represents a reliable workflow for microplastic analysis in marine biological matrices. The findings highlight the importance of particle size, spatial distribution, and standardized imaging strategies for accurate quantification and characterization. Future studies incorporating replicate samples and environmentally aged particles will further strengthen method validation under realistic environmental conditions.

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TOXIC METAL ACCUMULATION IN MEDICINAL PLANTS COLLECTED FROM ROADSIDE ENVIRONMENTS: IMPLICATIONS FOR HUMAN CONSUMPTION

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ABSTRACT

Medicinal plants can absorb heavy metals from the environment, which may pose risks to human health when consumed. These elements can negatively affect plant growth and metabolism by disrupting key physiological and biochemical processes. This study aimed to evaluate environmental conditions and determine the concentrations of selected minerals (Ca, K, Na and Mg) and heavy metals (Cr, Ni, Pb, Zn, Cu, Mn and Fe) in three medicinal plant species [*Silybum marianum* (L.) Gaertn., *Achillea millefolium* L. and *Convolvulus arvensis* L.], as well as to assess potential human health risks associated with their consumption. Plant samples were collected from roadside areas in the municipality of Prizren, Kosovo, and elemental concentrations were measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). The results revealed species-dependent variability in both mineral and heavy metal concentrations. *S. marianum* exhibited elevated levels of several essential minerals, whereas *C. arvensis* showed a higher accumulation potential for chromium and nickel, exceeding the permissible WHO/FAO limits. Correlation analysis indicated both synergistic and antagonistic relationships between minerals and heavy metals, suggesting shared sources of contamination and element-specific uptake mechanisms.
Health risk assessment based on estimated daily intake (EDI), target hazard quotient (THQ) and hazard index (HI) values indicated no significant non-carcinogenic risk for adult consumers under the assumed consumption scenario. Nevertheless, the accumulation of certain toxic elements highlights the importance of regular environmental monitoring and preliminary assessment of medicinal plants harvested from roadside environments. Future studies should also include heavy metal content and physicochemical properties of soils from harvesting sites to better understand plant–soil interactions and contamination pathways.

Keywords: environmental monitoring; human health risk; ICP-OES; roadside contamination; trace elements

Introduction

Medicinal plants have been used by humans for centuries in traditional medicine and culinary practices, serving as valuable sources of bioactive compounds for the prevention and treatment of various diseases (Hosseinzadeh et al. 2015). A growing body of scientific research has confirmed their therapeutic potential in both traditional and contemporary medical systems, particularly for managing a wide spectrum of health disorders (Yuan et al. 2016). In addition to bioactive constituents, medicinal plants may also provide essential minerals such as calcium (Ca), potassium (K), sodium (Na), and magnesium (Mg). These elements are involved in key metabolic processes, including protein synthesis and the regulation of water balance in the human body, thereby contributing to the medicinal and nutritional value of these plants (Shakya 2016).

Although herbal teas have historically been used for health-related purposes, their consumption increased markedly during the COVID-19 pandemic, when they were widely used as supportive approaches for managing and preventing infections (Luo et al. 2021). Several medicinal species have been investigated for their therapeutic potential. For example, *Silybum marianum* (milk thistle) has demonstrated anticancer properties and is widely used in the management of liver-related conditions (Porwal and Muath 2019; Emadi et al. 2022). *Achillea millefolium* (yarrow) is traditionally used to treat ulcers and inflammatory disorders of the gastrointestinal tract (Far et al. 2023). *Convolvulus arvensis* exhibits antibacterial and antioxidant activities (Salamatullah 2022), while other species within the genus *Convolvulus* have shown potential in improving cognitive functions and alleviating symptoms of neurodegenerative diseases, including Alzheimer's disease (Agarwal et al. 2014; Bihaqi et al. 2016).

In this context, *S. marianum* contains bioactive compounds such as silybin and silymarin, which exert hepatoprotective, anti-inflammatory, antioxidant, and anticancer effects, and have been implicated in the treatment of diseases affecting the liver, pancreas, prostate, and nervous system (Křen and Walterova 2005). In addition, *S. marianum* has been reported to act as an antifibrotic agent by neutralizing hepatic toxins (Abenavoli et al. 2010). *A. millefolium* contains constituents such as camphor, borneol, and eucalyptol, which contribute to its anti-inflammatory and antimicrobial properties (Candan et al. 2003). These effects have been associated with inhibitory activity against elastase and matrix metalloproteinases, enzymes involved in inflammatory processes (Benedec et al. 2007).

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Meanwhile, *C. arvensis* has been traditionally used for its laxative, diuretic, and anti-inflammatory properties (Salehi et al. 2020). Experimental studies have confirmed that *C. arvensis* exhibits anti-inflammatory, antimicrobial, and antifungal activities due to the presence of active compounds such as cuprenene, thymol, and chamazulene, which contribute to antioxidant activity and the inhibition of pathogenic microorganisms (Salamatullah 2022).

The documented therapeutic potential of medicinal plants may be hindered or compromised by environmental pollutants, particularly heavy metals. These toxic elements can interfere with metabolic pathways and disrupt the biosynthesis of active compounds, thereby diminishing the medicinal value of plants. Moreover, many medicinal species can accumulate substantial concentrations of heavy metals, occasionally exceeding thresholds considered safe for human health. For example, *S. marianum* has been reported to accumulate significant levels of heavy metals under natural field conditions, suggesting its potential use in environmental monitoring and phytoremediation strategies (Angelova et al. 2018), including possible applications within crop rotation systems (Angelova and Inhtyarova 2023). Similarly, *A. millefolium* has demonstrated the ability to accumulate heavy metals, which may alter the composition of essential oils and highlight its relevance in phytotoxicological assessments (Angelova and Inhtyarova 2023). In the case of *C. arvensis*, a high capacity to accumulate chromium (Cr), cadmium (Cd) and copper (Cu) has been reported, suggesting potential for phytoremediation of contaminated soils (Gardea-Torresdey et al. 2004). In addition, *C. arvensis* has been identified as a potential hyperaccumulator of chromium (Angelova and Inhtyarova 2023).

Certain metals such as copper (Cu), zinc (Zn), chromium (Cr), nickel (Ni) and manganese (Mn) are essential micronutrients for both plants and animals, contributing to numerous metabolic and enzymatic processes. However, at excessive concentrations these elements may become toxic. In contrast, metals such as lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg) do not serve known biological functions and are considered highly toxic even at trace levels (Luo et al. 2021). Due to their density, toxicity and reactivity, heavy metals can accumulate in plant tissues and may pose health risks when transferred through the food chain (Fahimirad and Hatami 2017).

Heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg), nickel (Ni) and chromium (Cr), even at low concentrations, as well as zinc (Zn) and copper (Cu) at elevated concentrations, have been shown to exert detrimental effects on both soft tissues (e.g. liver, kidneys and testes) and hard tissues (e.g. femur and tibia) in animals exposed to industrial pollution (Plakiqi Milaimi et al. 2016). In addition, exposure to moderate levels of these metals has been associated with anemia, erythropenia, leukocytosis and carcinogenic effects in human populations (Briffa et al. 2020), as well as with the inhibition of key enzymatic activities in plants (Gashi et al. 2024).

Although medicinal plants are often perceived as inherently safe due to their natural origin, several studies have shown that herbal products may contain toxic substances (WHO 2007). Given the increasing global consumption of medicinal herbs, ensuring their safety is essential, particularly with respect to contamination by heavy metals and, in cultivated species, pesticide residues. Such contaminants pose direct health risks and may contribute to their accumulation and potential biomagnification along the food chain.

Raising awareness and educating medicinal plant collectors regarding acceptable levels of heavy metals is therefore important for protecting public health. In Kosovo, previous studies have investigated heavy metal contamination in medicinal plants (Dreshaj et al. 2018); however, no specific research has been conducted on the three plant species examined in the present study. Many medicinal herbs available in local markets originate from both domestic and international sources. Nevertheless, species such as *S. marianum* and *C. arvensis*, which are commonly sold in informal herbal markets, may pose additional risks due to limited quality control, potential misidentification, uncertain dosing and regulatory gaps. The implementation of preventive measures, the establishment of safety standards, and rigorous contamination monitoring are critical steps in minimizing human exposure to heavy metals through medicinal plants.

Heavy metals can enter plants primarily through root uptake from contaminated soils, but also via foliar absorption following atmospheric deposition, making medicinal plants effective accumulators of environmental pollutants (Wild et al. 2005). The extent of accumulation depends on the type and chemical characteristics of each metal, as well as on the anatomical and physiological traits of the plant species involved (Sarma et al. 2011; Asiminicesei et al. 2024). Consequently, different plant species may accumulate varying concentrations of heavy metals even when growing under similar environmental conditions.

The main hypothesis of this study is that medicinal plants growing and being collected along roadsides accumulate higher levels of heavy metals compared to plants from uncontaminated areas. This is attributed to pollution at collection sites, from which these plants may subsequently enter formal and informal markets. Accumulation potential is expected to vary depending on environmental factors, such as contaminant levels in air, water and soil near high-traffic roads, as well as on intrinsic characteristics of each plant species.

This study focuses on the analysis of heavy metals and minerals, including chromium (Cr), nickel (Ni), lead (Pb), zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), calcium (Ca), potassium (K), sodium (Na) and magnesium (Mg), in medicinal plants collected along the Prizren-Prevallë roadside, a high-traffic area influenced by agricultural activity and tourism development. Specifi-

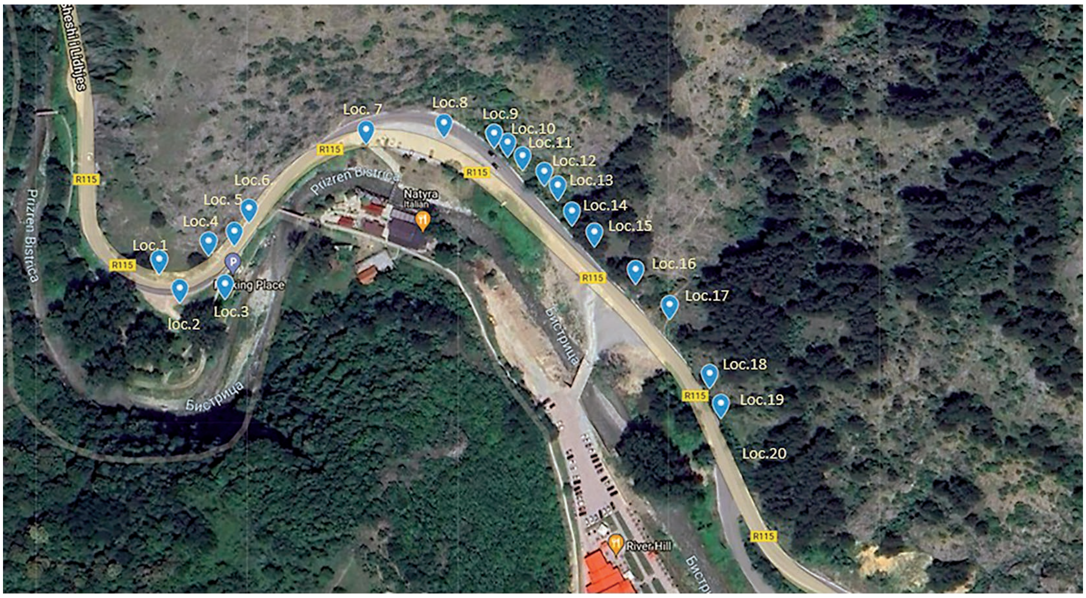

Fig. 1 Plant sampling locations in the study area (source: authors' GPS data and Google Maps, June 2025).

cally, the study aims to (1) identify interspecific differences in the capacity of medicinal plants to accumulate heavy metals and minerals under shared environmental conditions, (2) raise public health awareness in Kosovo and beyond regarding the risks associated with the identification, collection and sale of medicinal plants in unregulated markets, and (3) assess potential health risks arising from human consumption based on variations in mineral and heavy metal content among the investigated species.

Material and Methods

Study area

The study area is located along the Prizren-Prevallë road (R115) in southern Kosovo. This mountainous region is characterized by alpine forests, pastures, fertile lowland soils and rocky terrain at higher elevations. The area is geologically rich and contains mineral deposits including nickel (Ni), chromium (Cr), copper (Cu), zinc (Zn), lead (Pb), iron (Fe) and manganese (Mn), as documented in the Kosovo Geological Map (Kosovo Mining Agency 2024).

The predominant minerals in the region include quartz, olivine and serpentine, together with iron-bearing minerals such as hematite and magnetite. These geological characteristics influence soil chemistry and may contribute to the accumulation of heavy metals in plant tissues. In addition to natural geological factors, the region is influenced by anthropogenic activities such as intensive road traffic, agricultural practices and increasing tourism. The Prizren-Prevallë area is among the most frequently visited tourist destinations in Kosovo, which may increase environmental exposure to pollutants in both air and soil. Watercourses and natural springs may further contribute to pollutant transport and potential uptake by plants.

Local inhabitants are engaged in rural tourism and operate livestock and crop farms. Collection and sale of medicinal and aromatic plants is a longstanding tradition in the area, often conducted through informal roadside markets. However, this practice raises concerns regarding accurate plant identification and the absence of standardized quality control. Despite these limitations, local consumers continue to purchase and consume such products under the assumption that natural origin guarantees safety.

Plant sampling and sample handling

Samples of three medicinal plant species [*Silybum marianum* (L.) Gaertn., *Achillea millefolium* L. and *Convolvulus arvensis* L.] were collected from roadside areas along the Prizren-Prevallë road (R115) in June 2025, within an approximate stretch of 1 km. The sampling sites were located at a distance of 1.5–2.0 m from the main road to ensure comparable exposure conditions. The geographic coordinates of the sampling sites ranged from 42.20610° N to 42.207516° N and from 20.752597° E to 20.756254° E, with elevations between 439.6 m and 470.8 m a. s. l. Sampling locations are shown in Fig. 1.

For each plant species, 20 individual plants were collected. Aboveground parts (stems, leaves and flowers) were sampled, placed in sterile nylon bags, labelled and transported to the laboratory for further processing.

Sample preparation

Plant material was dried for 48 h at 50 °C in a drying oven. Dried samples were ground separately using a blender. The ground material obtained from the 20 in-

Table 1 Microwave digestion program used for plant samples.

Step	I	II	III	IV
Temperature (°C)	170	190	210	100
Pressure (bar)	30	30	30	0
Time (minutes)	15	10	15	10

dividuals per species was homogenized and subsampled. Approximately 10 g of each homogenized plant sample was placed into three sterile plastic cups to produce three analytical replicates per species.

Acid digestion

Sample digestion and elemental determination were conducted at the Agrovet Laboratory (Fushë Kosovë, Kosovo) using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). The analytical procedure followed the standardized U.S. EPA Method 6010C (EPA 2007).

Approximately 0.5 g of ground plant material was weighed using an analytical balance and transferred into Teflon digestion vessels. A mixture of 6 mL nitric acid (HNO_3, 65%) and 2 mL hydrogen peroxide (H_2O_2, 30%) was added. Samples were left at room temperature for 2 h before microwave digestion. Digestion was performed in a Berghof Speedwave MWS 3+ microwave system using a multi-step temperature and pressure program (Table 1).

After digestion, vessels were cooled for approximately 30 min at room temperature. Digests were then filtered and transferred to 50 mL containers.

ICP-OES measurement and wavelengths

Digested samples were diluted to the required volume using distilled water. Element concentrations were determined by ICP-OES by selecting appropriate analytical wavelengths according to EPA Method 6010C (EPA 2007). The wavelengths used for each element are shown in Table 2.

For each sample, three measurements were performed. Concentrations were determined for heavy metals (Cr, Ni, Pb, Zn, Cu, Mn and Fe) and minerals (Ca, K, Na and Mg).

Certified reference material (CRM) was applied during ICP-OES quality control procedures. However, the corresponding recovery (%) values were not retained in the archived analytical records from the period when the measurements were conducted.

Table 2 ICP-OES analytical wavelengths used for element determination.

Element	Wavelength (nm)	Element	Wavelength (nm)
Ca	317.933	Mn	257.610
Cr	267.716	Na	589.592
Cu	327.393	Ni	231.604
Fe	238.204	Pb	220.353
K	766.490	Zn	213.857
Mg	285.213		

Statistical analyses and human health risk assessment

Element concentrations were statistically processed using Sigma Stat 4.0 software. Arithmetic mean, standard error and Pearson correlation coefficients were calculated. Correlations were considered statistically significant at $p \leq 0.01$. Interspecific variation in metal and mineral concentrations among plant species was also evaluated.

To assess nutritional contribution, the percentage of the Recommended Daily Allowance (RDA%) was calculated for mineral elements, assuming plants were consumed as teas or herbs. Calculations were based on WHO recommendations (WHO 2004) using the following equation:

$$RDA\% = (C_{100}g / RDA) \times 100$$

where $C_{100}g$ is the mineral concentration in 100 g dry plant material (mg), and RDA is the recommended daily allowance for an adult (70 kg body weight).

Potential non-carcinogenic health risks associated with heavy metal intake through plant consumption were evaluated by calculating the Estimated Daily Intake (EDI), Target Hazard Quotient (THQ) and the overall Hazard Index (HI), following U.S. EPA methodology (US EPA 2024). EDI for each metal was calculated as:

$$EDI = (C \times IR \times EF \times ED) / (BW \times AT)$$

where C is the metal concentration in the sample (mg kg^{-1}), IR is the ingestion rate (kg day^{-1}), EF is exposure frequency (days $year^{-1}$), ED is exposure duration (years), BW is body weight (70 kg), and AT is averaging time (days).

THQ was calculated as:

$$THQ = EDI / RfD$$

where RfD is the reference dose (mg kg^{-1} day^{-1}) reported by regulatory agencies and literature sources (US EPA 1991; US EPA 1998; Khan et al. 2008; WHO 2022).

The Hazard Index (HI) was calculated as the sum of THQs for selected metals:

$$HI = THQ\ Cr + THQ\ Ni + THQ\ Pb$$

Values of THQ or HI < 1 indicate no significant non-carcinogenic risk, whereas values > 1 suggest potential long-term health effects.

Results and Discussion

Concentration of minerals and heavy metals in medicinal plants

The concentrations of heavy metals and essential minerals determined in the investigated medicinal plant species are presented in Table 3, while the relative percentage

Table 3 Concentration of heavy metals and minerals (mg kg^{-1}) in selected medicinal plants (*Silybum marianum*, *Achillea millefolium*, *Convolvulus arvensis*).

Parameter	*Silybum marianum* (L.) Gaertn.	*Achillea millefolium* L.	*Convolvulus arvensis* L.	WHO/FAO (2004) permissible limits (mg kg^{-1})	WHO/FAO toxic concentration (mg kg^{-1})
Ca	14215.53 ± 264.20	5946.23 ± 91.49	5641.99 ± 129.81	/	/
K	13884.84 ± 113.48	12630.56 ± 109.77	11219.38 ± 109.94	/	/
Mg	3008.99 ± 58.66	1259.42 ± 10.53	1741.99 ± 27.32	/	/
Na	1491.95 ± 61.98	1302.48 ± 38.31	1285.00 ± 17.77	/	/
Cr	2.88 ± 0.09	3.04 ± 0.12	7.23 ± 0.23	<0.1–1.0	2
Cu	6.88 ± 0.71	5.01 ± 0.09	4.67 ± 0.06	3–15	20
Fe	713.96 ± 23.73	281.83 ± 12.06	790.67 ± 50.37	50–250	>500
Mn	43.11 ± 1.49	63.46 ± 0.50	64.07 ± 2.41	15–100	400
Ni	3.99 ± 0.44	3.16 ± 0.27	5.17 ± 0.28	0.1–5.0	30
Pb	1.84 ± 0.02	1.50 ± 0.39	1.29 ± 0.14	1–5	20
Zn	41.78 ± 0.59	30.49 ± 0.67	20.97 ± 0.63	15–150	200

Note: Values are presented as mean ± SE; n = 20 individuals per species (composited sample), measured in three analytical replicates.

differences in elemental composition between the plant species are summarized in Table 5.

As shown in Table 3, *Silybum marianum* exhibited the highest concentrations of macrominerals among the analyzed species. Calcium (Ca) and potassium (K) were particularly abundant, reaching 14,215.53 mg/kg and 13,884.84 mg/kg dry weight, respectively. Magnesium (Mg; 3008.99 mg/kg) and sodium (Na; 1491.95 mg/kg) were also present at higher levels compared to the other studied plants. In addition, relatively elevated concentrations of copper (Cu; 6.88 mg/kg), chromium (Cr; 2.88 mg/kg), and zinc (Zn; 41.78 mg/kg) were detected in *S. marianum*.

Iron (Fe) concentration in *S. marianum* was high (713.96 mg/kg), exceeding the toxic threshold proposed by WHO/FAO (>500 mg/kg). However, both iron and manganese (Mn; 43.11 mg/kg) concentrations in *S. marianum* were lower than those recorded in *Convolvulus arvensis* (Fe: 790.67 mg/kg; Mn: 64.07 mg/kg).

Most of the analyzed heavy metals in *S. marianum*, including manganese (Mn; WHO/FAO toxic level: 400 mg/kg), nickel (Ni; 3.99 mg/kg; WHO/FAO toxic level: 30 mg/kg), and lead (Pb; 1.84 mg/kg; WHO/FAO toxic level: 20 mg/kg), were within permissible limits according to WHO/FAO guidelines and previously published studies (Stanojković-Sebić et al. 2017; Atta et al. 2023). Chromium was the only element exceeding the recommended toxic threshold (Cr >2 mg/kg), indicating potential environmental contamination.

In *Achillea millefolium*, potassium (12,630.56 mg/kg) and calcium (5946.23 mg/kg) were present at high concentrations, although lower than those observed in *S. marianum*. Magnesium concentration (1259.42 mg/kg) was the lowest among all investigated species, whereas sodium (1302.48 mg/kg) was slightly lower than in *S. marianum* but higher than in *C. arvensis*. Iron concentration (281.83 mg/kg) was markedly lower than in *S. marianum*, while manganese (63.46 mg/kg) was elevated but still lower than in *C. arvensis*. Zinc (30.49 mg/kg) and copper (5.01 mg/kg) were within normal plant concentration ranges and followed the decreasing trend *S. marianum* > *A. millefolium* > *C. arvensis*.

Nickel (3.16 mg/kg) and chromium (3.04 mg/kg) concentrations in *A. millefolium* were comparable to those found in *S. marianum*, whereas lead concentration (1.50 mg/kg) was lower. Similar to *S. marianum*, chromium was the only element exceeding the WHO/FAO toxic threshold (2 mg/kg), with values slightly higher than those observed in *S. marianum*.

In *Convolvulus arvensis*, potassium (11,219.38 mg/kg) and calcium (5641.99 mg/kg) concentrations were comparable to *A. millefolium* but remained lower than in *S. marianum*. Magnesium (1741.99 mg/kg) and sodium (1285 mg/kg) were detected at moderate levels.

Notably, *C. arvensis* showed the highest iron concentration among all studied species (790.67 mg/kg), exceeding the WHO/FAO toxic threshold (>500 mg/kg). In addition, this species exhibited the highest chromium (7.23 mg/kg) and nickel (5.17 mg/kg) concentrations, surpassing both the chromium toxic limit (>2 mg/kg) and the upper normal range for nickel (1–5 mg/kg). In contrast, lead (1.29 mg/kg), zinc (20.97 mg/kg), and copper (4.67 mg/kg) concentrations were lower than in the other two species and remained within acceptable ranges.

Overall, *S. marianum* was characterized by the highest concentrations of macrominerals (Ca, K, Mg, Na) and essential trace elements such as Cu and Zn, whereas *C. arvensis* accumulated the highest levels of potentially toxic metals, including Cr, Fe, Mn, and Ni. *Achillea millefolium* generally exhibited intermediate concentrations for most elements, with manganese levels comparable to those observed in *C. arvensis*.

Table 4 Comparison of toxicologically relevant heavy metal concentrations (mg kg⁻¹ DW) measured in this study with literature data from the region and beyond, including WHO/FAO guideline limits.

Species	Metal	Results in our study (mg kg⁻¹ DW)	Typical / average concentration in literature (mg kg⁻¹ DW)	WHO/FAO guideline limit (mg kg⁻¹ DW)	Plant toxicity range / reported toxic threshold (mg kg⁻¹ DW)	Literature sources
Silybum marianum	Cr	2.88	0.5–2.0	<0.1–1	>2	Kulhari et al. 2013; Stanojković-Sebić et al. 2017
	Cu	6.88	5–12 (seeds)	3–15	>20	Özcan 2021; Stanojković-Sebić et al. 2017
	Fe	713.96	50–250	50–250	>500	Stanojković-Sebić et al. 2017; Özcan 2021
	Mn	43.11	15–100	15–100	>400	Stanojković-Sebić et al. 2017
	Ni	3.99	1–3 (seeds); <5	0.1–5	>30	Stanojković-Sebić et al. 2017; Papadimou et al. 2024
	Pb	1.84	0.5–3	1–5	>20 *(WHO/FAO)*	Kulhari et al. 2013
	Zn	41.78	30–80 (seeds)	15–150	>200	Dghaim et al. 2015; Stanojković-Sebić et al. 2017
Achillea millefolium	Cr	3.04	0.1–1.0	<0.1–1	>2	Stanojković-Sebić et al. 2017
	Cu	5.01	4–10 (clean); 16–23 (polluted)	3–15	>20	Hlihor et al. 2022
	Fe	281.83	up to 100 (clean); up to 300 (polluted)	50–250	>500	Stanojković-Sebić et al. 2017
	Mn	63.46	16–163 (clean); up to 300 (polluted)	15–100	>400	Stanojković-Sebić et al. 2017; Abebe et al. 2024
	Ni	3.16	0–6 (rural); up to 15 (Ni-polluted)	0.1–5	>30	Hlihor et al. 2022
	Pb	1.50	~1 (clean); up to 15.9 (near traffic)	1–5	>20 *(WHO/FAO)*	Hlihor et al. 2022
	Zn	30.49	23–36 (rural); up to 211 (polluted)	15–150	>200	Hlihor et al. 2022
Convolvulus arvensis	Cr	7.23	Lack of data	<0.1–1	**30–50** *(plant toxicity range reported in literature)*	Kulhari et al. 2013
	Cu	4.67	5–15	3–15	>20	Stanojković-Sebić et al. 2017
	Fe	790.67	50–200	50–250	>500	Stanojković-Sebić et al. 2017
	Mn	64.07	20–150	15–100	>400	Stanojković-Sebić et al. 2017
	Ni	5.17	<5	0.1–5	>30	Stanojković-Sebić et al. 2017
	Pb	1.29	<5	1–5	>20 *(WHO/FAO)*	Kulhari et al. 2013
	Zn	20.97	20–70	15–150	>200	Dghaim et al. 2015; Stanojković-Sebić et al. 2017

Note: WHO/FAO guideline limits are provided for comparison based on reported permissible ranges (Table 3). "Plant toxicity range / reported toxic threshold" refers to concentrations associated with toxicity effects reported in plants or in environmental studies, and should not be interpreted as direct human safety limits. DW = dry weight.

The relative ranking of heavy metal concentrations (Ni, Cr, Pb) within each plant species followed distinct patterns:

S. marianum: Ni > Cr > Pb;
A. millefolium: Ni ≈ Cr > Pb;
C. arvensis: Cr > Ni > Pb.

The results presented in Table 3 were further evaluated through comparison with regional and international literature data, summarized in Table 4, which provides reference concentration ranges and threshold values for heavy metals commonly reported in medicinal plants. For instance, Kulhari et al. (2013) documented lead concentrations in *Achillea millefolium* reaching up to 15.9 mg/kg in samples collected from industrial areas, whereas Pb was absent or present at trace levels in plants harvested from uncontaminated environments. The same authors reported nickel concentrations as high as 42.9 mg/kg in *A. millefolium* growing on nickel-rich soils, highlighting the strong influence of soil geochemistry on elemental uptake by medicinal plants.

Comparative evaluation with literature data and interspecific differences

The concentrations of toxicologically relevant heavy metals determined in the medicinal plants investigated were further evaluated through comparison with data reported in regional and international literature, as summarized in Table 4. This table presents typical or average concentration ranges, WHO/FAO guideline limits, and reported plant toxicity thresholds for each metal, allowing a broader interpretation of the measured values.

For *Silybum marianum*, the concentrations of most trace elements were generally consistent with literature-reported values for medicinal plants. Chromium concentration (2.88 mg/kg) exceeded the WHO/FAO guideline limit (<0.1–1 mg/kg) and slightly surpassed the plant toxicity threshold (>2 mg/kg), suggesting environmental enrichment rather than species-specific hyperaccumulation.

Copper (6.88 mg/kg), manganese (43.11 mg/kg), nickel (3.99 mg/kg), lead (1.84 mg/kg), and zinc (41.78 mg/kg) remained within typical concentration ranges reported for *S. marianum*, particularly for seed material, and well below plant toxicity thresholds. Iron concentration (713.96 mg/kg), however, exceeded both the typical literature range (50–250 mg/kg) and the WHO/FAO toxic reference value (>500 mg/kg), indicating substantial iron uptake from the surrounding environment.

In *Achillea millefolium*, chromium concentration (3.04 mg/kg) also exceeded the WHO/FAO guideline limit and reported plant toxicity threshold (>2 mg/kg), consistent with findings from polluted or traffic-affected areas. Iron concentration (281.83 mg/kg) slightly exceeded the upper limit of the typical range for unpolluted sites but remained within values reported for plants growing under moderate environmental stress. Copper, manganese, nickel, lead, and zinc concentrations were comparable to literature values for *A. millefolium* collected from rural or lightly polluted environments and did not approach plant toxicity thresholds.

For *Convolvulus arvensis*, available literature data on heavy metal accumulation are limited, particularly for chromium. Nevertheless, the chromium concentration measured in this study (7.23 mg/kg) substantially exceeded WHO/FAO guideline limits and the reported plant toxicity threshold for Cr, indicating pronounced environmental contamination. Similarly, iron concentration (790.67 mg/kg) was markedly higher than typical literature values and exceeded the toxic reference level (>500 mg/kg). Nickel concentration (5.17 mg/kg) slightly exceeded the upper limit of the normal range (0.1–5 mg/kg) but remained well below the reported toxic threshold (>30 mg/kg). Other elements, including copper, manganese, lead, and zinc, were within ranges commonly reported for medicinal plants.

The interspecific differences in elemental composition are further illustrated by the percentage comparisons presented in Table 5. These data highlight pronounced variability among species, particularly for macrominerals and selected trace elements. *Silybum marianum* exhibited substantially higher concentrations of calcium, magnesium, potassium, sodium, copper, and zinc compared to both *A. millefolium* and *C. arvensis*. In contrast, *C. arvensis* showed markedly higher concentrations of chromium, iron, manganese, and nickel, emphasizing its greater tendency to accumulate potentially toxic metals.

Based on these findings, the investigated plant species can be considered valuable sources of essential minerals, although considerable interspecific variation was observed. The particularly high calcium concentration in *S. marianum* may be related to its more robust anatomical structure and well-developed root system, which could enhance mineral uptake efficiency. According to

Table 5 Percentage differences (%) in element concentrations among medicinal plant species.

Comparison (Plant 1 / Plant 2)	Ca	K	Mg	Na	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Silybum marianum / Achillea millefolium	139.07	9.93	138.92	14.55	−5.26	37.33	153.33	−32.07	26.27	22.67	37.03
Silybum marianum / Convolvulus arvensis	151.96	23.76	72.73	16.11	−60.17	47.32	−9.70	−32.71	−22.82	42.64	99.24
Achillea millefolium / Convolvulus arvensis	5.39	12.58	−27.70	1.36	−57.95	7.28	−64.36	−0.95	−38.88	16.28	45.40

Note: Values represent the percentage difference in element concentration between Plant 1 and Plant 2, calculated as: [(Plant1−Plant2)/Plant2]×100. Positive values indicate higher concentrations in Plant 1, whereas negative values indicate lower concentrations in Plant 1 relative to Plant 2.

Stanojković-Sebić et al. (2017), such differences in mineral composition are often driven by a combination of soil characteristics and species-specific physiological adaptations.

Importantly, the observed variation in mineral content among the studied plants does not imply toxicological concern with respect to essential elements. Instead, these differences are relevant for assessing the phytotherapeutic and nutritional potential of the species, especially in terms of their contribution to dietary mineral intake. The elevated concentrations of certain heavy metals, particularly chromium and iron, appear to reflect environmental conditions rather than intrinsic plant toxicity, underscoring the importance of site selection when harvesting medicinal plants.

Heavy metals

Chromium concentrations detected in the analyzed plant samples ranged from 2.88 mg/kg in *Silybum marianum* to 7.23 mg/kg in *Convolvulus arvensis* (Table 3). Although these values may appear relatively low in absolute terms, they exceed the toxic threshold defined by the World Health Organization and several authors, who consider concentrations above 2 mg/kg (dry weight) as potentially hazardous (WHO 2007; Kulhari et al. 2013; Stanojković-Sebić et al. 2017; Atta et al. 2023).

It is important to note that WHO toxicity limits primarily refer to hexavalent chromium (Cr^{6+}), which is highly toxic even at very low concentrations (≈0.02 mg/kg). The presence of Cr^{6+} in plant material is therefore considered undesirable (Oladeji et al. 2024). However, chromium in plant tissues generally occurs predominantly in the trivalent form (Cr^{3+}), which is less mobile and poorly absorbed by plant roots. Chromium is not considered an essential element for plants, and under unpolluted environmental conditions its concentration rarely exceeds 1 mg/kg (Stanojković-Sebić et al. 2017).

Consequently, the elevated chromium concentrations observed in the present study (2.88–7.23 mg/kg) strongly suggest environmental contamination. Similar chromium levels have been reported in medicinal plants growing near thermal power plants and industrial zones in Serbia and parts of Africa (Stanojković-Sebić et al. 2017; Oladeji et al. 2024). These findings support the hypothesis that chromium contamination in the study area is primarily associated with traffic-related emissions and resuspended roadside dust, rather than species-specific accumulation alone.

Although the chromium concentrations detected did not result in acute toxicological risk for consumers, as indicated by the EDI, THQ, and HI values (Table 8), they nevertheless point to environmental pollution. Given chromium's ability to bioaccumulate in the human body and its association with dermatological, respiratory, and carcinogenic effects under long-term exposure (Oladeji et al. 2024), careful selection of harvesting sites for medicinal plants remains essential.

Copper concentrations ranged from 4.67 mg/kg in *Convolvulus arvensis* to 6.88 mg/kg in *Silybum marianum* (Table 3). These values fall within the typical concentration range reported for medicinal plants (3–15 mg/kg) (Stanojković-Sebić et al. 2017). As all plants were collected from the same locality, the observed interspecific differences likely reflect species-specific physiological requirements rather than environmental variability. For example, *S. marianum* and *A. millefolium* may exhibit higher copper demand due to its role as an enzymatic cofactor.

Literature reports indicate that copper concentrations in *A. millefolium* may reach 14–16 mg/kg under normal conditions and up to 20 mg/kg in polluted or mining-affected areas (Hlihor et al. 2022), approaching phytotoxic levels. In the present study, none of the analyzed samples exceeded the plant toxicity threshold for copper.

From a human health perspective, the detected copper concentrations pose no concern. For instance, consumption of 10 g of plant material containing 10 mg/kg Cu would result in an intake of only 0.1 mg Cu, which is negligible relative to safe daily intake levels. Although certain plant species (e.g., *Cynodon dactylon*) are known copper accumulators in mining regions (Alvarez et al. 2023), no such hyperaccumulation behavior was observed in the investigated species.

Iron concentrations varied widely among the studied species, ranging from 281.83 mg/kg in *Achillea millefolium* to 790.67 mg/kg in *Convolvulus arvensis*, with *Silybum marianum* also showing elevated values (713.96 mg/kg) (Table 3). These concentrations exceed the typical range reported for plants (50–250 mg/kg) and, in the case of *S. marianum* and *C. arvensis*, surpass the WHO/FAO toxic reference value (>500 mg/kg).

Elevated iron concentrations of this magnitude have been reported previously in plants growing in polluted or traffic-affected environments (Stanojković-Sebić et al. 2017). Since iron is an essential nutrient, its accumulation in plant tissues largely depends on soil chemistry, redox conditions, and rhizosphere interactions. The observed interspecific differences (e.g., *C. arvensis* > *S. marianum* >> *A. millefolium*) may reflect differences in root architecture and iron availability at microscale soil patches.

From a nutritional perspective, iron deficiency is common in human populations, and these plants may represent supplementary dietary sources when consumed as infusions. However, iron bioavailability from plant matrices is typically low. Despite elevated iron concentrations, the health risk assessment based on EDI, THQ and HI values indicates no significant non-carcinogenic risk under the assumed consumption scenario.

Manganese concentrations ranged from 43.11 mg/kg in *Silybum marianum* to 64.07 mg/kg in *Convolvulus arvensis* (Table 3). These values fall within the typical range reported for medicinal herbs (50–100 mg/kg) (Stanojković-Sebić et al. 2017). Literature data indicate that medicinal plants may accumulate between 20 and 300 mg/kg

Mn depending on species and soil properties (Hlihor et al. 2022).

According to WHO guidelines, manganese concentrations up to approximately 500 mg/kg are considered safe in medicinal herbs (Abebe et al. 2024), while plant toxicity thresholds are reported around 400 mg/kg (Stanojković-Sebić et al. 2017). Therefore, manganese does not represent a toxicological concern in the present study.

Although excessive manganese exposure in humans may cause neurological disorders, the levels detected in the investigated plants are negligible compared to industrial exposure. Interspecific differences likely reflect physiological requirements, as manganese plays a key role in photosynthesis and enzymatic activation.

Nickel concentrations ranged from 3.16 mg/kg in *Achillea millefolium* to 5.17 mg/kg in *Convolvulus arvensis*, with *Silybum marianum* showing intermediate values (3.99 mg/kg) (Table 3). While *C. arvensis* slightly exceeded the upper limit of the normal range (0.1–5 mg/kg), all values remained well below the WHO/FAO toxic concentration threshold of 30 mg/kg.

Comparable nickel concentrations have been reported for medicinal plants growing in rural and moderately polluted environments, whereas substantially higher values occur in Ni-enriched soils or industrial areas (Stanojković-Sebić et al. 2017; Hlihor et al. 2022). The slightly elevated nickel concentration in *C. arvensis* may reflect greater uptake capacity or localized environmental enrichment, potentially linked to ultrabasic parent material or traffic-derived dust.

Although nickel may induce allergic reactions and chronic effects at high exposure levels (Nordberg et al. 2015; Milaimi et al. 2017), the calculated THQ values remained far below 1 for all species (Table 8), indicating low non-carcinogenic risk.

Lead concentrations were low across all samples, ranging from 1.29 mg/kg in *A. millefolium* to 1.84 mg/kg in *S. marianum* (Table 3). These values are well below the reported plant toxicity threshold (10 mg/kg) (Kulhari et al. 2013), suggesting minimal environmental lead contamination or limited uptake capacity of the studied species.

In contrast, literature reports from polluted areas indicate lead concentrations of 5–20 mg/kg, with values as high as 15.9 mg/kg reported for *A. millefolium* near traffic-intensive zones (Stanojković-Sebić et al. 2017). In the present study, THQ values for lead remained well below 1 (Table 8), confirming negligible health risk under the assumed consumption scenario. Nevertheless, secondary contamination during drying and processing cannot be excluded, and continuous monitoring is recommended due to lead's cumulative toxicity.

Zinc concentrations ranged from 20.97 mg/kg in *C. arvensis* to 41.78 mg/kg in *S. marianum*, with *A. millefolium* showing intermediate values (Table 3). These concentrations are typical for medicinal plants and fall below the WHO desirable limit of 50 mg/kg (WHO 2007). Similar values have been reported for *A. millefolium* collected from non-polluted areas (Hlihor et al. 2022).

Elevated zinc concentrations (>100 mg/kg) are generally associated with polluted environments and may approach phytotoxic levels (>200 mg/kg) (Stanojković-Sebić et al. 2017). The zinc concentrations observed in this study do not pose toxicological concern for plants or consumers. Given the recommended daily zinc intake (10–15 mg/day), the contribution from typical herbal consumption is minimal.

The relatively higher zinc concentration in *S. marianum* may reflect fertilizer inputs or adaptive physiological mechanisms. Interestingly, *S. marianum* also exhibited the highest lead concentration, which may suggest a protective role of zinc in mitigating metal stress (Milaimi et al. 2017). However, all zinc values remained far below levels associated with adverse effects.

Overall, comparison with literature data indicates that heavy metal concentrations in the investigated medicinal plants generally fall within permissible limits, reflecting low-level environmental contamination. Differences among species appear to be driven by both environmental factors and species-specific uptake mechanisms. While chromium and, to a lesser extent, nickel indicate traffic-related contamination, the calculated risk indices confirm that consumption of these plants under typical usage scenarios does not pose significant health risks.

Correlation analyses of heavy metal and minerals in medicinal plants

Detailed Pearson correlation matrices for each species are presented in Table 6a–c. Table 6 presents the Pearson correlation coefficients (r) describing the relationships between essential minerals and heavy metals in the investigated medicinal plant species. Overall, the correlation patterns indicate strong interactions between macroelements and potentially toxic metals, reflecting both shared uptake pathways and competitive absorption mechanisms.

Potassium (K) exhibited a strong positive correlation with chromium (Cr) in *Silybum marianum* (r = 0.998) and *Convolvulus arvensis* (r = 0.968), suggesting a possible co-uptake or common environmental source, such as traffic-derived dust or soil particles enriched in multiple elements. In contrast, potassium showed a strong negative correlation with copper (Cu) in *S. marianum* (r = −0.920), indicating potential antagonistic behavior during uptake or internal translocation.

Magnesium (Mg) was positively correlated with iron (Fe) across all investigated species (r = 1.000), reflecting their shared involvement in fundamental physiological processes and possible co-regulation within plant tissues.

Conversely, magnesium displayed negative correlations with nickel (Ni) in *S. marianum* (r = −0.664) and *Achillea millefolium* (r = −0.568), suggesting a competitive interaction that may limit nickel accumulation in the presence of higher magnesium availability.

Table 6 Pearson correlation coefficients (r) between minerals and heavy metals in the investigated medicinal plant species.

Table 6a *Silybum marianum* (Pearson r).

	Ca	K	Mg	Na	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Ca	1	0.931*	0.263*	0.999*	0.950*	−0.620*	0.280*	−0.636*	−0.896*	−0.874*	0.987*
K		1	0.596*	0.916*	0.998*	−0.920*	0.611*	−0.311*	−0.996*	−0.638*	0.978*
Mg			1	0.223*	0.551*	0.594*	1.000*	0.577*	−0.664*	−0.679*	0.417*
Na				1	0.936*	−0.652*	0.567*	−0.363*	−0.877*	−0.894*	0.979*
Cr					1	−0.344*	0.567*	1.000*	0.990*	−0.679*	0.988*
Cu						1	0.578*	1.000*	0.207*	0.923*	−0.484*
Fe							1	0.562*	−0.680*	0.220*	0.434*
Mn								1	0.227*	0.931*	−0.501*
Ni									1	0.568*	−0.956*
Pb										1	−0.783*
Zn											1

Table 6b *Achillea millefolium* (Pearson r).

	Ca	K	Mg	Na	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Ca	1	0.966*	0.793*	0.999*	−0.441*	0.843*	0.135*	0.990*	0.952*	0.885*	0.407*
K		1	0.609*	0.974*	−0.196*	0.953*	0.385*	0.993*	0.999*	0.975*	0.628*
Mg			1	0.772*	−0.897*	0.339*	−0.497*	0.969*	0.568*	0.417*	−0.234*
Na				1	−0.411*	0.860*	0.168*	0.994*	0.962*	0.900*	0.438*
Cr					1	0.112*	0.830*	−0.307*	−0.145*	−0.020*	0.640*
Cu						1	0.647*	0.911*	0.976*	0.996*	0.835*
Fe							1	0.277*	0.432*	0.581*	0.960*
Mn								1	0.986*	0.943*	0.535*
Ni									1	0.985*	0.668*
Pb										1	0.786*
Zn											1

Table 6c *Convolvulus arvensis* (Pearson r)

	Ca	K	Mg	Na	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Ca	1	1.000*	0.984*	0.649*	0.973*	−0.717*	0.862*	0.867*	0.884*	0.246*	0.910*
K		1	0.981*	0.663*	0.968*	−0.730*	0.956*	0.852*	0.875*	0.228*	0.917*
Mg			1	0.504*	0.998*	−0.581*	1.000*	0.942*	0.953*	0.414*	0.822*
Na				1	0.455*	0.455*	0.174*	0.183*	0.217*	−0.578*	0.906*
Cr					1	−0.536*	0.956*	0.959*	0.968*	0.464*	0.789*
Cu						1	−0.264*	−0.274*	−0.307*	−0.500*	−0.941*
Fe							1	1.000*	0.999*	0.703*	0.574*
Mn								1	0.999*	0.666*	0.582*
Ni									1	0.671*	0.610*
Pb										1	−0.179*
Zn											1

Note: Correlation is significant at the 0.01 level (2-tailed); Correlation is significant at the 0.05 level (2-tailed).

In *S. marianum*, sodium (Na) showed strong negative correlations with several potentially toxic metals, including lead (Pb; r = −0.894), copper (Cu; r = −0.652), and nickel (Ni; r = −0.887). These relationships may indicate a protective role of sodium in reducing heavy metal uptake or translocation, possibly through ionic competition at root uptake sites.

A pronounced positive correlation between chromium (Cr) and nickel (Ni) was observed in all studied species, particularly in *S. marianum* (r = 1.000) and *C.*

arvensis (r = 0.998). This consistent association strongly suggests a common source of contamination, most likely related to traffic emissions or soil material enriched with multiple metals. In *S. marianum*, chromium also exhibited a negative correlation with lead (Pb; r = −0.679), implying differential mobility or selective uptake mechanisms for these metals.

Copper (Cu) showed a strong positive correlation with manganese (Mn) in *S. marianum* (r = 0.911), a pattern also evident in *A. millefolium* and *C. arvensis*. This relationship may reflect their joint involvement in enzymatic systems related to photosynthesis and oxidative stress regulation. In contrast, copper was negatively correlated with zinc (Zn) in *S. marianum* (r = −0.484), suggesting competitive interactions between these two micronutrients.

A significant positive correlation between manganese (Mn) and nickel (Ni) was detected in *A. millefolium* (r = 0.986) and *C. arvensis* (r = 0.999), indicating that species accumulating higher manganese levels also tend to accumulate more nickel, possibly due to shared transport pathways or similar soil bioavailability.

Finally, a strong negative correlation between lead (Pb) and zinc (Zn) was observed in *S. marianum* (r = −0.783), which may indicate a protective or antagonistic interaction, where zinc accumulation limits lead uptake or vice versa.

Overall, the correlation analysis highlights complex interactions between essential nutrients and toxic metals, supporting the interpretation that both environmental factors (e.g., traffic-related pollution) and species-specific physiological mechanisms govern metal accumulation patterns in the studied medicinal plants.

The results presented in Table 6 demonstrate that calcium (Ca), potassium (K), magnesium (Mg), and sodium (Na) exhibit strong associations with the analyzed heavy metals, highlighting complex interactions between essential nutrients and potentially toxic elements. In particular, calcium and sodium showed significant positive and negative correlations with lead (Pb) and nickel (Ni), indicating possible competitive or antagonistic interactions during uptake and translocation within plant tissues.

Magnesium (Mg) was positively correlated with iron (Fe), reflecting their shared involvement in fundamental physiological processes, while both Mg and Na displayed negative correlations with several toxic metals. This pattern suggests a potential protective role of these macroelements, whereby higher availability of essential nutrients may limit the accumulation of certain heavy metals through competitive absorption mechanisms or physiological regulation.

A particularly strong correlation was observed between chromium (Cr) and nickel (Ni), supporting the hypothesis of a common source or shared pathway of entry into the plant system. Such associations are frequently reported in environments influenced by anthropogenic activities, especially traffic-related emissions and resuspended roadside dust (Ayinde et al. 2020). Although the absolute concentrations of these metals remain relatively low, their consistent co-occurrence across all investigated species suggests an environmental signal indicative of low-intensity but persistent contamination.

Metal accumulation in plants is strongly influenced by multiple interacting factors, including the total concentration and chemical speciation of metals in the soil. Elements such as nickel (Ni) and chromium (Cr) may originate from both natural sources (e.g., ultrabasic parent material) and anthropogenic inputs, including industrial activities and vehicular emissions (Papadimou et al. 2024). Soil properties such as pH and organic matter content further regulate metal bioavailability; for example, acidic conditions are known to enhance the mobility and uptake of certain metals, including cadmium (Cd) (Papadimou et al. 2024).

Plant species-specific traits also play a critical role in determining metal accumulation patterns. Genetic and physiological mechanisms control not only the efficiency of metal uptake but also their internal distribution among plant organs. For instance, *Silybum marianum* has been reported to accumulate metals such as cadmium predominantly in roots while restricting their transport to aerial parts, thereby reducing potential toxic effects in reproductive tissues (Papadimou et al. 2024). In general, metals tend to accumulate more strongly in vegetative organs (leaves and stems) than in flowers or seeds, a pattern that is consistent with the findings of the present study.

In addition to biological factors, both anthropogenic and geological sources contribute to metal enrichment in plants. Identifying the origin of contamination is essential for interpreting observed concentration patterns. Lead (Pb) is commonly associated with traffic emissions, whereas nickel (Ni) and chromium (Cr) may reflect inputs from metallurgical activities or naturally metal-rich substrates.

Within this context, *Achillea millefolium* and *Convolvulus arvensis* primarily mirrored the prevailing environmental conditions, with toxic metals detected mainly at trace levels, suggesting a lightly impacted environment. In contrast, *Silybum marianum* appeared to exhibit a more pronounced exclusion or retention mechanism, as reflected by comparatively lower heavy metal accumulation in its aerial parts. This observation is consistent with previous studies highlighting the potential of *S. marianum* for phytoremediation in metal-contaminated soils, where metals are preferentially immobilized in roots or non-harvested tissues (Papadimou et al. 2024).

Assessment of Daily Mineral Intake fulfillment from the investigated plants

Table 7 summarizes the estimated mineral intake derived from the consumption of 100 g dry weight (DW) of the investigated plant species and compares these values with established Recommended Dietary Allowance (RDA) reference values for adults. This scenario repre-

Table 7 Estimated mineral intake (mg) from 100 g dry weight (DW) of the investigated plant species, with recommended dietary allowance (RDA) reference values for adults.

Species	Ca (mg/100 g)	Cu (mg/100 g)	Fe (mg/100 g)	K (mg/100 g)	Mg (mg/100 g)	Mn (mg/100 g)	Na (mg/100 g)	Zn (mg/100 g)
Silybum marianum	142.16	76.48	892.45	29.54	71.64	187.45	9.95	37.98
Achillea millefolium	59.46	55.70	352.29	26.87	29.99	275.91	8.68	27.72
Convolvulus arvensis	56.42	51.96	988.35	23.87	41.48	278.58	8.34	19.06

RDA reference values (adult): Ca = 1000 mg/day; Cu = 0.9 mg/day; Fe = 8 mg/day; K = 4700 mg/day; Mg = 420 mg/day; Mn = 2.3 mg/day; Na = 1500 mg/day; Zn = 11 mg/day.

Note: Mineral values are expressed as mg per 100 g DW for standardized comparison among species; typical daily intake of medicinal plants as herbal tea is considerably lower (commonly 2–10 g/day).

sents a theoretical maximum intake and is used to evaluate the potential contribution of medicinal plants to daily mineral supply rather than actual consumption patterns.

The results indicate that *Silybum marianum* is particularly rich in several essential minerals. Consumption of 100 g DW would provide approximately 142 mg of calcium (Ca), 71.6 mg of magnesium (Mg), and nearly 892 mg of iron (Fe). Notably, the calculated iron and copper contents substantially exceed daily nutritional requirements, reflecting the naturally high mineral density of the plant material rather than realistic dietary exposure. Potassium (K) and sodium (Na) contributions were comparatively low, accounting for only minor fractions of the recommended daily intake.

In *Achillea millefolium*, mineral contributions followed a similar pattern but at generally lower levels for most elements. Calcium (59.5 mg), magnesium (30.0 mg), and iron (352 mg) were present in moderate amounts, while manganese (Mn) showed particularly elevated values, consistent with the known tendency of this species to accumulate Mn. As with *S. marianum*, potassium and sodium intake remained negligible in relation to daily dietary needs.

Convolvulus arvensis exhibited the highest estimated iron intake (approximately 988 mg per 100 g DW), along with moderate levels of magnesium and manganese. Zinc concentrations were comparatively lower than in the other two species. Despite these elevated mineral contents, it is important to emphasize that medicinal plants are typically consumed in small quantities (e.g., as infusions or extracts), and thus actual dietary intake would be substantially lower than the values calculated under this hypothetical scenario.

Overall, the data presented in Table 7 demonstrate that the investigated medicinal plants can serve as concentrated sources of essential minerals, particularly iron, magnesium, and manganese. However, due to the unrealistically high consumption level assumed (100 g DW), these values should be interpreted with caution and regarded as indicators of mineral richness rather than direct dietary recommendations.

Assessment of risk factors (EDI, THQ and HI) in the study plants

Table 8 presents the Estimated Daily Intake (EDI), Target Hazard Quotient (THQ), and Hazard Index (HI) for heavy metals such as chromium (Cr), nickel (Ni), and lead (Pb) in adults consuming the four-plant species included in the study. These values are evaluated for a 70 kg adult person and represent potential non-carcinogenic risks associated with plant consumption.

S. marianum – The EDI for chromium (Cr) was 1.97E-02 mg/kg/day, and the THQ value was 9.85E-03, both of which are below the threshold of 1, indicating acceptable non-carcinogenic risk. The EDI for nickel (Ni) was 1.95E-02 mg/kg/day, and the THQ was 1.30E-02, also below the threshold of 1. The EDI for lead (Pb) was 5.94E-03 mg/kg/day, and the THQ value was 5.94E-04, again below 1.

The sum of the THQs for the three metals resulted in an HI value of 2.34E-02, which is also below the threshold of 1, indicating no significant risk.

A. millefolium – The EDI and THQ values for all three metals (Cr, Ni, Pb) were below the threshold of 1, suggesting low risk. Specifically, the EDI for chromium was 2.08E-02 mg/kg/day and THQ was 1.04E-02, for nickel 1.54E-02 mg/kg/day and THQ 1.03E-02, and for lead 4.84E-03 mg/kg/day with a THQ of 4.84E-04.

Table 8 Estimated daily intake (EDI), target hazard quotient (THQ) and hazard index (HI) values for adults associated with the consumption of investigated medicinal plant species.

Species	Cr EDI (mg kg^{-1} day^{-1})	Cr THQ	Ni EDI (mg kg^{-1} day^{-1})	Ni THQ	Pb EDI (mg kg^{-1} day^{-1})	Pb THQ	HI
Silybum marianum	1.97E-02	9.85E-03	1.95E-02	1.30E-02	5.94E-03	5.94E-04	2.34E-02
Achillea millefolium	2.08E-02	1.04E-02	1.54E-02	1.03E-02	4.84E-03	4.84E-04	2.12E-02
Convolvulus arvensis	4.95E-02	2.47E-02	2.53E-02	1.68E-02	4.16E-03	4.16E-04	4.20E-02

Note: EDI = estimated daily intake; THQ = target hazard quotient; HI = hazard index (sum of THQs). Values were calculated for a 70 kg adult.

The total HI value for this plant was 2.12E-02, which is also below the threshold of 1.

C. arvensis – The EDI for chromium was 4.95E-02 mg/kg/day, and the THQ was 2.47E-02, both of which are above the values seen in the other plants but still below the threshold of 1. The EDI for nickel was 2.53E-02 mg/kg/day, with a THQ value of 1.68E-02, and for lead, the EDI was 4.16E-03 mg/kg/day with a THQ of 4.16E-04.

The HI value for *C. arvensis* was 4.20E-02, which is higher than the other plants but still below the critical threshold of 1.

The results from the analysis suggest that all plant species (*S. marianum*, *A. millefolium*, *C. arvensis*) have heavy metal EDI, THQ, and HI values below the threshold of 1, indicating no significant risk for non-carcinogenic effects from their consumption. However, *C. arvensis* exhibited slightly higher values, particularly for chromium and nickel, compared to the other species. Therefore, while all plants are considered safe based on these criteria, *C. arvensis* should be monitored more closely for potential exposure to higher levels of heavy metals.

The health risk assessment yields favorable results but advises caution regarding long-term exposure. The analysis was based on the consumption of 5–10 g of dried herbs per day by a person weighing approximately 70 kg. The Target Hazard Quotient (THQ) for the metals (Ni, Cr, and Pb) and the combined Hazard Index (HI) were calculated.

The THQ values for chromium (Cr), nickel (Ni), and lead (Pb) were very low for all plants (Cr THQ up to 2.47E-02; Ni THQ up to 1.68E-02; Pb THQ up to 5.94E-04), all of which are well below the value of 1. The THQ values remained completely under 0.1, indicating an acceptable non-carcinogenic risk under the assumed exposure scenario. These findings are consistent with other reports on medicinal herbs where exceedances (THQ > 1) are typically driven by Pb, Cd, As, or Hg, whereas Cu and Zn rarely exceed the threshold (Luo et al. 2021).

The Hazard Index (HI) values were all below 1 for each plant, indicating that if a person were to consume any of the plants regularly, the risk of adverse effects from the toxic metals would be low. In general, HI ranged from nearly 0.0212 for *A. millefolium* (which had lower concentrations of toxic metals) to approximately 0.0420 for *C. arvensis*, which was affected by Ni and Pb.

However, these values are still below the threshold of 1 and are thus considered acceptable (Luo et al. 2021). This outcome was expected, as none of the metals were present at concerning levels. Consequently, their combined effect resulted in a value well below the threshold of 1, indicating that their overall toxicological effect remains within acceptable tolerance level (Ozyigit et al. 2023).

According to the literature, certain heavy metals such as chromium (VI), nickel (Ni), cadmium (Cd), and lead (Pb) are classified as carcinogenic following long-term exposure. However, in our study, the levels of these metals were found to be very low, which significantly reduces the carcinogenic risk. Long-term exposure (over years) is typically required for the concentrations of these metals to reach levels where the carcinogenic risk exceeds 1 (Luo et al. 2021). Therefore, the carcinogenic risk in our case is negligible.

Based on these findings, it can be concluded that the primary concern with heavy metals in the plants studied is not carcinogenicity but rather chronic non-carcinogenic toxicity. This could lead to health issues such as kidney damage from Ni (Milaimi et al. 2017) or nerve damage from Pb, among other potential effects. The THQ/HI data indicate that the consumption of these plants does not pose a significant health risk for an average user (e.g., a cup of tea, rather than consuming tens of grams per day). Therefore, the use of these plants in moderate amounts, without long-term continuous consumption, is considered safe.

Even though the HI values are below 1, it is important to minimize unnecessary exposure to heavy metals. This is particularly crucial as heavy metals have the tendency to bioaccumulate in the body over time. For instance, Pb has a very low renal excretion rate (Singh and Kalamdhad 2011), meaning that even small, daily doses can accumulate in bones and other tissues (Milaimi et al. 2017). This bioaccumulation is of particular concern for sensitive groups, such as children, pregnant women, and individuals with chronic illnesses, as they are more vulnerable to the neurotoxic effects of Pb. The WHO suggests that even low chronic exposure to Pb can impair mental development in children (Luo et al. 2021).

Thus, maintaining HI levels well below 1 should be a primary objective when developing herbal medicinal products. It is important to note that not all of the metal content in plants transfers to tea during brewing, as some metals remain bound to plant fibers and do not dissolve completely in water, reducing consumer exposure. However, commercial herbal preparations present a higher risk, as they could deliver the entire amount of metal to the consumer. Therefore, strict testing of commercial medicinal plant products for heavy metals, as recommended by the WHO, is essential. Supporting this concern, authors Dghaim et al. (2015) found exceedances of the permissible limits for Pb, Cd, and other metals in 81 spice/herb samples in the United Arab Emirates.

In our study, the primary risk is environmental, as the plants grow in natural habitats that are exposed to pollutants. The aim was to assess whether the environment in Prizren is polluted and evaluate the safety of plants sold in street markets, considering the additional exposure to metal dust from traffic. In Kosovo, most plants, including those in our study, are commonly found in street markets rather than processed outlets, increasing the potential risk of contamination. Beyond the environmental exposure, another risk lies in the correct identification of the plants. Misidentification can further compromise the safety of these plants.

Research by Stanojković-Sebić et al. (2017) indicates that plants collected near roads, particularly those near industrial activities, may contain toxic levels of chromium (Cr) and copper (Cu). Specifically, species like *Taraxacum officinale* L. and *Trifolium pratense* L. have been shown to accumulate dangerous concentrations of these metals, making them unsafe for human use.

Medicinal herbs have a known ability to accumulate heavy metals, and some are even used for soil remediation. In our study, while none of the plants demonstrated high metal accumulation, *S. marianum* stood out as having higher levels of Cu, Pb, and Zn compared to the others. This finding is consistent with Papadimou et al. (2024), who noted that *S. marianum* can thrive in contaminated soils, particularly those with high levels of cadmium (Cd). Interestingly, *S. marianum* accumulates Cd in the roots and stems without transporting it to the seeds, indicating that its seeds, when harvested from contaminated soil, remain safe for consumption. This characteristic makes *S. marianum* a promising candidate for phytoremediation, where its roots and stems could be discarded, and its main products (e.g., oil and seed extract) could remain free from contaminants.

A similar case can be made for *A. millefolium*, which is not a prominent heavy metal accumulator, but it shows tolerance to polluted soils, growing without significant damage despite not accumulating metals in large amounts (Murtiç et al. 2021). Additionally, *C. arvensis*, a wild grass resistant to harsh growing conditions, has been recommended in some countries for phytoremediation purposes, especially for Pb and Hg, alongside other species like *Convolvulus prostratus* Forssk. and *Convolvulus pluricaulis* in India (Kulhari et al. 2013).

From a public health perspective, the plants studied in our research present a low risk of heavy metal exposure. However, it is critical to ensure the control of heavy metals in phytotherapeutic products. As symptoms of heavy metal toxicity typically arise only after prolonged exposure, many countries have established rigorous control measures for medicinal plants. These measures include mandatory testing for heavy metals with each shipment, as highlighted by Dghaim et al. (2015). This underscores the importance of sourcing plants from clean areas, and performing regular heavy metal analysis in accordance with WHO guidelines for permissible limits (Kulhari et al. 2013).

Conclusions and Recommendations

The present study assessed the concentrations of selected heavy metals and essential minerals in *Silybum marianum*, *Achillea millefolium*, and *Convolvulus arvensis* collected from a roadside environment. Overall, the results indicate low to moderate environmental contamination, with most measured elements occurring within ranges commonly reported for medicinal plants. The calculated risk indicators (EDI, THQ and HI) remained consistently below the critical threshold value of 1, indicating no significant non-carcinogenic health risk for adults under the assumed consumption scenario.

Although certain elements, particularly chromium and iron, exceeded guideline or typical background or guideline values in specific species, these elevations did not translate into increased health risk according to the applied risk assessment framework. Lead, nickel, manganese, zinc and copper concentrations were generally within acceptable limits, supporting the safe use of the plants investigated for phytotherapeutic or culinary purposes, provided that harvesting and processing practices are properly controlled. The observed interspecific differences in metal accumulation – such as higher Cu, Pb and Zn concentrations in *S. marianum* and elevated Cr, Fe, Mn and Ni levels in *C. arvensis* – highlight species-specific uptake patterns and suggest differing physiological responses to environmental metal availability.

Based on these findings, several practical recommendations can be proposed:

- Medicinal plants should be collected and cultivated in environmentally safe areas, away from intensive traffic and potential industrial sources, to minimize the risk of metal contamination.
- Species showing higher accumulation capacities, particularly *S. marianum* and *C. arvensis*, may be further explored for phytoremediation purposes in soils moderately contaminated with heavy metals, provided that their use for human consumption is avoided in such contexts.
- Public awareness should be raised regarding the importance of proper plant identification and controlled harvesting, especially when plants are collected from wild or roadside habitats.
- Regular environmental and toxicological monitoring of medicinal plants is recommended to ensure product quality and protect public health.

The results of this study provide valuable information for environmental monitoring, public health protection and sustainable management of medicinal plant resources. They contribute to interdisciplinary fields including environmental science, toxicology, pharmacology and sustainable agriculture, and support the development of regulatory measures for the safe and sustainable use of medicinal plants in Kosovo and comparable regions.

Despite its contributions, this study has several limitations. It was conducted at a single sampling location, included a limited number of plant species, and did not incorporate soil or detailed environmental parameter analyses. In addition, the absence of long-term exposure data restricts conclusions regarding potential chronic health effects. Future research should address these limitations by integrating soil physicochemical properties (e.g. pH, texture, organic matter and elemental composition), biological factors (such as plant genotype and soil microorganisms), and multi-site sampling designs to better elucidate the mechanisms governing metal uptake and accumulation in medicinal plants.

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SOCIAL PERCEPTIONS OF THE PRESENT AND FUTURE OF POND LANDSCAPES FROM INHABITANTS AND STAKEHOLDERS: RESULTS AND PERSPECTIVES IN EUROPE, TÜRKIYE AND URUGUAY

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ABSTRACT

Despite the crucial importance of pond landscapes for biodiversity conservation, they are less studied, especially in terms of their impacts on people and society. This paper presents the results of a survey carried out on the perceptions of inhabitants and stakeholders across 17 pond landscapes in six countries in Europe, as well as Türkiye and Uruguay. We collected 117 and 590 answers from stakeholders and local inhabitants, respectively, through questionnaires, including questions about their perceptions and preferences. Our results show that pondscapes are widely valued for their benefit to the quality of life and biodiversity. Three Nature's Contributions to People are considered important by both groups: 'creation and maintenance of habitats', 'physical and psychological experiences' and 'maintenance of options' (i.e. potential opportunities offered by nature to ensure resilience). Similar perceived threats related to 'climate change' and 'pollution' have been identified by stakeholders and inhabitants in all countries and have a direct impact on the maintenance of the most important contributions. The perceptions of potential solutions to identify threats are quite similar for most pondscapes, with conservation and maintenance actions being the most important for enhancing ecosystems and societal resilience to climate change and other societal challenges.

Keywords: nature-based solutions; nature's contributions to people; pond conservation and management; pondscape

Introduction

Ponds represent an estimated 304 million standing waterbodies of less than 10 hectares worldwide (Céréghnio et al. 2014). The number of ponds has fallen dramatically in many places. In Europe, the reasons (Hill et al. 2018) are related to land use change (Curado et al. 2011; Smith et al. 2022), urbanization (Brans et al. 2018) and climate change (Fahy et al. 2024). Additional anthropogenic pressures that affect biodiversity and ecosystem functioning (Stamenković et al. 2019) include pollution and the appearance of exotic and invasive species (Smith and Buckley 2020; Macedo et al. 2024). Ponds are environments that can make a significant contribution to aquatic biodiversity, for example, by harbouring 70% of the pool of freshwater species in European landscapes (Davies et al. 2008). They also make other contributions, such as the provision of water for livestock, pollination,

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carbon sequestration, fish production and recreational facilities (Oertli et al. 2023). Depending on the context, ponds can also contribute to cultural heritage (Delpero and Volpato 2022), flood regulation and tourist attractiveness (Turkelboom et al. 2021; Pereira-Lindoso et al. 2025). All the life that these ponds support makes a general contribution to human well-being (Jiang et al. 2023; Vasco et al. 2024). Over the last decade, pond landscapes, also known as pondscapes (Boothby 1997; Borthagaray et al. 2023), have gained traction in the field of pond science and management due to interest in thinking about them as networks and better integrating them with land uses. In a way, this concept overlaps with the other notion of waterscape (Karpouzoglou and Vij 2017), which invites us to think about the interplay of social and biophysical components of the landscapes.

In light of this perspective, better management of ponds at the pondscape level is increasingly promoted to achieve integrated management actions at the crossroads of water management, biodiversity conservation, and land planning (Hill et al. 2021; Cuenca-Cambronero et al. 2023). As mentioned by de Necker et al. (2024), it is essential to understand the opinions and knowledge of individuals on pondscapes when making any decisions regarding the conditions of these water bodies. In the face of growing societal demand for information and involvement, the identification of pond values provides the basis to prioritize the most important social and environmental issues for local actors (López-Rodríguez et al. 2015), improve their condition, fine-tune their management, and better integrate their management plans with regulatory and planning frameworks in many countries (Ryfisch et al. 2024).

Although largely focusing on Europe, ponds are spaces that have been studied in recent years by social science, whether concerning fishpond management (Popp et al. 2019; Lasner and Antje 2024; Zdeněk et al. 2025), their uses in relation to socio-economic interests (Blicharska et al. 2017; Vo et al. 2023; Bartrons et al. 2024), implementation of public policy (Ryfisch et al. 2023) and maximizing their ecosystem services (Jiang et al. 2023; Rey-Valette et al. 2024). Several studies show that pondscapes are highly valued by people, bringing psychological benefits (Zhang 2021), as well as cultural services (Oertli and Parris 2019). However, it is possible to collect some contrasting perceptions of pondscapes as spaces intended either exclusively for human use with anthropogenic pressures (Bouahim et al. 2014) or for uses oriented towards biodiversity conservation (Zamora-Marin et al. 2021). The social perception approach has also been mobilized recently to understand the contribution of ponds to well-being (Rey-Valette et al. 2022; Vanhöfen et al. 2025), the relationship of inhabitants with biodiversity (Vasco et al. 2024), their role in supporting local identities and water quality in urban environments (Mitroi et al. 2022). The social perception approach examines how individuals process information using their senses, experiences, and cognitive activity to make sense of what they understand and observe (Zebrowitz 1990). Perceptions are themselves influenced by knowledge of the environment and depend on social, economic and cultural context (Cauberghe et al. 2021). The analysis of perception can help take social and geographic perspectives into account (Castro et al. 2014; Quintas-Soriano et al. 2018) by proposing strategic ecological intervention measures. For example, meeting the specific needs of local actors can contribute to cultural heritage, pond restoration or biodiversity conservation, educational intervention, or preventing area degradation.

Cuenca-Cambronero et al. (2023) and Hill et al. (2021) identified research priorities for ponds and pondscapes that the social perceptions approach can strongly contribute to cover, such as climate change, water quality deterioration, biodiversity decline, and the delivery of environmental benefits. In this vein, we used two concepts, Nature-based Solutions (NbS) and Nature's Contributions to People (NCP), in order to assess perceptions of these challenges by linking them to the biophysical properties and functions of the pondscapes. The NCP concept (Díaz et al. 2018) represents nature's key positive and negative contributions to people and considers the socio-cultural dimension by identifying all links between society and nature. To interpret what nature is and understand its benefits for human societies, the NCP perspective emphasizes culture as one of the main factors in identifying bonds between society and nature (Pedersen et al. 2019). Specifying the relationship with nature is crucial to understanding the context-specific viewpoints and different knowledge systems (Pascual et al. 2017). The same NCP may be perceived as beneficial or detrimental depending on the cultural, socioeconomic, temporal, or spatial context. Within the NCP framework, nature contributes to the quality of life through material (e.g. food and feed), nonmaterial (e.g. recreational activities), and regulating processes (i.e. regulation of hazards), which are affected by natural or anthropogenic drivers. The concept of NbS (Nesshover et al. 2017) refers to measures based on or inspired by nature that are implemented to modify the functioning of environments and deliver specific outcomes to people while providing benefits to biodiversity and ecosystems. The NbS concept includes various forms, from creating, protecting, or restoring natural ecosystems to management measures. Therefore, NbS and restoration are not identical (Waylen et al. 2024). NbS is grounded on two logical follow-up ideas: (1) natural and artificial ecosystems must be managed to secure the production of a diverse range of services that impact the quality of life (Mell and Clement 2019); (2) this implies understanding and producing knowledge on socio-ecological processes and determining the most appropriate way of addressing environmental challenges.

In this study, using a survey, we explored social perceptions of different pondscapes in Europe, Türkiye and Uruguay to identify common patterns despite local variations and different respondents. The paper highlights the importance of studying several types of study sites in areas with different histories and uses to gather as many different perceptions as possible. It is thus important to fill a knowledge gap concerning the variability of perceptions according to the contexts of the study sites and the types of respondents. The study areas have never been studied from the perceptions point of view. Analysis of responses can provide important insights on eliciting motivation for pond management measures and the design of public policies.

This paper takes a global and comparative view by examining the responses of stakeholders and inhabitants. By stakeholder, we mean anyone who keeps abreast of pondscape news and is professionally, politically or associatively involved in its management. This framing allows us to draw up an exhaustive inventory of the existing expectations of inhabitants and stakeholders regarding current and potential future contributions. In doing so, we provide a step toward valuing the role of pondscapes for the quality of life, estimate the importance of certain challenges, assess the perceptions of different NCPs, and determine which NbS measures should be prioritized. It is likely that a large part of the perceptions could be in favor of the conservation of pondscapes. In light of the results, we discuss this hypothesis using the literature in order to identify ways of making a positive contribution to the management of these pondscapes.

Materials and Methods

Questionnaire content and dissemination

We distributed questionnaires in six European countries (Belgium, England, Denmark, Germany, Spain/Catalonia, Switzerland), Türkiye and Uruguay between 2021 and 2023. Questionnaires were developed with the specific purpose of assessing the inhabitants' and stakeholders' perception of pondscape offered by each different case study. Two different questionnaires were used for stakeholders and inhabitants. While both questionnaires largely contain the same questions for all pondscapes, they also include different questions relating to the profile of the inhabitants and stakeholders regarding their activities and their role in pondscape management. These specific questions were tailored to each type of respondent to better understand the relevance of their answers in relation to their profiles, were informative and had no connection to their social perceptions of pondscapes. In this article, through a comparative study, we present the results of the common questions asked of the two types of actors surveyed. Data from stakeholders and inhabitants have been analyzed to determine convergences and divergences between them. The questionnaires included several common questions covering topics such as: profile of the respondents, relation to nature and pondscape, the self-reported rating on the role of pondscapes on quality of life, visual perception of changes over time, perception about the environmental condition of the pondscape, perception of NCPs, perception of the most important threats and impact of threats to the pondscape in future and perception on the selected measures of NbS. Perceptions of the contribution of pondscapes to life quality may depend on familiarity and intensity of use, but also on connection and commitment to nature. Therefore, we have included these questions to understand the respondents' profiles. We selected closed questions, including questions concerning preferential choice (ranking) and rating. Several questions comprised close-ended dichotomous and multiple-choice questions to gain structured data on specific topics. A Likert rating scale was chosen to measure respondents' personal attitudes toward particular topics (Brown 2000). As interval data, we use the Likert scale where 1 is the lowest and 5 is the highest rating. The template of the common questions of the questionnaire is attached as Annex 1.

Templates of both questionnaires were written in each local language (Catalan, Danish, Dutch, English, French for Switzerland, German, Spanish for Uruguay, and Turkish). The translation of the questionnaire was standardized across countries, so that questions and answers were exactly the same. Only the unit of measurement varied (km-mile, hectare-acre) and was considered in calculating the results (Table S1). Before starting the questionnaire, a brief presentation of the research project was provided, along with a confidentiality statement and a guarantee of anonymity. Responses to the questionnaires were obtained via the internet and on-site during times spent on the pondscape.

The completed questionnaires were entered into a LimeSurvey database (http://www.limesurvey.org) that we developed for this study. Online questionnaires were collected via LimeSurvey and the paper were later entered to LimeSurvey by the authors.

Approach regarding the NCP and NbS framework

Díaz et al. (2018) proposed 18 different types of NCPs, of which not all are necessarily relevant to ponds. The list of 18 types of NCPs was therefore filtered according to their relevance for pondscapes and reduced to a final list of 11 NCPs (Table 1), selected to determine the contributions they provide and how they impact quality of life.

As suggested in the study by Gonzalez-Ollauri et al. (2023) on the selection of NbS concerning hydro-meteorological hazards, the list of NbS was also filtered according to their relevance for pondscapes. The NbS considered in the paper are:

- Pond creation (e.g. digging a pond in a place where there was formerly no waterbody);
- Pond restoration (e.g. digging a pond in a place where formerly a pond existed; regenerating a landfilled pond; undertaking important transformations on an existing pond that was functionally lost);

Table 1 List of the 11 types of NCPs selected in this survey based on IPBES (Díaz et al. 2018).

		Explanations
Regulation of environmental processes	Habitation creation and maintenance	Diverse habitats (shelter, nesting, breeding, refuge…) for numerous freshwater species such as aquatic plants, benthic invertebrates, amphibians, fish…
	Pollination	Favourable habitats for beneficial insects, such as wild bees and syrphid flies, due to the surrounding vegetation and the water supply in the pond landscape.
	Regulation of climate	Ponds can influence the microclimate by cooling or warming the surrounding air. Ponds also have the potential to sequester greenhouse gas and capture carbon through wetland vegetation.
	Regulation of water quantity, location and timing	A pond, being a water reservoir, can contain a certain level of stormwater and serve to reduce the amount of water delivered downstream.
	Regulation of water quality	Ponds can be important in purifying water against pollutants, by retaining them through algae, plants and other organisms present in the environment.
	Regulation of hazards and extreme events	Prevent flooding during heavy rainfall events.
Materials and assistance	Food and feed	Fishponds, livestock watering…
Non-materials	Physical and psychological experiences	Place open to the public and providing a great environment in which people can exercise and relax.
	Learning and inspiration	People can learn and be inspired by contact with nature. This space can also be used and studied for educational programmes.
	Supporting identities	Cultural heritage, local identity.
Other	Maintenance of options	Potential opportunities offered by nature to ensure resilience in the future. Pondscape's ability to keep options open for the future.

- Pond infrastructure and local management actions (e.g. removing some vegetation or tree shade; removing or introducing species; water management);
- Landscape management actions (protective status, changing land use around the landscape of ponds, enhancing the connectivity between ponds or the landscape of ponds).

Presentation of the studied pondscapes

The seventeen pondscapes where the questionnaires were distributed are shown in Table 2, along with their main characteristics. They have not been chosen to be representative of all European, Turkish or Uruguayan pondscapes but rather to represent a diversity of areas in terms of bioclimatic zone (Continental, Mediterranean, Subtropical, Arid context, Atlantic), surface area of the pondscapes (less than 1 km² to 30 km²) and pond age (from newly created ponds to naturally occurring ponds that have existed for thousands of years). The pondscapes are integrated into watersheds with a variety of land uses (arable, urban and suburban zone, grassland, pasture, nature reserve) and have different land tenure relationships. Pondscapes have all seen measures implemented in recent years to improve their condition. On the one hand, across all study areas, the creation of ponds has been the most implemented measure, followed by management actions, and then measures to restore ponds (Table 3). These features may directly affect the relationship of stakeholders and inhabitants to the pondscapes because the issues and challenges linked to them may be different. This selection of study sites with natural or artificial nature of a pond is explained by the context of each country. While in European countries, pondscapes tend to be located in recreational areas, dedicated to biodiversity or no intended purpose of use, the context in Uruguay and Türkiye is a little different. In Uruguay, man-made ponds are located in isolated areas and mostly dedicated to livestock watering and secondarily for low scale irrigation support to boost rural production. In Türkiye, the three pondscapes are close to a large lake and flats (Dolcerocca et al. 2024) with no officially identified use for the ponds, but which are currently used as nature areas, landfill sites, informal pleasure gardens with water pumping system.

Samples of respondents

In all countries combined, we collected 117 completed answers from stakeholders and 703 completed answers from the different inhabitants, of which 590 have visited the pondscapes at some point (Table 3). Given that these pondscapes are located in different countries, their management mode is not as institutionalized as other water bodies such as large lakes and rivers. There is no exclusive legal responsibility (uncertain status of ownership) for the ponds in some countries, which presents the challenge of identifying the stakeholders. This explains the limited number of responses from stakeholders.

There are large differences in the number of responses from inhabitants between the seventeen pondscapes studied. We identified four groups of pondscapes with a similar number of answers:

Table 2 Characteristics of the studied pondscapes.

Country	Name of the pondscape	Bioclimatic zone	Landscape and surrounding land use	Land tenure	Area (km²)	Number of ponds in the pondscape	Pond sizes (m²)	Activities in the pondscapes
Switzerland	**Bois de Jussy**	Continental	Rural with woodland and agriculture	Mostly public	7	25	100 to 4000	Wildlife watching Hiking
	Rhône Genevois		Suburban (agricultural, woodland, partly nature reserve)	/	16	40	50 to 20 000	Hiking Wildlife watching
Germany	**Schöneiche**	Continental	Suburban with agriculture	Mostly private	16	12	100 to 20000	Relaxation Wildlife watching
Türkiye	**Dikkuyruk**	Central-Anatolian arid-cold steppe climate	Peri-urban (wheat field, landfill, ornamental garden with water pumping system, near a lake)	Mostly private	0.58	4	4000 to 80000	Wildlife watching Hiking
	Gölbasi			Mostly public	0.26	23	100 to 10.000	Hiking Relaxation
	Imrahor			Private	2.51	12	225 to 57000	Informal uses
England /UK	**Pinkhill Meadows**	Atlantic	Rural and floodplain (Thames)	Private	0.1	50–60	20 to 3000	Wildlife watching Relaxation
	Water Friendly Farming		Rural with farmland	Mostly private	30	120	100 to 5000	Wildlife watching Relaxation
Catalonia /Spain	**Albera**	Mediterranean	Rural with agriculture	Mostly private	25	23	450 to 62000	Wildlife watching Hiking
	La Pletera		Suburban and coastal marshes	Public	0.6	20	100 to 3600	Wildlife watching Cycling
Belgium	**Gete Vallei**	Atlantic	Rural with grassland/ arable land	Mostly private	>10	41	100 to 150	Hiking Cycling
	Pikhakendonk		Rural with grassland	Mostly private	0.1	62	100 to 150	Hiking Relaxation
	Tommelen		Nature reserve near urban area	Public	0.12	144	100 to 150	Hiking Relaxation
Uruguay	**Sierra de los Caracoles**	Subtropical/ temperate humid	Rural with pastures and grasslands for grazing cattle	Private	>10	25	400 to 5000	Agriculture Education purpose
	La Pedrera				>10	18		Hiking Picnic
Denmark	**Lystrup**	Continental	Suburban	Mostly public	8–30	14+	100 to 1500	Wildlife watching Relaxation
	Fyn		Rural with pasture/ arable	Mostly private	8–250	30+	100 to 1500	Hiking Wildlife watching

- Three pondscapes with more than 80 responses: Rhône Genevois in Switzerland, Albera and La Pletera in Spain;
- Four pondscapes between 30 and 60 responses: Pinkhill Meadows, Schöneiche in Germany, Gölbasi in Türkiye, Bois de Jussy in Switzerland;
- Four pondscapes between 15 and 30 responses: Pikhakendonk and Tommelen in Belgium, Lystrup in Denmark, Water Friendly Farming in England;
- Six pondscapes between 2 and 12 responses: Fyn in Denmark, the Uruguayan pondscapes, Gete Vallei in Belgium, Imrahor and Dikkuyruk in Türkiye.

Given these discrepancies and the low number of responses per pondscape for the last group of six pondscapes, we will place less emphasis on their results when presenting comparisons between pondscapes but we will keep them for the figures including all pondscapes combined. However, it is important to explain the low number of responses due to the difficulty of collecting data on ponds. Many are not located in tourist or frequented areas. Population density in the immediate vicinity of some ponds (particularly in Uruguay, Turkey, and Funen, Denmark) is low, with some ponds having a small surface area. Other ponds are difficult to access (Uruguay, Funen, Denmark, Water Friendly Farming in England, Turkey), with some areas being geographically isolated. The temporary or permanent disappearance of ponds (in Spain and Germany) does not attract particu-

Table 3 NbS implementend of the studied pondscape in recent years.

Country	Name of the pondscape	Pond creation	Pond restoration	Pond infrastructure and local management actions (e.g. digging, vegetation clearing)
Switzerland	**Bois de Jussy**	X	X	X
	Rhône Genevois	X	X	X
Germany	**Schöneiche**	X		X
Türkiye	**Dikkuyruk**	X	X	
	Gölbasi	X	X	
	Imrahor		X	X
England/UK	**Pinkhill Meadows**	X		X
	Water Friendly Farming	X		X
Catalonia/Spain	**Albera**		X	X
	La Pletera	X	X	
Belgium	**Gete Vallei**	X	X	X
	Pikhakendonk	X	X	
	Tommelen		X	
Uruguay	**Sierra de los Caracoles**	X		X
	La Pedrera	X		X
Denmark	**Lystrup**	X		X
	Fyn	X	X	X

Table 4 Number of answers per respondent type (i.e., inhabitants and stakeholders) and pondscape.

Country	Pondscapes	Number of answers	
		Inhabitants	Stakeholders
Switzerland	**Bois de Jussy**	57	7
	Rhône Genevois	84	7
Germany	**Schöneiche**	44	5
Turkey	**Dikkuyruk**	8	8
	Gölbasi	41	9
	Imrahor	2	6
England	**Pinkhill Meadows**	32	11
	Water Friendly Farming	18	6
Spain	**Albera**	92	17
	La Pletera	118	9
Belgium	**Gete Vallei**	8	5
	Pikhakendonk	22	5
	Tommelen	23	7
Uruguay	**Sierra de los Caracoles**	12	4
	La Pedrera	4	3
Denmark	**Lystrup**	17	3
	Fyn	8	5

lar attention. As shown in Table S1 (supplementary data), the profile of stakeholders varies from one pondscape to another. The stakeholders see themselves mostly in the role of counselling, public authority and civil society. Regarding the profile of the inhabitants (supplementary data), some pondscapes attract a very local population on average with less than 5 kilometers away whereas half of the pondscapes in this study are visited by people who live more than 10 kilometers away. That distance explains why inhabitants do not visit the pondscapes more frequently.

Statistical analyses

All the graphical representations and statistical analyses were carried out with R and RStudio software (R Development Core Team 2025). Graphics have been made using the *ggplot2* package (Wickham 2016) or associated packages such as *ggradar* (for certain types of graphic representation, Fig. 1). All results based on these questionnaires are expressed with mean scores (from 0 to 5, and accompanied by standard deviations) or as percentages. These responses are presented by pondscapes, by type of respondents or for all respondents combined (main figures and supplementary data), according to the most readable and useful way of presenting them. Our aim was to analyze the data as a whole, comparatively by study site, but also by type of respondents. Where results vary between pondscapes, we also highlight comparisons between study sites. Hence, the results are presented at one or two levels of analysis: by pondscape and all pondscapes combined, to show both specific features and overall trends. Comparisons between the responses provided by inhabitants and stakeholders were carried out using t-tests. Differences between pondscapes were analyzed using ANOVA. For both types of tests, the alpha risk was set at 5%. For the questions on threats and impacts, we have chosen to retain only the top-3 responses, to make it easier to read the results, but also to easily identify general trends and compare both between pondscapes and between types of respondents.

Results

Relation to pondscapes and the environmental condition of the pondscapes

We asked participants a series of questions to find out about their relationship with nature, the pondscape that concerns them and the importance of this same pondscape for their quality of life. We included a table in the supplemental data (S2).

Inhabitants and stakeholders gave both high scores to express their connection with nature (i.e. replies to the question: how would you describe your relationship with 'nature'?) with a mean of, respectively, 4.4 (± 0.8) and 4.7 (± 0.6) out of 5. The maximum average score was 5 for La Pedrera in Uruguay, and the minimum was 3.5 for the Imrahor pondscape in Türkiye (Fig. 1). Both respondent types also gave high marks to their relationship with the pondscapes (between 3 and 4.2 for inhabitants with a mean value of 3.8 (± 1.0) and between 3.2 and 4.6 for stakeholders, with a mean value of 4.0 (± 1.1) and quality of life (between 3.1 and 4.1 for inhabitants, with a mean value of 3.8 (± 1.1) and between 3 and 4.8 for stakeholders, with a mean value of 4.2 (± 1.0). We note that the given scores are higher for the relation with nature than for the relation with pondscapes (Fig. 1; t test, $t = 13.83$, $df = 1202.8$, $p < 0.001$). Stakeholders gave higher ratings than inhabitants for the connection to nature and the quality of life (t tests, respectively; $t = -4.94$, $df = 188.45$, $p < 0.001$ and $t = -3.41$, $df = 175.92$, $p < 0.001$). Despite

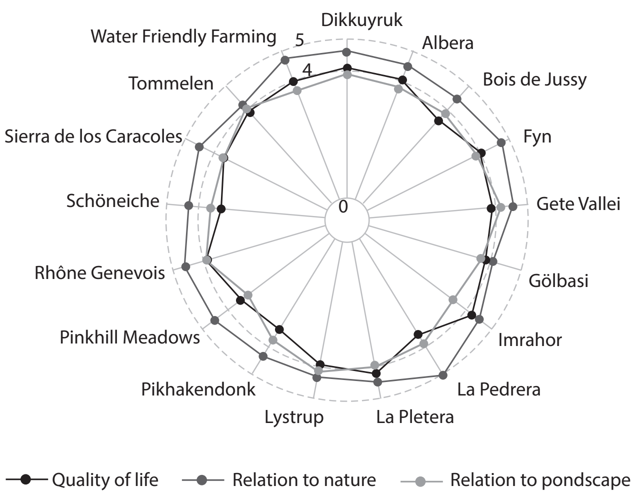

Fig. 1 Representation of the average scores given by the two types of respondents (inhabitants and stakeholders) on their relationship with nature (intermediate grey line), the pondscape (grey line) and the pondscape's contribution to their quality of life (black line), for each pondscape.

a higher average score, this difference is not significant for the relationship with the pondscape ($t = -1.44$, $df = 156.02$, $p = 0.07$). This trend is reversed for the Uruguayan pondscapes, and to some extent in Türkiye ones. Despite the variability of the results obtained, the average scores given to these three questions are high (>3), regardless of the pondscape or type of respondents (Fig. 1).

To get an overview of the condition of pondscapes from the respondents' point of view, we asked them how they assess the environmental status of pondscapes (Fig. 2) and what changes they noticed over the last ten years. Across all the pondscapes, the inhabitants gave an average score of 3.6 (± 0.9), while the stakeholders gave a score of 3.4 (± 1.0), with a significant difference (t test, $t = 2.06$, $df = 138.01$, $p < 0.05$). Except for the Pinkhill pondscape in England, Albera in Spain, Bois de Jussy in Switzerland and Dikkuyruk in Türkiye, the inhabitants gave a higher score than the stakeholders about the environmental status of the pondscape (Fig. 2), contrary to the trend observed for the first questions. Concerning this environmental status, there were divergent perceptions between stakeholders and inhabitants in Gete Valei (± 0.9), Pink Hill, Gölbasi and Dikkuyruk (± 0.8), Lystrup, Pikhakendonk, Fyn, Rhône Genevois and the Uruguayan pondscapes (± 0.6). In contrast to the first three questions, there was a slightly greater variability between pondscapes, with a minimum mean score of 1.6 given by Schöneiche stakeholders in Germany (2.4 by inhabitants) (Fig. 2). Conversely, Pinkhill stakeholders gave the highest mean score of 4.7. Considering both types of respondents, the pondscape effect is significant (ANOVA, $df = 16$, $F = 14.11$, $p < 0.001$). Except for Schöneiche (Germany), environmental status appeared satisfactory from the respondents' point of view.

To further address the perception of each landscape's status, we asked respondents to select the main visual changes observed during the last decade. With multiple-choice questions, we have retained here the most selected answers according to the number of responses selected by respondents (Table 5). Some negative changes ('decrease of pondscape surface area', 'more frequent drying pond' and 'lower pond water level') were mainly selected by both types of respondents. Conversely, only one positive change, 'increase in the number of ponds', was selected. When combining all pondscapes and answers, the negative changes highlighted exceed the positive ones. The perceptions of the changes observed were similar for residents and stakeholders in Switzerland and Water Friendly Farming (England) with positive changes, Germany, Spain, Tommelen (Belgium) with negative changes.

Analysis conducted to understand perceptions of threats and their impacts

Perceptions of threats and their impacts on pondscapes are represented in Figures 3 and 4. The threat 'climate change' was selected in the top-3 in 13 out of 17 pond-

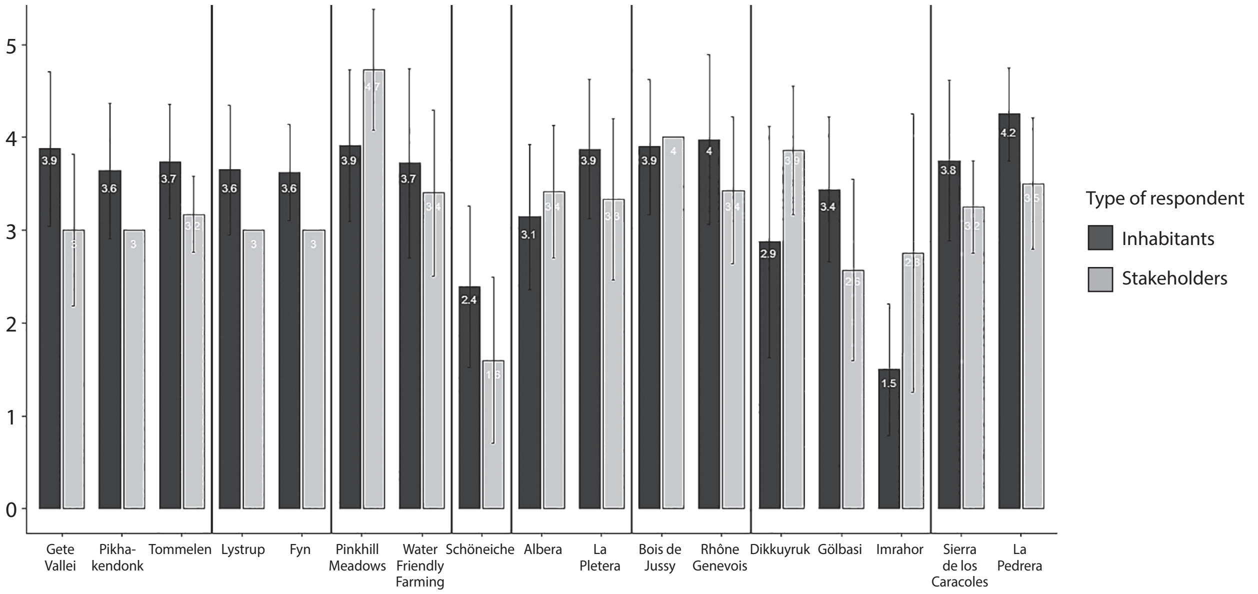

Fig. 2 The environmental status per pondscape according to the type of respondent (inhabitants in black and stakeholders in gray). The values represent the averages of the various responses obtained, and the bars represent the standard deviations.

Table 5 Comparison between inhabitants and stakeholders on the changes observed during the last decade (S = Stakeholder. I = Inhabitants). The term "citations" refers to responses that were quoted at least twice by respondents to ensure congruence between responses.

Country	Pondscape	Decrease of pondscape surface area	More frequent drying ponds	Increase in the number of ponds	Lower pond water level	Colonization of new plant species	Colonization of new plant species	Deterioriation of water quality	Improvement of water quality	Higher pond water level
Switzerland	Bois de Jussy			S & I		I	S & I			
	Rhône Verbois			S & I		S & I	I			
Germany	Schöneiche	S & I	S & I							
Turkiye	Dikkuyruk	S			I			S & I		
	Gölbasi	S			S			I		
	Imrahor	S & I						S & I	I	
England	Pinkhill M.			S		S	I			
	Water Friendly F.					S & I	S & I			
Spain	Albera	I	S & I		S & I					
Belgium	Gete Vallei		I	I				S	I	
	Pikhakendonk		S & I		S					I
	Tommelen	S & I	S & I				I			
Uruguay	Sierra de los C.			S		I	S			
	La Pedrera			I		I	I			
Denmark	Lystrup					I				
	Fyn	S			S	I				
Total number of citation		6S & 3I	4S & 5I	4S & 4I	4S & 2I	3S & 7I	3S & 6I	3S & 3I	2I	1I

scapes (global average for the 13 pondscapes in S1 in supplementary data: 4.1), followed by 'pollution' (9 out of 17 pondscapes), and 'tourism' (9 out of 17 pondscapes) according to inhabitants (Fig. 3). Their responses on impacts highlight that the 'impact on biodiversity' is the primary concern (selected 14 times out of 16 pondscapes with a global average available in Fig. S2 in supplementary data: 4.3). 'Impact on water quantity' (11 out of 16 pondscapes with a global average of 4) was selected as second, followed by 'impact on water quality' (selected 11 times out of 16 pondscapes with global average: 3.9). Interestingly, the data from stakeholders (Fig. 4) are fairly similar, with 'climate change' (15 out of 17 pondscapes, global average: 4.4) and 'pollution' (10 out of 17 pondscapes, global average: 3.8) chosen by the majority as the main threats (Figure 4). Concerning the impact of threats, stakeholders have given priority in top-3 to 'impact on biodiversity' (13 out of 17 pondscapes, global average:

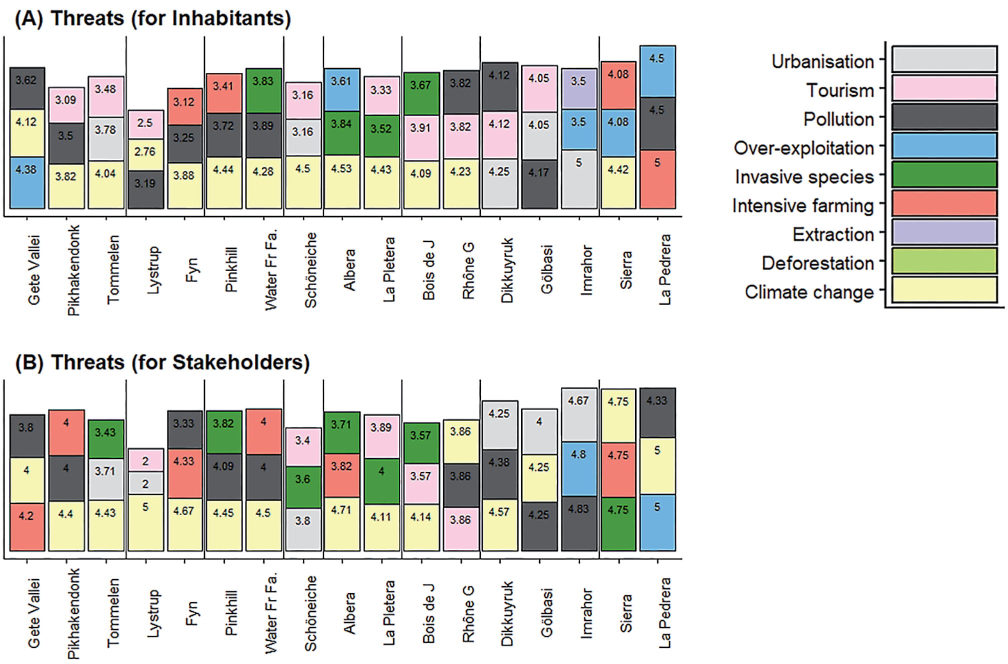

Fig. 3 Threats assessment in inhabitants' and stakeholders' responses per pondscape. Mean scores are given for the top three for each pondscape.

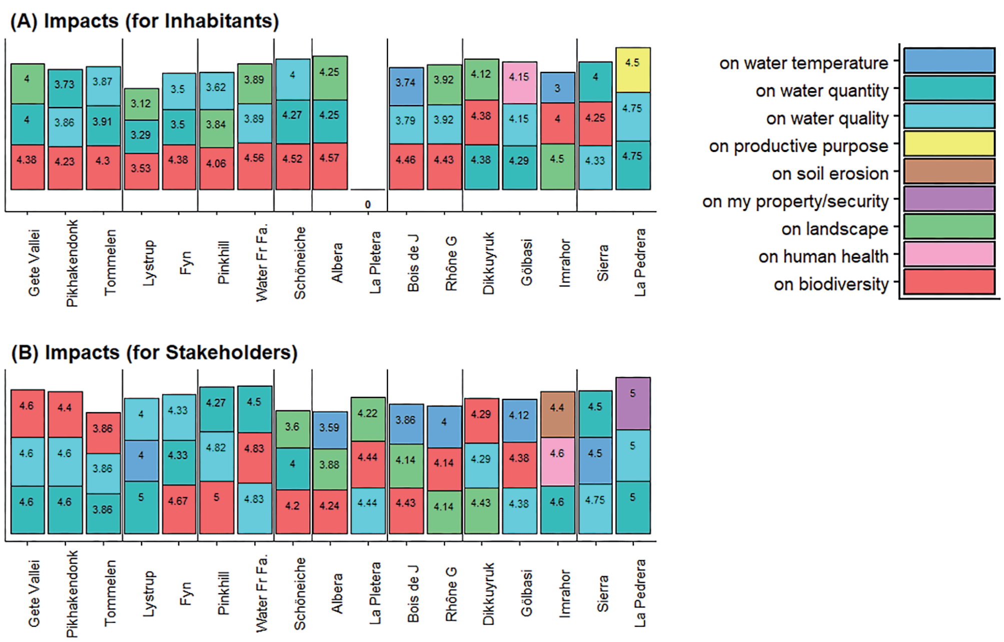

Fig. 4 Impacts of threat assessment in stakeholders' and inhabitants' responses per pondscape. Mean scores are given for the top three for each pondscape.

4.4), 'impact on water quality' (12 out of 17 pondscapes, global average: 4.4) and 'impact on water quantity' (11 out of 17 pondscapes, global average: 4.4). This means that the views are quite aligned between both samples.

About differences between pond landscapes, the Turkish and Uruguayan respondents selected slightly different answers compared to the other pondscapes, given their agricultural context. In particular, they perceived "overexploitation" and "urbanization" as significant threats (Fig. 3 and 4). We also observed that Turkish and Uruguayan pondscapes received the highest average scores on both questions when all responses to threats were combined. Conversely, Danish pondscapes received the lowest overall average score for all threats. The rest of the pondscapes scored in the mid-range.

NCP assessment

The ranking of NCP made by inhabitants and stakeholders is proposed in Fig. 5. The data (Fig. S3, S4, S5, S6 in supplementary data) clearly show similar results

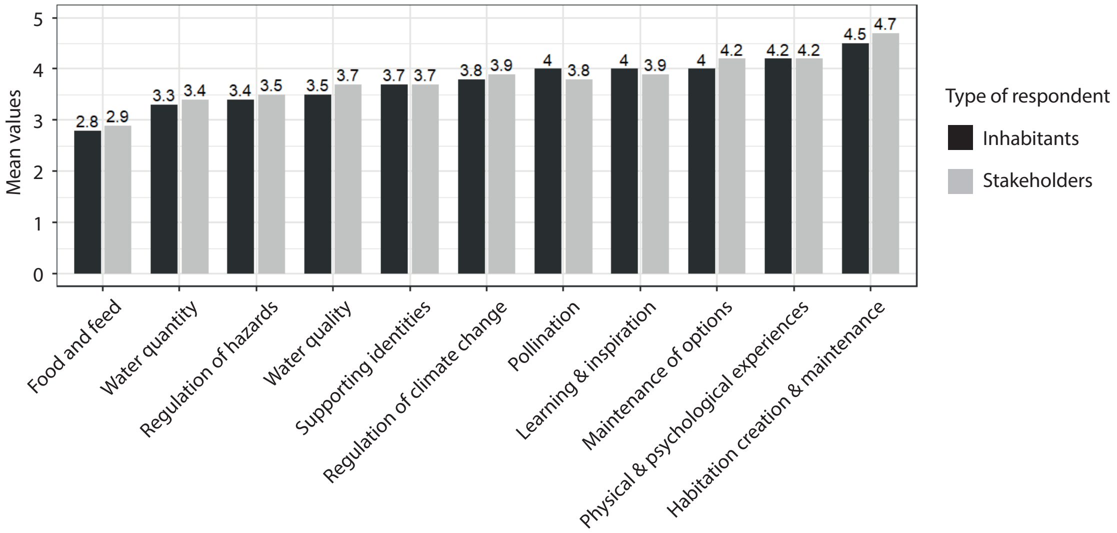

Fig. 5 Mean scores of NCPs assessment from all pondscapes combined, according to the type of respondent (stakeholders in black and inhabitants in grey).

with a clear top-three for a large part of the European pondscapes: 'creation and maintenance of habitats' relating to biodiversity (4.6), 'physical and psychological experiences' (4.2) and 'maintenance of options' as potential opportunities offered by nature to ensure resilience (4.1). The three contributions are important for both types of respondents. Similarly, a certain consensus emerges concerning NCPs that are perceived as more secondary such as 'learning and inspiration', 'regulation of climate' and 'regulation of water quality'. For all NCPs, the two types of respondents give almost identical average scores (t-tests for each, $p > 0.05$), with differences of no more than 0.2 (Fig. 5). This means that the NCP expectations are aligned between them despite some slight differences, resulting in homogeneous perceptions of the NCPs, shared between inhabitants and stakeholders.

Some perceptions of all NCPs are more noticeable than others among the sites studied. For example, we can mention Uruguay and Türkiye with the highest scores for 'food and feed' and 'regulation of water quantity' (supplementary data), although these two NCPs have the lowest scores all sites combined (2.9 and 3.4, respectively). The prioritization of the NCPs is the same between stakeholders and inhabitants.

NbS' assessment

We also compared the results from inhabitants and stakeholders about the perception of the NbS measures that should be implemented in the future (Table 6). Several pondscapes with a lack of data were not included. Analyzing by pondscape, there is often agreement on at least 2 types of NbS measures to be implemented for pondscapes among the 9 proposed, except for Bois de Jussy, Pinkhill, la Pedrera and Tommelen. Therefore, the views of both types of respondents on NbS are more or less aligned without being exactly the same. If we read this table by NbS measures, there are clearly four of them that were selected the most: 'restoration measures', 'maintenance of biodiversity', 'better/more environmental education', 'improving water quality'. On another level, compared to stakeholders, inhabitants chose distinctly the NbS measures 'limitation of certain uses' and 'better/more environmental education'.

Discussion and Perspectives

Pondscapes and quality of life: connecting people to nature

Our results demonstrate that stakeholders and inhabitants both show strong interest in pondscapes, with high mean scores. The stakeholders gave higher scores for the items "quality of life" or "connection to nature" than the inhabitants. The results on "relation to pondscape" show no significant difference between respondents. Except for Germany and England, this high link for stakeholders can be explained by their professional profiles with activities in management and public policy monitoring, and backgrounds focused on biology and ecology. Inhabitants visiting the pondscapes mostly once a month for activities (hiking, wildlife watching and relaxation) demonstrate a strong relationship with ponds thanks to nature exposure and connectedness. This can also be explained by the fact that all pondscapes have seen measures implemented in recent years to improve their conditions. This is consistent with studies on the same subject (Lumber et al. 2017; Rey-Valette et al. 2022; Vasco et al. 2024). These pondscapes are thus widely appreciated by respondents for their contribution to their quality of life, which has also been highlighted by other authors (Xie et al. 2021; McDougall et al. 2022; Vo et al. 2023). In addition to our survey, it would be interesting to find out whether this is linked to the aesthetic quality of the landscape (Hill et al. 2021), the presence of emblematic species (Oertli et al. 2005) and the *naturalness* of the vegetation and the *land-*

Table 6 Comparison between inhabitants and stakeholders on NbS measures (S = Stakeholder and I = Inhabitants). The term "citations" refers to responses that were quoted at least twice by respondents to ensure congruence between responses.

Country	Pondscape	Restoration measures	Maintenance of biodiversity	Better/more environmental education	Improving water quality	Limitation of certain uses	Creating new pond	Increasing water volume	Monitoring of ponds	Abandonment of certain uses
Switzerland	Bois de Jussy	S	S & I	I			I			
	Rhône Verbois	S	S & I	S & I		I				
Germany	Schöneiche	S & I	S	S & I				S & I		
Turkiye	Dikkuyruk	I		S & I	S & I	I				S
	Gölbasi	I		S & I	S & I	I				S
England	Pinkhill M.		S & I	I			I		S	
	Water Friendly F.		S & I		S & I		S & I			
Spain	Albera	S & I	S	S & I		S & I				
Belgium	Gete Vallei	S & I	S & I		S & I					
	Pikhakendonk	S & I		I	S & I	S	S			
	Tommelen	I	S	I		I		S		
Uruguay	La Pedrera	I		S	I	S & I				
Denmark	Fyn	S	S & I		S & I		I			
Total number of citations		7S & 8I	9S & 6I	6S & 9I	6S & 7I	3S & 6I	2S & 4I	2S & 1I	2S	2S

scape structure (Szilassi et al. 2017; Hermes et al. 2018), or the right conditions for practising recreation activities. This would need to be correlated with other nearby green spaces to identify the specificity of the pondscapes in relation to other waterscapes in particular (Karpouzoglou and Vij 2017; Borthagaray et al. 2023).

Perceptions of changes, threats and environmental status

Based on the responses obtained and within the framework of pondscapes that have already undergone measures to improve their condition, positive changes such as the 'increase in the number of ponds' were perceived in seven pondscapes out of seventeen. These pondscapes most often concerned recently restored sites shown in Table 3, such as Bois de Jussy and Pinkhill Meadows. The effects of restoration have, therefore, been perceived and made visible and do not seem to have generated any form of resistance to restoration strategies (van Marwijk et al. 2012; Rajput et al. 2023), as evidenced by the high assessment awarded to the relationship with the pondscape and their contribution to quality of life. Given these positive post-restoration perceptions in certain pondscapes, the restoration frameworks applied seem to converge in socially acceptable and ecologically feasible (Petursdottir et al. 2013).

A wide consensus was found on a range of threats and impacts of threats with significant negative changes such as "more frequent drying of ponds", "lower pond water level", "degradation of water quality", and 'decrease of pondscape surface area' selected. An interpretation could be made to link these main negative changes observed in the majority of the pondscapes (9/16) with the main threats identified, namely 'climate change' and 'pollution', and the main impacts identified by respondents on 'biodiversity' and 'water quantity'. This perception of deterioration over the last ten years are in line with the threats mentioned by de Necker et al. (2024), including climate change and habitat degradation.

It is important to consider the links between social perceptions and the monitoring of pondscapes as the documentation of temporal changes by authorities, which does not exist to date for some of these pondscapes. This is why future research is needed to determine how the different respondent types perceive visible and invisible elements of the landscape's biogeophysical processes. Acceptance of the management of the pondscapes could be affected by not considering the habits (visual, auditory, practical) of the inhabitants. Similarly, it would be interesting to explore why "colonization of new plant species" and "degradation of water quality" were frequently cited as threats, in order to identify the causes from the respondents' point of view. A multitude of anthropogenic disturbances could affect pondscapes (Brönmark and Hansson 2002), and it is important to identify them for each study site so that they can be incorporated into management plans (Biggs et al. 2024). Making visible the types of change and their causes could be a useful decision-making tool in environmental education initiatives (Ardoin et al. 2020).

NCP and NbS assessment

Both stakeholders and inhabitants generally ranked the various proposed NCPs in the same order, suggesting that this is not necessarily dependent on the context of local uses. The NCPs 'habitat creation and maintenance' was considered a priority. This means that respondents are very attached to the pondscapes because they can either derive a source of satisfaction as a place to connect with nature or alternatively enjoy leisure activities on pondscapes that have a fairly direct impact on their quality of life: contemplating nature, relaxation and hiking are important recreational activities for people (Schafft et al. 2021). Two other NCPs, 'physical and psychological experiences' and 'maintenance of options', were also perceived as important. NCP 'physical and psychological experiences' refers to the direct uses of humans with leisure activities (cycling and walking, picnic areas) with beneficial effects on physical and mental health (Lopez-Haro et al. 2024), particularly in urban areas (Vasco 2024). The NCP 'maintenance of options' is interesting and original and was not expected to be so important, especially for inhabitants. This highlights the importance of maintaining or improving the current situation to avoid losing potentially useful options for the future. This NCP makes it possible to link existing nature with future options for use and benefit. There is a convergence of social perceptions between stakeholders and residents revealing the acuteness of risks and impacts in the future with a felt need to think about the future and anticipate the management of pondscapes. The prominence given to this NCP testifies to the importance of intergenerational justice and the quality of life of future generations (Faith 2021).

While our results highlighted that the most selected NCPs are focused on biodiversity, the numerous non-material NCPs (Methorst et al. 2020; Hill et al. 2021) are consistent with the literature on the multifunctionality of ponds (Popp et al. 2019; Hambäck et al. 2023). It is important to study in greater depth how these various contributions can be combined. Two recent articles on ponds and pondscapes have contradictory results on this subject. According to the review from Necker et al. (2024), some European restoration projects have regrettably failed to take account of different contributions and services in the initial ecological objectives of the restoration. However, in their analysis, Bartrons et al. (2024) mention combinations of NCPs that have implemented NbS in ponds/pondscapes in 24 countries. According to this paper, the NCP 'creation of habitat for biodiversity' was combined with the NCPs 'learning and inspiration', 'regulation of water quantity' or 'physical and psychological experiences'. The integration of various objectives on the NbS measures is central to achieving the inter-relationship between biodiversity, aquatic ecosystem functioning and human activities, in knowing whether these objectives are compatible or contradictory. This brings us back to the scientific debates specific to ecosystem service bundles (Raudsepp-Hearne et al. 2010; Meacham et al. 2022), in terms of whether contributions can be cumulative, synergetic, antagonistic or neutral (Hambäck et al. 2023). The maintenance of the quality of the environment and of contributions provided by the environment (Streimikiene 2015) are ways of strengthening stakeholders' attention (Smyth et al. 2021) and public awareness (Sousa et al. 2016), in this case to pondscapes. Maintaining or improving the environmental condition of a pondscape is a milestone in fostering the commitment of inhabitants and stakeholders to preserve it. This preservation, in whatever form (creating, managing or restoring), is achieved by integrating various contributions such as visitor activities, the aesthetic quality of the landscape, the landscape or cultural identity of the site, or the presence of emblematic species.

Across countries, most opinions on NbS were quite similar for a majority of pondscapes with the same options of responses selected: 'restoration measures', 'maintenance of biodiversity', 'improving water quality' and 'better/more environmental education'. Views of both types of respondents on NbS were more or less aligned. This may be explained by the large number of suggested answers (12; detail in supplementary data), which may have dispersed the responses. Alongside the best way to integrate the different contributions, the same applies to the co-benefits to be implemented during an integrated approach to NbS planning (González-García et al. 2025). In facing the challenge of water quantity, respondents from the Belgian, German, Turkish and Uruguayan pondscapes have selected other options, such as "increasing water volume", "limitation of certain uses", and "abandonment of certain uses" with action both on the supply and demand of water. This refers to the causes of the deterioration of the pondscapes, which implies a limitation of activities (industrial, agricultural or touristic) to maintain or improve the situation or preserve water resources in the face of climate change. However, abandoning certain practices and uses, such as agriculture, is not always a panacea, as shown by Erös et al. (2020). Monitoring the dynamics of pondscapes requires close cooperation between those involved in habitat conservation and management, in order to develop strategies that encourage closer socio-ecological links and improve the impact of research on decision-making (Fisher et al. 2020). Another main research priority moving forward should be to better understand applying (Arnautu and Dagenais 2021) these results to the management and policy of pondscapes (Ryfisch et al. 2024). The broad-based knowledge exchange collaborations can assist local actors including particularly interested inhabitants, and actors at the meso-level (e.g. regional actors and civil society) and macro-level (e.g. legal and regulatory systems) that together shape management decisions in implementing NbS measures.

Limitations

This survey has limitations. Firstly, we did not obtain a similar number of responses for all pondscapes with a low number of responses linked to certain pondscapes in Türkiye, Belgium and Uruguay. Although the low number of responses can be justified by the characteristics of these pondscapes, a larger sample of responses would have been required to study more precisely certain variables (age, gender) in greater statistical depth. Therefore, when comparing inhabitants-stakeholders, we thus paid more attention to pondscapes with the strongest response.

Secondly, we are unable to explain the reasons that led different respondent types to favor specific answers. While most responses often converge, the interpretation of the main differences between the population and stakeholders remains limited. As a result, the sum of individual points of view cannot be representative of the collective point of view, since this approach does not allow for the emergence of discussion and negotiation processes leading actors to change their perceptions through contact with others (Zerbe 2023). This approach is also biased towards the profile of respondents (S1 in supplementary data). For example, we sent the questionnaire to stakeholders, with the indication to share it with people likely to be able to respond. An initial orientation was therefore carried out. This way of dissemination may have led to more responses from existing small networks of actors and specific epistemological communities in pond management, perhaps to the exclusion of other actors with fewer institutional links but with potentially greater expertise (Arango-Quiroga et al. 2023).

With the face-to-face surveys and the sign boards dedicated to inhabitants, the respondents likely included people already interested in ponds and pondscapes, as we targeted respondents who live near/within or use the pondscapes. Social networks, which could have broadened the spectrum of respondents, were not used, as none corresponded to the perimeter of these pondscapes. It should also be stressed that the representativity of the inhabitant sample was not an issue in terms of gender, income, geographical location or age group.

All the pondscapes studied exhibit a diversity of uses (biodiversity and recreation, and sometimes agricultural and informal uses linked to a lack of management and maintenance) and land-use contexts (urban, peri-urban, agricultural). These mixed-use ponds not allow for the deduction of specific perceptions for each use due to the multifunctionality provided by the ponds. Selecting study sites with very different and less multifunctional uses would allow us to determine whether potentially different perceptions would facilitate or reinforce the synergies or antagonisms of management objectives. This would lead to specific strategies for prioritizing or not multifunctionality at the pond landscape scale in order to consider the synergies between NCPs.

Conclusion

Our survey describes and compares perceptions within and across pondscapes and explores to what extent perceptions vary between stakeholders and inhabitants. The examination of perceptions on a variety of topics (biodiversity, nature conservation, environmental condition, environmental change, threats assessment) gives interesting insights into the role of these small water bodies and their importance. Overall, with regard to the questions asked, the perceptions of stakeholders and inhabitants are fairly similar (threats and impacts, NCPs), but may also differ (quality of life, relationship with nature, environmental status), with high scores given by both types of respondents nonetheless. A central conclusion of our study is that pondscapes are widely valued by inhabitants and stakeholders in all pondscapes in all studied countries, and that this largely stems from the benefits that the pondscapes provide for the quality of life. Biodiversity was overall highly valued, and the importance for the conservation and protection of pondscapes was strongly acknowledged. Social, cultural, and recreational activities were also perceived as beneficial and promoting well-being and integration into the community. Respondents also rated the 'maintenance of options' NCP particularly highly, referring to long-term management to ensure resilience. Stakeholders and inhabitants identified 'climate change' and 'pollution' as the most important threats in the investigated pondscapes. Regarding the various ways of addressing the problems identified, the perceptions on NbS are quite similar for a large majority of pondscapes. The most frequently selected NBS were "restoration", "maintenance of biodiversity" and "better/more environmental education".

In light of this, by analyzing perceptions in several pondscapes, we gain insight into ways of understanding and acting upon pondscapes as Nature-based Solutions. These results are not necessarily representative of the European and South American context, but they illustrate how attachment to ponds, geospatial changes over time, and the issues surrounding NCPs inform management projects. Future actions should aim to ensure that these pondscapes continue to play a key role in biodiversity conservation and improving people's quality of life through recreational activities. Our social data can be used to analyse synergies and trade-offs in future policies and management of pondscapes, provided that broad-based knowledge exchange collaborations and educational and dissemination campaigns are put in place.

Acknowledgements

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Data availability

The dataset on pondscape features is available in the Zenodo repository (DOI: 10.5281/zenodo.14017011).

Any additional raw data used in the manuscript are available from the corresponding author upon reasonable request.

Consent to participate

We conducted this survey in accordance with ethical standards, and informed consent was obtained from all respondents. Participants were adults whom we asked exclusively in relation to pondscapes and not on sensitive subjects (e.g. health, sexual orientation, politics, etc.). The questionnaire explained the objectives of the study, the scientific context and required the consent of the respondents. All the data gathered during the survey will be only used for the research purposes and will be stored and used in line with GDPR. Any results will be presented only in aggregated form, so it will not be possible to identify particular participants.

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Annex 1 (template of the common questions asked to the inhabitants and stakeholders)

How would you describe your relationship with 'nature'?
Please select a score from 1 to 5, where 1 means 'very weak' and 5 means 'very strong'.

How would you describe your relationship with pondscapes?
Please select a score from 1 to 5, where 1 means "very weak" and 5 means "very strong".

Are pondscapes important for your quality of life?
Please respond using the five-point scale, where 1 means "not important at all" and 5 means "very important".

Have you observed significant changes in this pondscape during the last ten years?
- Yes
- No

And, if so, which one(s)?
Check any that apply
- Colonisation of new animal species
- extinction of local animal species
- colonisation of new plant species
- extinction of local plant species
- increase of bad odours
- decrease of bad odours
- increase of pondscape surface area
- decrease of pondscape surface area
- increase in the number of ponds
- decrease in the number of ponds
- more rubbish
- less rubbish
- higher pond water level
- lower pond water level
- improvement of water quality
- deterioration of water quality
- more frequent drying of ponds
- less frequent drying of ponds
- other:

In your opinion, what are the contributions provided by this pondscape?
Please rank the following contributions on a scale from 1 to 5, where 1 means "not important at all" and 5 means "very important".

	1	**2**	**3**	**4**	**5**
Food and feed (productivity of food: fish, waterfowl, livestock)					
habitat creation and maintenance (preservation of desired species, for biodiversity conservation)					
pollination (diversity of plants to be pollinated)					
regulation of water quality (water purification)					
regulation of water quantity (reservoir of irrigation, water supply)					
regulation of hazards (flooding regulation, fire protection)					
regulation of climate (carbon storage, maintaining an acceptable temperature)					
physical and psychological experience (calm, freshness, sociability, activities)					
learning and inspiration (aesthetic, art, education, science)					
supporting identities (cultural heritage, local identity)					
maintenance of options (potential opportunities offered by nature to ensure resilience in the future)					

In your opinion, what is the environmental condition of this pondscape?
Please respond using the five-point scale, where 1 means "very bad" and 5 means "very good".

What do you perceive are the most important threats to this pondscape in future?
Please, rank the following threats on a scale from 1 to 5, where 1 means "not important at all" and 5 means "very important".

	1	2	3	4	5
Climate change					
Deforestation					
extraction of materials (gravel, sediment, sand…)					
intensive farming (trampling by cattle for example)					
invasive species					
over-exploitation (water abstraction, irrigation)					
Pollution					
tourism (rubbish, damage to vegetation by trampling, disturbance of wildlife)					
Urbanization					

For you, what are the impacts of these threats in future?
Please, rank the following impacts on a scale from 1 to 5, where 1 means "minor impact" and 5 means "major impact".

	1	2	3	4	5
impact on the productive purpose					
impact on human health					
impact on water quantity					
impact on water quality					
impact on biodiversity					
impact on water temperature					
impact on soil erosion					
impact on the landscape					
impact on my property/my security (nuisance species and flooding for example)					

In order to mitigate these threats and impacts, what changes would you propose to improving the environmental state of the most visited pondscape?
Tick the following propositions on a scale of 1 to 5, where 1 means "not at all important" and 5 means "very important".

- better/more environmental education
- creating new ponds
- increasing biodiversity (species, populations, or on a genetic level)
- improving water quality
- increasing the volume of water
- limitation of certain uses
- abandonment of certain uses
- restoration measures
- maintenance of biodiversity
- monitoring of ponds
- developing public ownership
- developing environmental regulation

Supplementary data

Table S1 Dominant profile of stakeholders and inhabitants per pondscape.

Pondscapes	Stakeholders			Inhabitants	
	Dominant area education	Prominent role	Sense of professional responsibility (1–5)	Most selected visit frequency	Mean distance from their home (km)
Bois de Jussy	Biology	Consultancy	3.2	Once a month	7.4
Rhône Genevois	Biology	Consultancy	3.8	Once a month	7.2
Schöneiche	Administration	Local authority	2.6	Once a month	2.4
Dikkuyruk	Engineering	Civil society	4.0	Once a week	12.0
Gölbasi	Engineering	Civil society	4.1	Once a week	9.7
Imrahor	Engineering	National authority	4.3	/	15.0
Pinkhill Meadows	Ecology	Civil society	3.0	Once a month	12.5
Water Friendly Far.	Ecology	National authority	3.0	Once a week	9.6
Albera	Biology	Regional authority	3.1	Once every six months	17.6
La Pletera	Biology	Research / Local authority / Regional authority	2.5	Once every six months	19.2
Gete Vallei	Ecology	Civil society	3.6	Once a month	9.3
Pikhakendonk	Ecology	Research	3.4	Once a month	4.2
Tommelen	Ecology	Civil society	3.0	Once a month	3.8
Sierra de los Car.	Agronomy	Research	3.5	Once a month	18.7
La Pedrera	Ecology	Research	4.6	/	18.8
Lystrup	Biology	National authority	3.6	Once a day	2.2
Fyn	Biology	National authority	4.0	Once every six months	23.1

Table S2 Mean scores obtained by respondent types for their relations to nature, to the pondscape and quality of life. The values are presented by pondscapes, by country, and also for all data obtained.

Country	Pondscapes	Relation to nature		Relation to pondscape		How important are the pondscapes for your quality of life?	
		Inhabitants	Stakeholders	Inhabitants	Stakeholders	Inhabitants	Stakeholders
Switzerland	Bois de Jussy	4.4	4.8	3.8	4.2	3.5	4.2
	Rhône Genevois	4.5	4.8	3.9	4.2	3.8	4.2
Germany	Schöneiche	4.2	4.8	3.6	3.6	3.2	4.2
Turkey	Dikkuyruk	4.5	4.6	4.2	3.6	3.8	4.3
	Gölbasi	3.9	4.6	3. 8	3.3	3.7	4.2
	Imrahor	3.5	4.6	3.0	3.4	3.5	4.5
England	Pinkhill Meadows	4.4	4.7	3.0	3.9	3.1	4.3
	Water Friendly Farming	4.7	5.0	3.3	4.5	3.7	4.3
Spain	Albera	4.5	4.5	3.8	3.9	4.0	4.0
	La Pletera	4.1	/	3.9	/	4.1	/
Belgium	Gete Vallei	4.3	4.8	3.8	4.6	3.8	4.2
	Pikhakendonk	4.2	4.6	3.5	4.4	3.2	4.0
	Tommelen	4.0	4.5	3.9	4.2	3.8	4.1
Uruguay	Sierra de los Caracoles	4.5	4.5	3.8	3.2	3.9	3.0
	La Pedrera	5.0	5.0	3.7	4.0	4.0	3.0
Denmark	Lystrup	4.1	5.0	4.1	4.3	3.8	4.6
	Fyn	4.6	5.0	3.7	4.2	3.5	4.8
Average		4.4	4.7	3.6	3.9	3.6	4.1

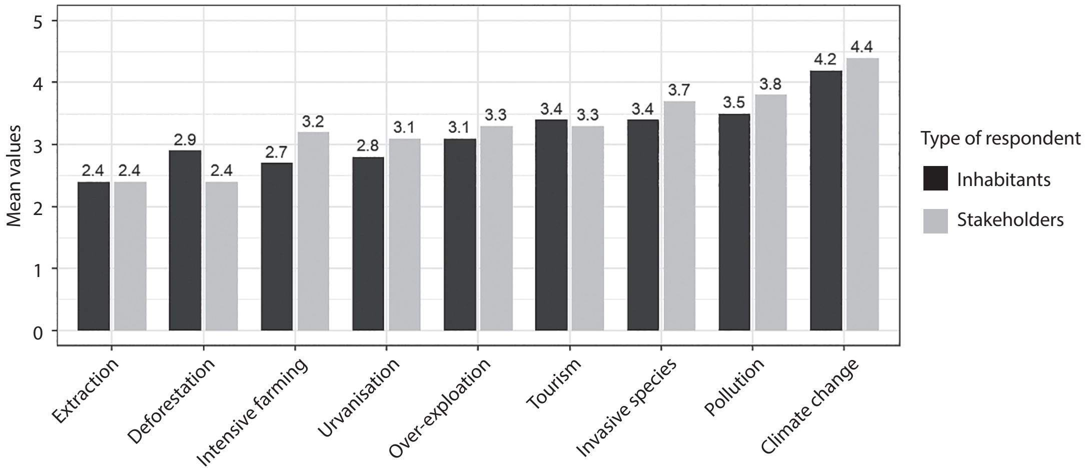

Fig. S1 Assessment of threats with mean scores given by both respondent types (inhabitants in black and stakeholders in grey), with all pondscapes combined.

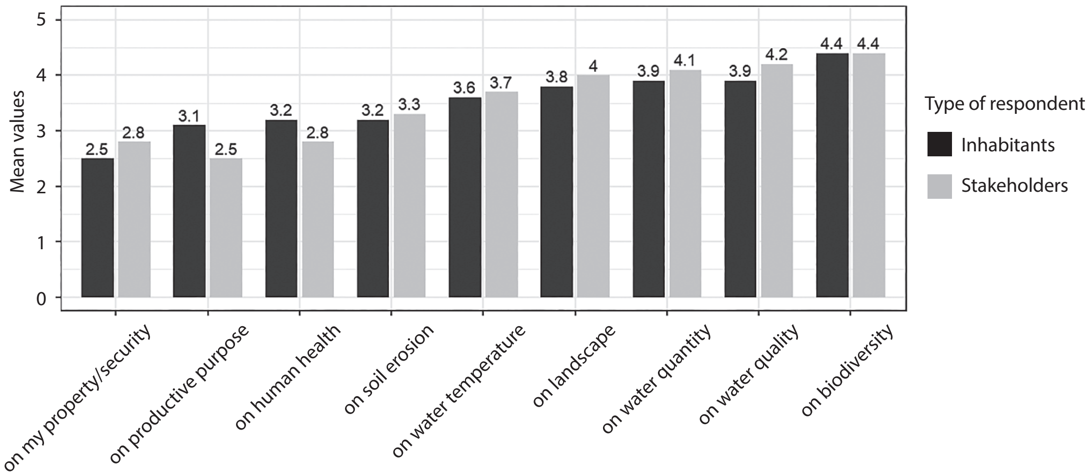

Fig. S2 Assessment of impacts of threats with mean scores given by both respondent types (inhabitants in black and stakeholders in grey), with all pondscapes combined.

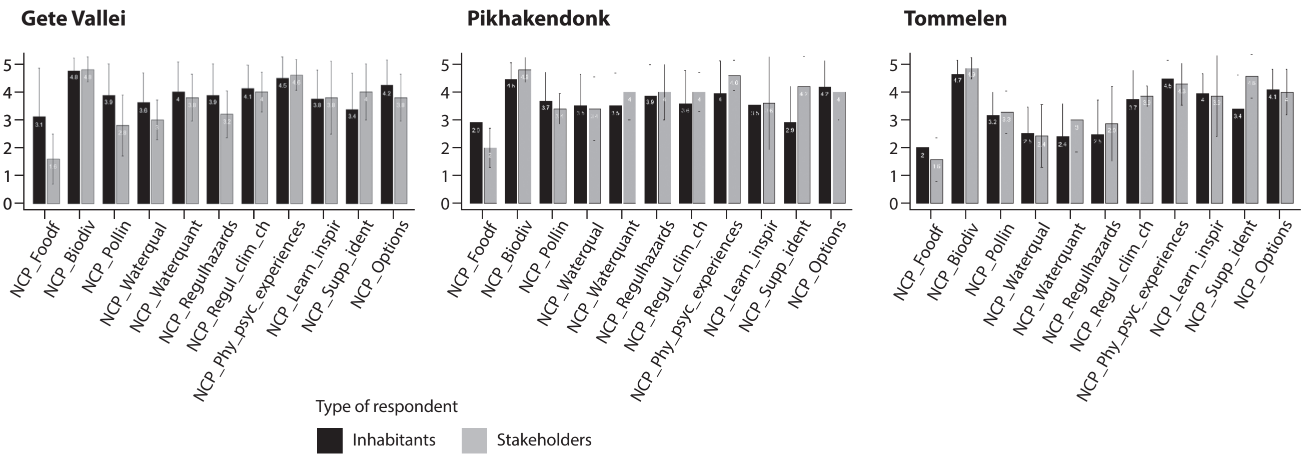

Fig. S3 NCP assessment for inhabitants (in black) and stakeholders (in grey) in the Belgium pondscapes.

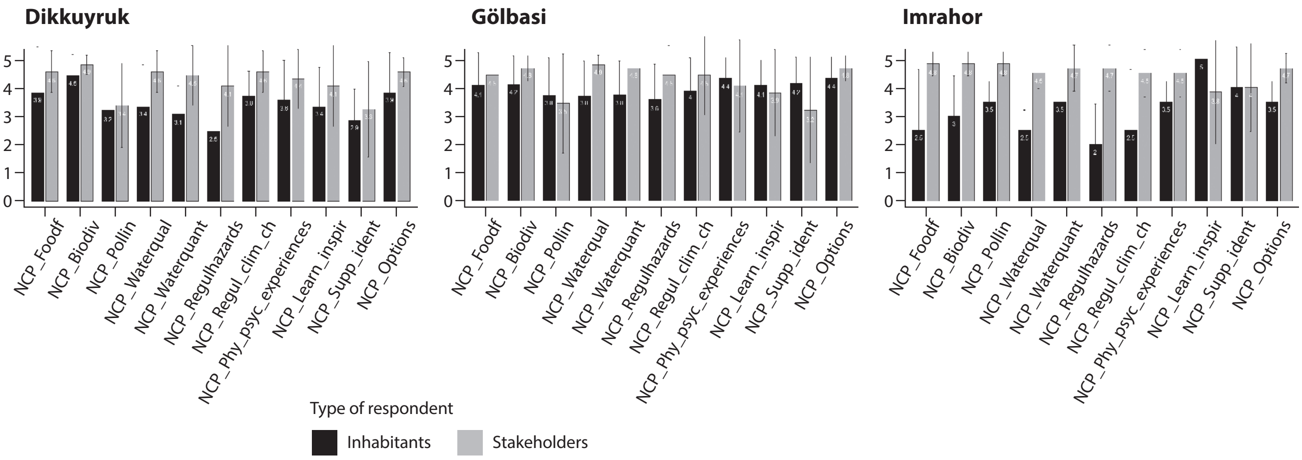

Fig. S4 NCP assessment for inhabitants (in black) and stakeholders (in grey) in the Turkish pondscapes.

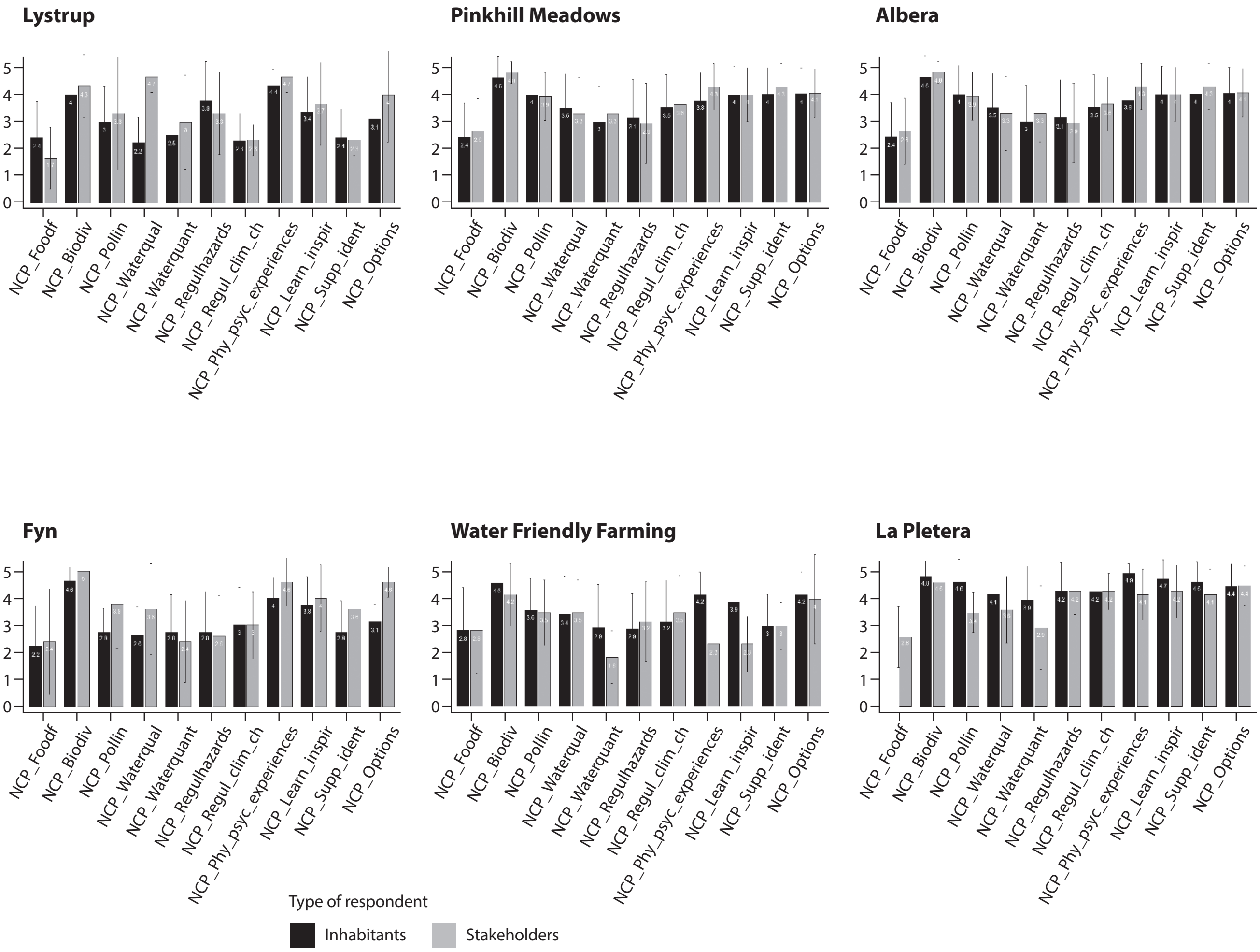

Fig. S5 NCP assessment for inhabitants (in black) and stakeholders (in grey) in the English, Danish and Spanish pondscapes.

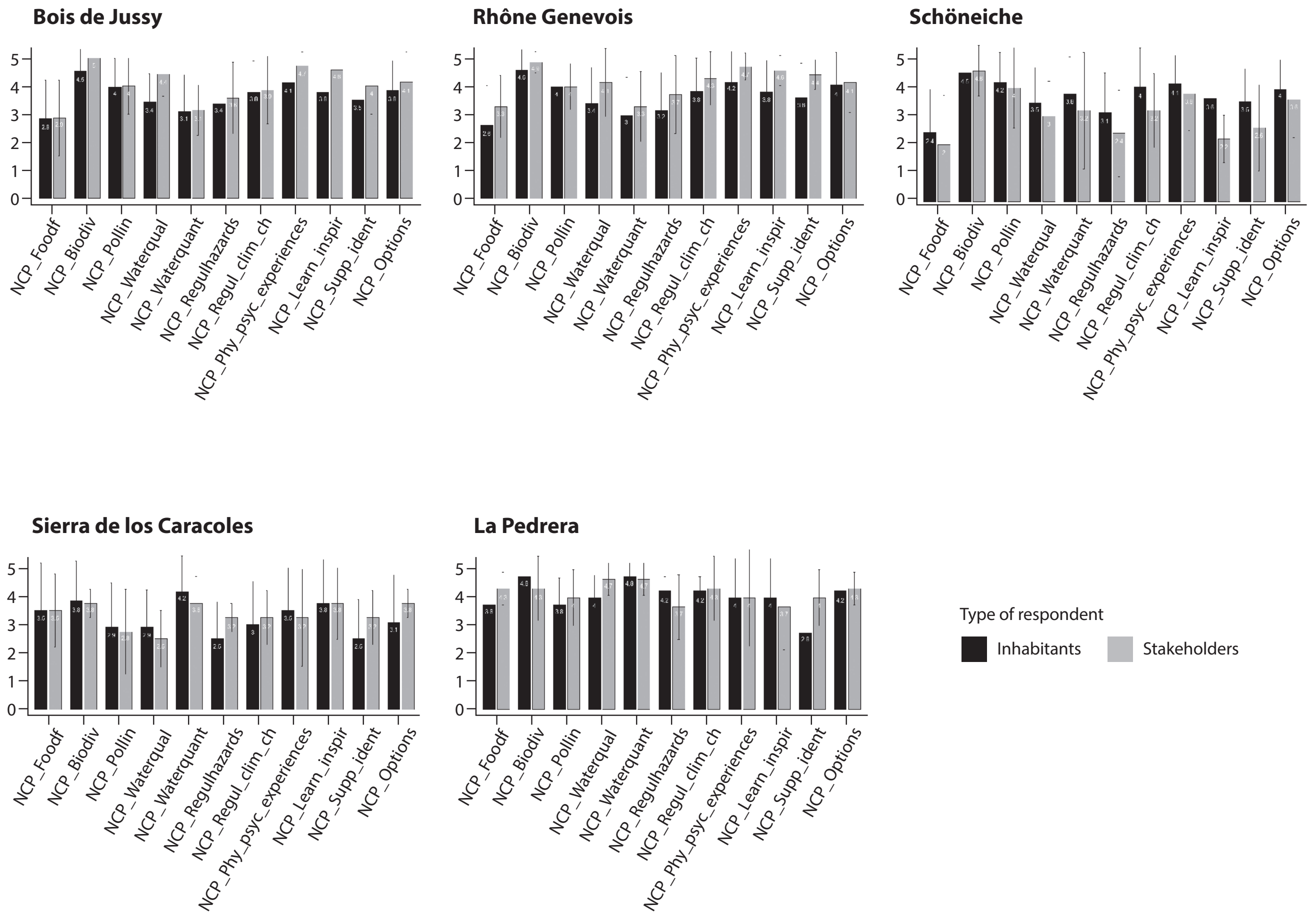

Fig. S6 NCP assessment for inhabitants (in black) and stakeholders (in grey) in the Uruguayan, Swiss and German pondscapes.