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Chronosequence of two forest disturbances recorded in the soils of the Tatra Mountains (Carpathians)

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Abstract. Over recent decades, the frequency of forest disturbances such as windthrow events and bark beetle outbreaks has increased in mountainous regions of Europe. These disturbances often co-occur; however, they alter soil conditions in different ways and consequently affect soil microorganisms through distinct mechanisms. Understanding their effects on soil microorganisms is crucial, as soil biological processes play a fundamental role in carbon sequestration and nutrient cycling. This study investigates the response of soil microbial communities to forest disturbances under active forest management, where tree trunks were removed, in the Tatra Mountains. We assessed soils affected by windthrow and bark beetle outbreaks across a chronosequence spanning 9 to 57 months after disturbance.

The analyses included soil chemical properties, soil respiration, SIR induced microbial biomass, the activity of hydrolytic enzymes (β-D-cellobiosidase, β-glucosidase, β-xylosidase, N-acetyl β-glucosaminidase, phosphatase, arylsulfatase), and the catabolic profiles of bacteria and fungi (Biolog® ECO and FF). Microbial responses varied depending on disturbance type and time since disturbance. Indicators of microbial activity did not change linearly over time, suggesting a rapid microbial response during the initial post disturbance period. However, soils affected by bark beetle outbreaks exhibited less abrupt changes than windthrow affected soils, likely due to the continuous input of organic matter, the absence of an initial lag phase in decomposition, and more gradual shifts in environmental conditions.

Substrate utilisation patterns (catabolic profiles) differed over time between windthrow and bark beetle affected soils, indicating distinct trajectories of microbial succession. Microbial responses also varied between soil horizons. The litter horizon was more sensitive to changes in enzymatic activity and substrate utilisation than the humus horizon, likely because it depends strongly on inputs of fresh organic matter. In contrast, variability in the humus horizon was more closely associated with soil chemical properties, particularly nitrogen availability and soil pH.

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1 Introduction

Forest disturbances caused by strong winds (windthrows) and insect infestations are natural processes that shape forest ecosystems worldwide (Schaetzl et al. 1989; Peterken 1996; Ulanova 2000; Zielonka and Malcher 2009; Grodzki et al. 2010). Although such disturbances are part of natural forest dynamics, their frequency and intensity have increased in recent decades, largely due to anthropogenic pressures. Since the mid-20th century, forest disturbances in Europe have intensified for two principal reasons. The first involves inappropriate forest management practices, particularly the widespread conversion of natural mixed forests into even aged spruce monocultures during the 19th and early 20th centuries (Grodzki et al. 2010). The second relates to climate change, which has increased forest vulnerability to drought and intensified windstorm activity (Usbeck et al. 2010; Mezei et al. 2017; Pretzsch et al. 2020).

Paradoxically, natural forest disturbances can act as drivers of ecological development. Disturbances are widely recognised as key factors shaping forest regeneration and creating environmental niches that promote biodiversity (Fleischer et al., 2009; Zielonka and Malcher, 2009; Urbanovičová et al., 2013). Among the most common disturbance types—windthrows and insect infestations—interactions are frequent, and one disturbance may reinforce the other. Windthrows generate large amounts of deadwood, which facilitates bark beetle outbreaks in spruce stands (Grodzki et al., 2006; Økland et al., 2016; Grodzki and Fronek, 2017; Havašová et al., 2017). Furthermore, wind damage can weaken the remaining forest stand, making trees on disturbance margins more vulnerable to subsequent strong and turbulent winds (Palm-Hellenurm et al., 2024). Although windthrows and insect outbreaks appear similar in terms of their landscape-level effects, it is highly likely that they elicit markedly different responses in soil biota. This notion is supported by the results of previous studies; however, some of these investigations primarily emphasize changes in soil-forming conditions and environmental drivers, while lacking direct evidence derived from measurements of soil biological properties.

Disturbances caused by windthrow events induce more abrupt changes in the soil environment than those resulting from insect infestations. Tree uprooting and sudden canopy loss alter solar radiation, humidity, and other environmental conditions, thereby modifying habitat structure, including the composition of flora, fauna, and soil microorganisms (Ulanova 2000; Egli et al. 2002; Coyle et al. 2017; Čuchta et al. 2019). Such disturbances also initiate rapid secondary succession processes (Ulanova 2000). In contrast, insect infestations typically cause a gradual decline of forest stands over several years, leading to slower changes in environmental conditions, including tree mortality, organic matter inputs, and microclimatic characteristics (Edburg et al. 2012). The two disturbance types also differ in the nature of the substrates supplied to soil microorganisms, with insect infestations contributing substantial amounts of organic material derived from fallen needles and other fine plant residues (Edburg et al. 2012).

Understanding the effects of forest disturbances on soil microorganisms is essential, as soil biological processes play a key role in regulating carbon sequestration and nutrient cycling (Pietrzykowski et al. 2025). Although numerous studies have examined the effects of forest harvesting on soil biological activity (e.g., Jones et al. 2003; Adamczyk et al. 2015; Cambi et al. 2017; Settineri et al. 2018), research focusing specifically on naturally occurring disturbances remains relatively limited,

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despite the increasing frequency of such events in recent years (e.g., Gömöryová et al. 2014, 2017; Holden and Treseder 2013; Šťursová et al. 2014; Borkhuu et al. 2015; Mikkelson et al. 2016, 2017; Custer et al. 2020). Furthermore, none of these studies has explicitly addressed the potentially contrasting responses of the litter and humus horizons, which differ substantially in their composition and in their exposure and susceptibility to environmental factors. Given the increasing prevalence of natural disturbances, further research is urgently needed to clarify their effects on soil microbiota.

To fully understand the complex interactions between forest disturbances and soil properties, a broad range of research methods is required, as the relationships involved are multifaceted. Studies focusing on the effects of natural forest disturbances on soil biological activity often produce ambiguous results. Moreover, the response of soil microorganisms may depend on several factors, including the time elapsed since disturbance and, in the case of bark beetle infestations, the degree of tree mortality (Köster et al. 2011; Gömöryová et al. 2014, 2017; Holden and Treseder 2013; Borkhuu et al. 2015; Mikkelson et al. 2016, 2017; Wasak et al. 2019).

The aim of this study was to examine how soil microbiota respond to forest disturbance, taking into account both the disturbance type (windthrow vs. insect outbreak) and the duration of post-disturbance succession. We hypothesised that forest disturbances cause non-linear changes in soil biological activity, with trajectories varying between disturbance types and soil horizons. We further hypothesised that windthrow effects would be stronger than those of bark beetle infestation due to the abrupt nature of wind disturbance, but that soil biological activity would gradually recover with natural succession. Finally, we expected that disturbance-related changes in vegetation and substrate inputs would alter not only overall microbial activity but also the functional structure of microbial communities.

2 Materials and methods

2.1 Research settings

To investigate the impact of different types of forest disturbance on soil microbiota, the Tatra Mountains were selected as the study area, as this region has experienced repeated disturbances of various origins in recent decades (Fabijanowski and Dziewolski 1996; Koreň 2005; Grodzki and Guzik 2009; Grodzki et al. 2010). Moreover, the environmental background of the Tatra Mountains—including its well-studied soil cover—has been extensively documented, making this region an excellent natural laboratory for research on soil microbiota and disturbance ecology(Skiba et al. 2015;Miechówka and Drewnik 2018; Stolarczyk et al., 2024; Wasak-Sęk et al., 2025a,b).

The study focused on both the litter and humus horizons (formally referred to in soil science as the O and A horizons, respectively). These horizons were analysed separately due to the contrasting nature of the organic substrates entering the soil and the distinct microclimatic conditions characteristic of each horizon, which are expected to produce differing biological responses.

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To minimise the effect of short term climatic variability, all sampling was conducted simultaneously across the study plots during the second half of September. The close proximity of the plots further reduced the influence of extraneous environmental factors on the processes being examined.

The methods employed in this study provided complementary insights into multiple aspects of microbial functioning. Soil respiration (respC-CO_2) and SIR induced microbial biomass (SIR-C_{mic}) were used as measures of overall microbial activity, without distinguishing between ecological or functional groups. SIR-C_{mic} reflects the pool of microorganisms capable of responding rapidly to the addition of an easily available substrate, such as glucose (Anderson and Domsch 1978). Enzymes constitute the essential biochemical machinery that enables microorganisms to decompose organic matter. Several common hydrolytic enzymes (ectoenzymes) play key roles in nutrient cycling processes: the C cycle (β-D-cellobiosidase, β-glucosidase, β-xylosidase), the N cycle (N-acetyl-β-glucosaminidase), the P cycle (phosphatase), and the S cycle (arylsulfatase). Their primary function is the degradation of complex organic polymers such as cellulose, hemicelluloses, and chitin (Parvin et al. 2018).

The catalytic activity profiles of microorganisms, assessed using Biolog® ECO and FF plates, reflect the availability of different substrates serving as energy sources for specific bacterial and fungal groups (Garland and Mills 1991; Preston Mafham et al. 2002). Some of these substrates are themselves products of enzymatic degradation (e.g. xylose, cellobiose). Catalytic profiles also enable differentiation between bacterial and fungal activity. The availability of substrates—whether for enzymatic reactions or catabolic activity—depends on the type of organic material entering the soil (e.g., decomposing plant tissues, microbial biomass, secretions, or root exudates) and on the stage of decomposition (Stevenson et al. 2004; Sinsabaugh et al. 2014; Bourget et al. 2023; Tavares et al., 2026).

2.2Study area

The study areas are located in the lower parts of the Javorova Valley (Slovakia) and the Lejowa Valley (Poland), both situated in the Tatra Mountains (Figure 1a). The two areas share similar geographical characteristics and comparable histories of forest management. They lie at elevations of 950–1200 m on north-east and east-facing slopes with inclinations ranging from 5° to 15°. The geological substrate consists of Jurassic and Eocene sedimentary rocks rich in carbonates, including limestones, dolomitic sandstones, and conglomerates (Szczegółowa mapa geologiczna Tatr, 2025). As a result, inherently fertile loamic and siltic soils have developed on these carbonate-rich parent materials (Skiba et al. 2015).The mean annual air temperature in both valleys ranges between 4 and 6 °C (Żmudzka et al. 2015), while mean annual precipitation (MAP) varies between 1,200 and 1,400 mm, with rainfall maxima occurring during summer (Ustrnul et al. 2015).

The potential natural vegetation of the study areas consists of broadleaf forests, predominantly fertile Carpathian beech forest (*Dentario glandulosae-Fagetum*), in which *Fagus sylvatica* is the dominant species, accompanied by *Abies alba*, *Acer pseudoplatanus*, and *Picea abies*. Locally, sycamore forests may also occur, composed of *Acer pseudoplatanus*, *Picea abies*, *Sorbus aucuparia*, and *Fagus sylvatica* (Myczkowski and Lesiński 1974; Myczkowski 1975; Piękoś-Mirkowa and Mirek

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1996). However, extensive spruce (*Picea abies*) monocultures were established during the 19th century (Fabijanowski and Dziewolski 1996).Before the disturbance events, mature spruce monocultures (approximately 80 years old) dominated both study areas. In the Javorova Valley, windthrow events occurred at the turn of 2015/2016 (20 months before sampling; WD20 — Table 1) and again in early 2018 (9 months before sampling; WD9). In the Lejowa Valley, a large-scale windthrow occurred in winter 2013 (57 months before sampling; WD57). Although fragments of mature forest survived, they were subsequently affected by two bark beetle infestation phases: in 2016/2017 (32 months before sampling; BB32) and approximately 12 months prior to sampling in 2018 (BB12). By the time of fieldwork, no undisturbed forest remained in the Lejowa Valley study area (Figure 1b).

Windthrow-affected areas were characterised by the near-complete uprooting of trees; no standing trees remained (Figure 1b,c). Although stems had been removed, roots and numerous branches were left on site. In forest stands affected by bark beetle infestation in 2016/2017 (BB32), most trees were already broken, with only isolated individuals remaining, all of them exfoliated (Figure 1c; Table 1). By the time of sampling, most trunks in this area had been removed. In the younger infestation stage (2018; BB12), most trees were still standing, although most were exfoliated and partly debarked, with approximately 30% remaining green (Table 1; Figure 1c).

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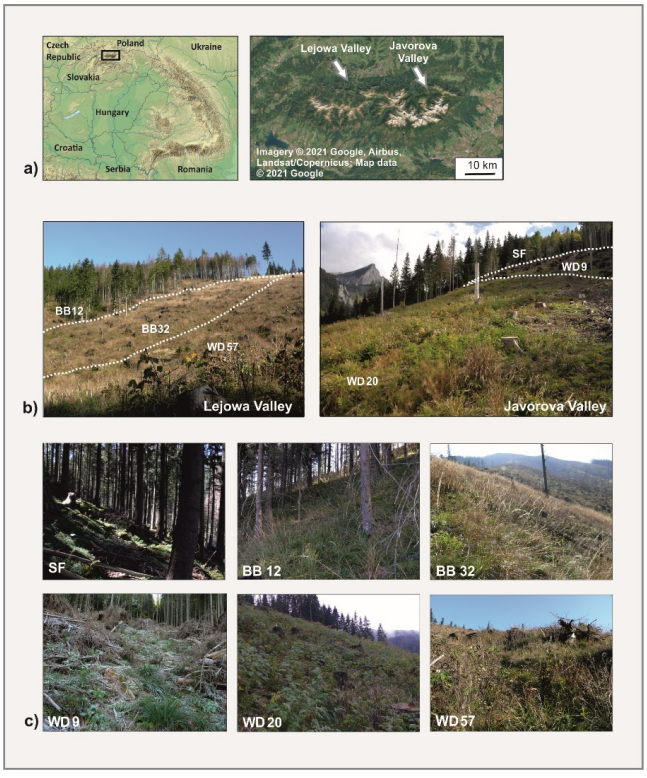

145 **Figure 1. Study area; a) location; b) general view; c) experimental plots.Satellite imagery source: Imagery © 2021 Google, Airbus, Landsat/Copernicus, Map data © 2021 Google.**

In the undisturbed spruce forest (SF), forest-floor vegetation cover was relatively sparse (40–50%) and consisted mostly of species typical of fertile lower-montane beech forest communities, including *Oxalis acetosella*, *Soldanella carpatica*,
150 *Homogyne alpina*, *Aruncus sylvestris*, *Deschampsia* sp., *Luzula* sp., *Vaccinium myrtillus*, various ferns, and mosses. The youngest disturbances exhibited denser ground vegetation (50–60% in WD9 and 90–100% in BB12), dominated by typical forest-floor species such as *Oxalis acetosella*, *Soldanella carpatica*, *Homogyne alpina*, *Viola reichenbachiana*, *Aruncus sylvestris*, *Euphorbia amygdaloides*, *Geranium phaeum*, *Vaccinium myrtillus*, *Calamagrostis villosa*, *Deschampsia* sp., and *Luzula* sp. In addition, species typical of open habitats—such as *Fragaria vesca*—appeared. Pioneer species associated with
155 secondary succession, especially *Rubus idaeus* and *Calamagrostis villosa*, also became increasingly abundant.Intermediate-aged disturbances (WD20 and BB32) represented subsequent stages of secondary succession, with 100% ground-vegetation cover and dominance of *Rubus idaeus* and *Calamagrostis villosa*, while forest-floor specialists

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became marginal. In WD20, young *Sorbus aucupariaand* *Acer pseudoplatanus* had already begun to regenerate. In the oldest windthrow (WD57), *Calamagrostis villosa* and *Rubus idaeus* predominated, and young trees such as *Sorbus aucuparia*, *Acer* 160 *pseudoplatanus*, and *Picea abies* were abundant, whereas typical forest-floor species had largely disappeared (Table 1; Figure 1c).

Table 1. Characteristics of the experimental plots.

Experimental plot	Description	Soil	Vegetation
SF (3 sampling sites)	Reference forest: spruce (Picea abies) monoculture aged approx. 80-90 years; Javorova v.	Medium-depth, fertile soils developed on the parent material forming the slope cover; WRB2022: Skeletic Eutric/ Dystric Cambisol (Loamic/ Siltic, Humic/ Hyperhumic); bedrock beneath the solum: limestones, calcareous shales and mudstones	Canopy cover 100%: entirely even-aged *Picea abies*; Forest floor cover 40-50% – *Oxalis acetosella, Soldanella carpatica, Homogyne alpina, Aruncus sylvestris,Deschampsia* spp., *Luzula* spp., *Rubus idaeus, Vaccinum myrtyllis*, ferns, mosses.
BB12 (3 sampling sites)	Area affected by bark beetle infestation approximately 12 months* prior to sampling; Lejowa v.	Medium-depth, fertile soils developed on the parent material forming the slope cover; WRB2022: Skeletic Calcaric/ Eutric Cambisol Loamic/ (Siltic, Humic/ Hyperhumic); bedrock beneath the solum: dolomitic sandstones and calcareous conglomerates and shales	Canopy cover 70-80%, green canopy cover 30%: *Picea abies*; Ground vegetation cover 90–100% – *Oxalis acetosella, Soldanella carpatica, Homogyne alpina, Viola reichenbachiana, Aruncus sylvestris, Euphorbia amygdaloides, Geranium phaeum, Fragaria vesca, Vaccinium myrtillus, Calamagrostis villosa, Deschampsia* spp., *Luzula* spp., ferns, mosses.
BB32 (3 sampling sites)	Area affected by bark beetle infestation approximately 32 months* prior to sampling; Lejowa v.	Medium-depth, fertile soils developed on the parent material forming the slope cover; WRB2022: Skeletic Calcaric/ Eutric Cambisol (Loamic/ Siltic, Humic/ Hyperhumic); bedrock beneath the solum: dolomitic sandstones and calcareous conglomerates and shales	Canopy: mostly fallen trees (isolated trees has left: exfoliated *Picea abies*); Ground vegetation cover 100% – dominated by Calamagrostis villosa and Deschampsia spp., Aruncus sylvestris, Euphorbia amygdaloides, Viola reichenbachiana, ferns, moses,
WD9 (3 sampling sites)	Area affected by windthrow 9 months prior to sampling; Javorova v.	Medium-depth, fertile soils developed on the parent material forming the slope cover; WRB2022: Skeletic Calcaric/ Eutric Cambisol (Loamic/ Siltic, Humic/ Hyperhumic); bedrock beneath the solum: limestones, calcareous shales and mudstones	Canopy: trunks absent; numerous branches remaining after stump removal; abundant pine needles on soil surface; Ground vegetation cover 50-60% – *Oxalis acetosella, Soldanella carpatica, Homogyne alpina, Aruncus sylvestris, Petasites albus, Sanicula europaea, Deschampsia* spp., *Luzula* spp.,

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			Rubus idaeus, ferns, mosses.
WD20 (3 sampling sites)	Area affected by windthrow 20 months prior to sampling; Javorova v.	Medium-depth, fertile soils developed on the parent material forming the slope cover; WRB2022: Skeletic Calcaric/ Eutric Cambisol (Loamic/ Siltic, Humic/ Hyperhumic); bedrock beneath the solum: limestones, calcareous shales and mudstones	Canopy: trunks absent; ground vegetation cover 80-100% – Young trees: *Sorbus aucuparia, Acer pseudoplatanus*; Ground vegetation: *Rubus idaeus, Calamagrostis villosa, Deschampsia* spp., *Soldanella carpatica, Oxalis acetosella, Vaccinum myrtyllis*, ferns, mosses
WD57 (3 sampling sites)	Area affected by windthrow 57 months prior to sampling; Javorova v.	Medium-depth, fertile soils developed on the parent material forming the slope cover; WRB2022: Skeletic Calcaric/ Eutric Cambisol (Loamic/ Siltic, Humic/ Hyperhumic); bedrock beneath the solum: limestones, calcareous shales and mudstones	Canopy: trunks absent; ground vegetation cover 100% – Young trees: *Sorbus aucuparia, Acer pseudoplatanus, Picea abies*; Ground vegetation: *Calamagrostis villosa, Deschampsia* spp., *Rubus idaeus, Fragaria vesca, Chamaenerion angustifolium, Petasites albus, Sanicula europaea, Salix* sp., mosses

*) calculated from the moment the first signs of tree decline were observed

2.3 Soil sampling

Six experimental plots (70 m × 70 m) were established for the purposes of this study (Figure 2). Soil sampling was conducted in late September 2018. The plots represented the following disturbance categories: undisturbed spruce forest (SF); windthrow from early 2018 (WD9); windthrow from early 2016 (WD20); windthrow from winter 2013 (WD57); bark beetle infestation from 2016/17 (BB32); and bark beetle infestation approximately 12 months after the first symptoms of dieback (BB12) (Table 1; Figure 1c). All plots exhibited comparable slope exposure and inclination. Each experimental plot was divided into a grid of 10 m × 10 m squares, and three sampling sites within each plot were selected using a simple random sampling strategy (Pennock 2004), resulting in a total of 18 sampling locations (Figure 2). Sampling points were positioned in areas where the soil had not been mechanically disturbed, despite the presence of uprooting pits formed by windstorms.

Soil profiles were excavated down to the lithic contact. Profile descriptions followed the WRB classification system (IUSS Working Group WRB 2022). Samples were collected throughout the depth of the solum, according to soil horizons, defined as horizons formed by the same set of pedogenetic processes (Canarche et al. 2006). From each horizon, samples were taken to determine soil pH, total carbon (TC), total nitrogen (TN), and soil texture.Samples intended for analyses of enzymatic activity, respC-CO_2, SIR-C_{mic}, and mineral nitrogen forms were collected specifically from the litter and humus horizons

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using a sterile scoop and placed in sterile polyethylene bags. All samples were stored at 4 °C prior to laboratory analysis. Laboratory procedures were completed within one week of collection to minimise potential alterations in biological activity.

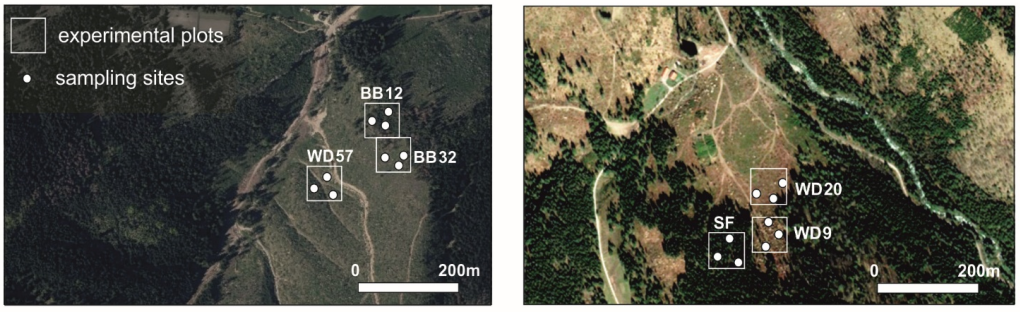

Figure 2. Location of sampling sites on the experimental plots, shown on an aerial photomap.Source of orthophoto:Geoportal of Poland (GUGiK), Geodetic and Cartographic Authority of the Slovak Republic (ZBGIS).

2.4 Laboratory methods

Soil samples intended for the analysis of pH, equivalent $CaCO_3$, total carbon (TC), and total nitrogen (TN) were air-dried, gently crushed with soft tools, and sieved through a 2 mm mesh. Soil pH was measured in deionised water at a soil-to-water ratio of 1:2.5 (pHw) (Thomas 1996). Carbonates ($CO_{2(carb)}$) were quantified using a volumetric calcimeter, and $CaCO_3$ content was calculated accordingly. TC and TN were determined using a CHN analyser (Elementar Vario MICRO Cube elemental analyser). Soil organic carbon (SOC) was calculated by subtracting inorganic carbon (SIC; i.e., carbon derived from $CO_{2(carb)}$) from TC when necessary (Wasak and Drewnik 2015). The C/N ratio was calculated as SOC/TN. Soil dry weight (DW) was determined by drying samples at 105 °C for 24 h. Field moisture (FM) was calculated as the percentage of mass lost upon drying fresh samples at 105 °C for 24 h (Smykla et al. 2015).

Prior to the determination of NH_4^+-N and NO_3^--N, field-moist samples were sieved through a 4 mm mesh and extracted using 1% K_2SO_4 solution at a ratio of 4:1 for mineral soils and 50:1 for organic soils (Sapek 2006). Extracts were filtered using Chemland qualitative cellulose filters. NH_4^+-N was determined using the Nessler method (Siepak 1992; Gąbka et al. 2007), while NO_3^--N was measured colorimetrically using sodium salicylate (PKNMiJ 1982). Analyses were conducted using a Specord spectrophotometer. Extracts were stored at 4 °C and analysed within 24 h of preparation. Soil samples were dried at 105 °C to constant weight to determine DW, and concentrations of NH_4^+-N and NO_3^--N were expressed on a dry-weight basis.

For soil respiration (respC-CO_2) and microbial biomass(SIR-C_{mic})analyses, samples were moistened with deionised water to 60% of the maximum water-holding capacity (WHC). A subsample equivalent to 8 g DW was placed in plastic containers

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and incubated for 24 h at 22 ± 1 °C in a climate chamber. RespC-CO_2 was measured using CO_2 trapping. Each sample was sealed in an airtight glass jar containing a beaker with 5 ml of 0.2 M NaOH. After incubation, 2 ml of $BaCl_2$ was added, and the remaining NaOH was titrated with 0.1 M HCl using a digital burette and phenolphthalein as an indicator (Laskowski et al. 2003). Respiration rates were expressed as mM CO_2 per kg soil organic matter over 24 h (mM CO_2 kg OM^{-1} 24 h^{-1}). Duplicate measurements were averaged.Microbial biomass-C (SIR-C_{mic}) was determined using the substrate-induced respiration (SIR) method in the same samples used for soil respiration. A glucose solution delivering 10 mg glucose per g DW soil was added (Anderson and Domsch 1978). Samples were mixed gently, sealed, and incubated for 4 h at 22 °C. SIR-C_{mic} was calculated from SIR using the equation proposed by Anderson and Domsch (1978):

$$〚SIR - C〛\_mic = 40.04y + 0.37 \quad (1)$$

where y is ml CO_2 h^{-1} g^{-1} OM.SIR measurements were performed once per sample, as glucose addition cannot be repeated.

Enzymatic activities involved in C, N, and P cycling were determined using fluorogenic substrates (Pritsch et al. 2004; Sanaullah et al. 2016). Extracellular enzymes analysed included β-glucosidase (BG), β-D-cellobiosidase (CB), xylanase (XYL), N-acetyl-β-D-glucosaminidase (NAG), phosphatase (PH), and arylsulfatase (SP). A soil suspension was prepared by combining 2.75 g of soil with 92 ml of universal buffer solution (pH 6.0) and pipetting it into microwell plates containing substrate solutions and modified buffer. Plates were incubated for 1.5 h at 35 °C (Puregrade, Germany). Fluorescence was measured immediately afterward using a BioTek Synergy multidetector plate reader with excitation at 355 nm and emission at 460 nm (Turner 2010).

Biolog® ECO and FF plates were used to assess the catabolic activity and functional diversity of bacteria and fungi, respectively. ECO plates are 96-well microplates containing common carbon substrates and a redox dye that changes colour in response to microbial metabolism (http://www.biolog.com). The degradation of substrates in individual wells results in colour development, which is quantified as an increase in absorbance. The substrates included in the plates represent various chemical groups (Campbell et al. 1997).

Soil samples (3 g dry-weight equivalent) were shaken in 30 mL of 0.9% NaCl solution at 200 rpm for 30 minutes. The resulting microbial suspension (100 µL) was diluted in 9.9 mL of 0.9% NaCl. For the FF plates, the dilution included oxytetracycline to inhibit bacterial growth. Subsequently, 100 µL of the appropriate suspension was inoculated into each well of the Biolog® ECO and FF plates. To prevent contamination, all tools were sterilised before use, and all manipulations involving Biolog® plates were performed in a laminar-flow chamber.Absorbance readings for each well were taken immediately after inoculation, and then daily over a period of seven days using a Tecan i-control microplate reader at 590 nm (ECO plates) or 490 nm (FF plates). Average Well Colour Development (AWCD) values were assigned to substrate guilds and used to describe bacterial functional diversity for each plate and replicate (Preston-Mafham et al. 2002). The overall rate of substrate utilisation by bacteria (AUC_{bact}) and fungi (AUC_{fungi}) was expressed as the area under the curve, calculated according to the formula:

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$$AUC = \sum_{i=1}^{N} \sum_{t-1}^{n-1} \frac{(A_n + A_{n+1})}{2} \times (t_{n+1} - t_n) \quad (2)$$

where A_n and A_{n+1} represent absorbance values of a given well (substrate n) at two consecutive measurement times t_n and t_{n+1}. Functional diversity was expressed as the number of utilised substrates (R_{bact}, R_{fungi}) and by the Shannon diversity index (H'_{bact}, H'_{fungi}), calculated as:

$$H' = \sum_{i=1}^{N} p_i \, (log_{10} p_i) \quad (3)$$

where p_i is the proportion of activity on substrate i relative to the sum of activities across all substrates.

2.5 Statistical methods

To determine the statistical significance of differences in soil properties (SOC, TN, C/N, pHw, FM, NH_4^+-N, NO_3^--N), overall biological activity parameters (respC-CO_2, SIR-C_{mic}), enzymatic activities (CB, BG, XYL, NAG, SP, PH), and Biolog®-derived indices (AUC_{bact}, AUC_{fungi}, H'_{bact}, H'_{fungi}, R_{bact}, R_{fungi}, and utilisation of individual bacterial and fungal substrates) across land-use types and soil horizons, a two-way ANOVA was applied. Statistical significance was assessed at two thresholds: $p < 0.1$ and $p < 0.05$. Post-hoc comparisons between individual plots and soil horizons were conducted using the Least Significant Difference (LSD) test ($p < 0.05$).

Principal component analysis (PCA; $N = 36$) was employed to identify differences in enzymatic activity (CB, BG, NAG, XYL, SP, PH) and in the utilisation of substrate groups by bacteria and fungi (ECO and FF plates), analysed separately for the litter and humus horizons. A one-way ANOVA was used to test differences in the utilisation of individual bacterial and fungal substrates ($p < 0.05$), followed by LSD post-hoc tests ($p < 0.05$).

Correlation analyses were conducted to examine relationships between biological indicators and soil properties separately for the litter and humus horizons.

Prior to all statistical analyses, data were tested for normality using skewness and kurtosis criteria (Watkins 2021). Variables that did not meet the assumptions of normality were transformed by logarithmic or square root transformation. All variables were subsequently standardized.

All statistical analyses were performed using Statistica 13.3 software.

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3 Results

3.1 Soil morphology and properties

The soils in the study area are of medium depth, with solum thickness reaching up to 80 cm, underlain by carbonate rocks and calcareous shales. All examined profiles exhibited similar morphology, characterised by the following sequence of horizons: Oi/Oe–Ah–Bw–B/C–R (Figure 3). With increasing depth, the proportion of coarse fragments (stones and gravel) increased from approximately 0–5% (vol.) in the uppermost part of the mineral soil to nearly 90% (vol.) in the transitional B/C horizon (data not shown). The soils displayed a well-developed pedogenic structure, and roots were present throughout the entire solum.

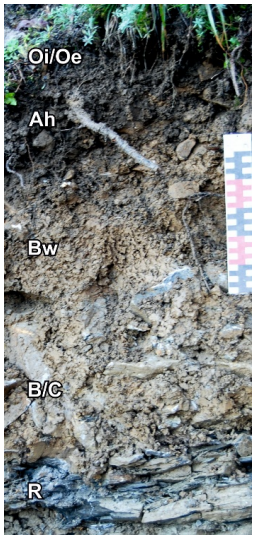

Figure 3. Soil profile from the study area (soil horizon symbols indicated). Beneath the litter horizon in varying stages of organic matter decomposition (Oi/Oe) lies a humus horizon with relatively high organic matter content (Ah), followed by a transformation horizon (Bw) and a transitional horizon to the parent rock (B/C). A well developed pedogenic structure and numerous roots are visible throughout the soil profile, including the B/C horizon. Sedimentary carbonate rocks appear at the base.

The litter horizons were thin and morphologically poorly differentiated (Oi/Oe). They contained high SOC concentrations (on average 35–45%) and exhibited C/N ratios exceeding 20.The mean pHw values ranged from 4.1 to 5.4. Beneath the litter, the humus horizons contained moderately high SOC levels (approximately 7–10%) and C/N ratios ranging from 12 to 16. The mean pHw values ranged from 3.4 to 6.6(Table 2).

According to the IUSS Working Group WRB (2022), the studied soils were classified as Cambisols based on their morphology and key diagnostic properties. Specifically, they fell into the categories ‘Hypereutric Skeletic Cambisols

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(Loamic, Humic)' and 'Dystric Skeletic Cambisol (Siltic, Humic)', with some pedons additionally meeting the criteria for the qualifiers 'Calcaric', 'Dolomitic', and 'Leptic' (Table 1).

In general, the litter horizons displayed higher values of SOC, TN, C/N, FM, NH_4^+-N, and NO_3^--N compared with the humus horizons. Significant differences in TN, C/N, pHw, and NO_3^--N were observed among litter samples, whereas in the humus horizons differences were detected in C/N, FM, pHw, and NO_3^--N. The WD20 soils had the highest TN content and the lowest C/N ratio in both the litter and humus horizons. The forest litter (SF) was characterised by the lowest pHw and NO_3^--N levels, whereas the litter at the BB32 plot showed the highest pHw and NO_3^--N concentrations (Table 2).The oldest windthrow plot (WD57) exhibited the highest NO_3^--N concentrations in the humus horizons.

Table 2. Mean values and standard deviations for soil properties: soil organic carbon (SOC), total nitrogen (TN), C/N ratio, field moisture (FM), soil pH measured in water (pHw), NH_4-N, NO_3-N. Two-way ANOVA test results (p value) were presented at the bottom of the table. Statistical significance levels are indicated: * p<0.1; ** p<0.05. Differences between sites within the soil horizon are indicated with small letters (LSD test, p<0.05). Differences between soil horizons are not indicated in the table.

Site	SOC	TN	C/N	FM	pHw	NH_4-N	NO_3-N
	%	%	-	%DW	-	mg kg^{-1}	mg kg^{-1}
Litter (organic O horizons)							
SF	35.44 (5.68)	1.56 (0.18)**b**	22.6 (1.2)**ab**	290.1 (79.4)	4.1 (0.6)**c**	218.1 (62.9)	33.4 (9.2)**b**
BB 12	37.14 (7.87)	1.30 (0.37)**b**	29.2 (3.4)**a**	269.8 (75.3)	4.7 (0.8)**bc**	199.6 (46.8)	34.5 (11.3)**b**
BB 32	36.74 (7.44)	1.40 (0.13)**b**	26.1 (3.3)**ab**	326.6 (41.2)	5.4 (0.2)**a**	295.8 (62.7)	108.7 (16.0)**a**
WD 9	43.05 (9.85)	1.47 (0.33)**b**	31.5 (15.0)**a**	329.3 (26.2)	5.0 (0.3)**ab**	228.4 (158.3)	92.5 (62.2)**ab**
WD 20	39.41 (6.24)	1.95 (0.37)**a**	20.3 (1.6)**b**	346.8 (91.5)	5.2 (0.3)**ab**	208.2 (93.4)	51.6 (18.9)**ab**
WD 57	43.01 (9.09)	1.40 (0.18)**b**	31.0 (7.3)**a**	354.3 (9.9)	5.2 (0.5)**ab**	311.0 (92.6)	59.2 (12.0)**ab**
Humus horizons (A horizons)							
SF	9.60 (2.57)	0.60 (0.18)	16.0 (0.6)**ab**	97.4 (74.0)**ab**	3.4 (0.1)**bc**	20.9 (19.1)	10.7 (9.7)**c**
BB 12	6.50 (2.29)	0.37 (0.11)	17.3 (1.2)**a**	152.8 (11.3)**a**	6.1 (0.7)**a**	4.9 (3.1)	13.0 (8.9)**bc**
BB 32	6.55 (0.91)	0.45 (0.04)	14.6 (2.8)**ab**	167.6 (4.8)**a**	6.6 (0.0)**a**	2.9 (0.3)	20.6 (0.2)**b**
WD 9	8.44 (2.39)	0.56 (0.08)	14.9 (1.9)**ab**	63.1(12.1)**ab**	3.8 (0.3)**bc**	12.8 (7.6)	10.8 (1.4)**bc**
WD 20	7.45 (0.96)	0.60 (0.03)	12.4 (1.0)**b**	67.2 (9.7)**b**	4.1 (0.6)**b**	10.5 (3.0)	10.7 (3.9)**bc**
WD 57	10.44 (2.92)	0.60 (0.03)	17.2 (4.2)**a**	180.0 (13.2)**a**	6.4 (0.1)a	3.3 (0.4)	208.5 (10.0)**a**
Site	ns	**0.0284** *	**0.0244****	**0.0850** *	**0.0000****	ns	**0.0000****
Soil horizon	**0.0000****	**0.0000****	**0.0000****	**0.0000****	**0.0323** *	**0.0000****	**0.0000****
Interaction	ns	ns	ns	**0.0593** *	**0.0000****	ns	**0.0003****

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3.2 Overall biological activity

295 SIR-C_{mic}exhibited a significant interaction effect ($p < 0.5$), indicating that vegetation-related impacts differed between the litter and humus horizons, although no significant differences were observed among individual plots within each soil horizon (Table 3, Figure 4). In the litter, mean SIR-C_{mic} values were highest in the older disturbance plots (WD57, WD20, and BB32). In the humus horizons, mean SIR-C_{mic} values were lowest in the windthrow-affected plots (WD20 and WD57).

Mean respC-CO_2 rates in the litter were lowest in the youngest disturbance plots (WD9 and BB12), whereas in the humus

300 horizons respC-CO_2 was higher in bark beetle-affected plots than in windthrow-affected plots (Table 3), but there were no statistically significant differences in soil respC-CO_2 between the plots, either in the litter or the humus horizons.

Table 3. Mean values and standard deviations for overall soil respiration rate (respC-CO2), microbial SIR biomass (SIR-Cmic). Two-way ANOVA test results (*p* value) were presented at the bottom of the table. Statistical significance levels are indicated: * *p* <

305 **0.1; **p< 0.05. Differences between sites within the soil horizon are indicated with small letters (LSD test, p < 0.05). Differences between soil horizons are not indicated in the table.**

Site	Overall microbial activity	
	respC-CO_2 $mMCO_2\ kgOM^{-1}24\ h^{-1}$	SIR-C_{mic} $mg\odot gOM^{-1}$
	Litter (organic O horizons)	
SF	69.30 (2.01)	5.88 (1.31)
BB 12	56.20 (7.20)	6.39 (3.87)
BB 32	92.28 (30.83)	9.97 (2.67)
WD 9	48.06 (42.00)	6.09 (2.89)
WD 20	81.69 (65.01)	9.26 (5.73)
WD 57	92.76 (17.13)	8.95 (0.88)
	Humus horizons (A horizons)	
SF	54.45 (42.59)	7.02 (4.01)
BB 12	79.75 (38.15)	8.99 (4.38)
BB 32	57.48 (19.73)	8.13 (2.60)
WD 9	35.56 (26.18)	6.80 (2.67)
WD 20	32.71 (20.57)	1.01 (0.80)
WD 57	45.08 (10.80)	3.48 (2.46)
Site	ns	ns
Soil horizon	**0.0442****	**0.0398****
Interaction	ns	**0.0158****

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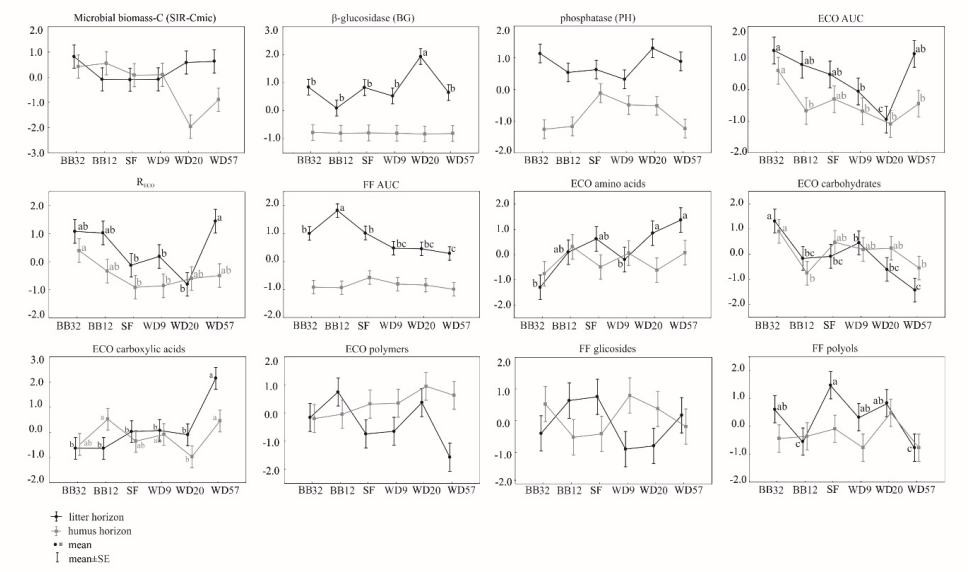

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Figure 4. Differences in soil biological properties and interaction effects for experimental plot and soil horizon (two-way ANOVA results). Significant differences (where present) between plots are indicated by letters (a, b) (LSD test, $p< 0.1$ and $p< 0.05$). Significant differences between soil horizons are listed in Table 4 and are not marked on the figure. Values on the y axis do not represent raw measurement means, as data were transformed (logarithmic or square root, when required) to approximate a
315 **normal distribution and subsequently standardised.**

3.3 Enzymatic activity

Mean enzymatic activity differed significantly between soil horizons, with substantially higher activities in the litter than in the humus horizons ($p < 0.05$). Differences among plots were comparatively weaker, and only β-glucosidase (BG) exhibited significantly higher activity in the litter of the WD20 plot ($p < 0.1$) (Figure 4). A significant interaction effect between the
320 litter and humus horizons indicated differing responses for BG ($p < 0.1$) and phosphatase (PH) activity ($p < 0.5$). For BG, no significant differences were observed among humus horizons, in contrast to the litter. For PH, mean activity was highest in the litter of WD20 and BB32, whereas in the humus horizons it was highest in SF, WD9, and WD20; however, these differences were not statistically significant (Table 4).

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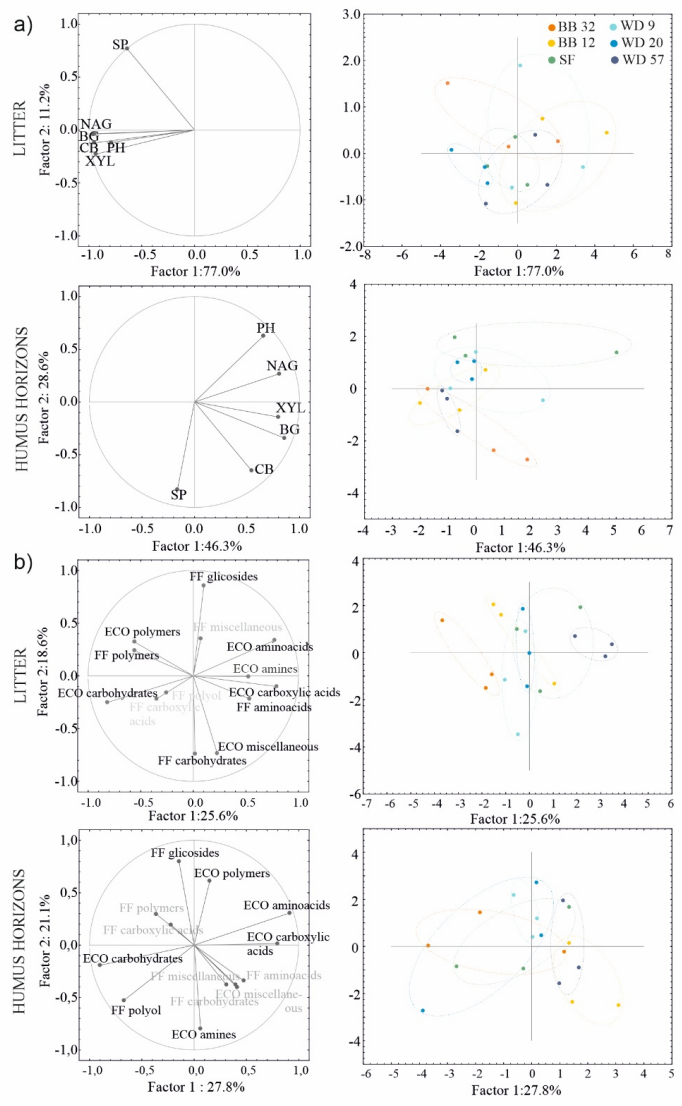

325 **Figure 5. Projection of variables (soil biological properties) and experimental plots in the space of the first two principal components of PCA. O horizons and A horizons are analysed separately. a) activity of extracellular enzymes; b) utilisation of bacterial and fungal substrate groups (ECO and FF).**

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PCA results indicated a positive relationship among all analysed enzymes (Figure 5a). In the litter horizon, the first principal component (PC1) explained 77.0% of the variance in enzymatic activity, while PC2 accounted for 11.2% and was primarily associated with arylsulfatase (SP). PCA clearly separated the youngest disturbances (WD9 and BB12) from WD20 along PC1.In the humus horizons, PC1 explained 46.3% of the variance and indicated positive correlations among all enzymes except SP (Figure 5a). PC2 explained 28.6% of the variance and reflected a negative relationship between SP and CB versus PH. Based on PC2, PCA differentiated the SF and WD20 plots from the oldest disturbances (BB32 and WD57).

3.4 Catabolic activity

The catabolic activity of bacteria (AUC_{bact}) and fungi (AUC_{fungi}) varied among the studied soils depending on both the type and the stage of disturbance, and was significantly higher in the litter than in the humus horizons ($p < 0.05$) (Table 4). AUC_{bact} was significantly highest in the oldest disturbances: in the bark beetle-affected plot BB32 (in both litter and humus horizons) and in the oldest windthrow plot WD57 (in the litter). In contrast, the lowest AUC_{bact} values were recorded in WD20, although this difference was statistically significant only in the litter horizons (Figure 4).The lack of a statistically significant interaction indicates a similar pattern of AUC_{bact} changes in both horizons (Table 5). AUC_{fungi} was significantly higher in the undisturbed spruce forest (SF) and in bark beetle-affected plots (BB12, BB32) than in windthrow-affected plots (WD9, WD20, WD57), however this pattern occurred only in the litter horizons, not in the humus (Figure 4). In the litter horizons, R_{bact} was highest in the oldest disturbance (WD57) and lowest in the younger windthrow plots (WD9, WD20). However, H'_{bact} did not differ significantly between plots. Both R_{fungi} and H'_{fungi} were significantly higher in litter than in humus horizons, but neither index differed significantly between disturbance types or plots (Table 5; Figure 4).

Table 4. Two-way ANOVA test results (p value) for soil enzymes and substrate use (ECO and FF). Statistical significance are indicated by * ($p < 0.1$) and ** ($p < 0.05$).

	Site	Soil horizon	Interaction
Soil enzymes			
CB	ns	**0.0000****	ns
BG	**0.0751***	**0.0000****	**0.0616 ***
XYL	ns	**0.0000****	ns
NAG	ns	**0.0000****	ns
PH	ns	**0.0000****	**0.0483****
SP	ns	**0.0000****	ns
ECO substrate use			
Amino acids	**0.0410****	ns	ns
Amines	ns	ns	ns
Carbohydrates	**0.0038****	ns	ns
Carboxylic acids	**0.0030****	ns	**0.0596***

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Polymers	ns	**0.0287****	**0.0984***
Miscellaneous	ns	ns	ns
FF substrate use			
Amino acids	ns	ns	ns
Carbohydrates	ns	ns	ns
Carboxylic acids	ns	ns	ns
Polymers	ns	ns	ns
Glicosydes	ns	ns	**0.0593***
Polyols	**0.0323****	**0.0312****	ns
Miscellaneous	ns	ns	ns

Clear differences were observed in the utilisation patterns of specific chemical groups of BIOLOG® substrates by bacteria (Figure S1) and fungi (Figure S2), with stronger differentiation among bacterial substrates (Table 4).These patterns distinctly separated bark beetle-affected plots from windthrow-affected plots (Figure 5b). Utilisation of ECO amino acids was highest in WD20 and WD57 and lowest in WD9 and BB32, with statistically significant differences in the litter ($p < 0.05$). However, the lack of a statistically significant interaction indicates a similar pattern of changes in both soil horizons (Table 4). Utilisation of carboxylic acids was also highest in WD57 in both litter and humus horizons, although the trends differed among other plots between the two horizons (significant interaction effect, $p < 0.1$) (Figure 4, Table 4). In the litter horizons, the lowest utilization of carboxylic acids was observed in the remaining plots, whereas in the humus horizons, the highest utilization was recorded in WD52 and BB12, and the lowest in WD20 (Figure 4). In contrast, utilisation of carbohydrates showed an opposite pattern: carbohydrate use was highest in WD57 and lowest in BB32, with similar trends in both horizons (the lack of significant interaction) (Figure 4, Table 4). The utilization of ECO polymers differed significantly between the litter and humus horizons, being higher in the humus horizon; however, trends among plots varied between the two horizons, as indicated by a significant interaction effect ($p < 0.1$) (Figure 4, Table 4). In the litter, polymer utilisation was lowest in WD57 and highest in the BB12 plot, in the humus horizon, polymer utilisation was lowest in the bark beetle-affected plots (BB32 and BB12), although differences between plots were not statistically significant (Figure 4).

Table 5. Mean values and standard deviations for soil bacteria (ECO) and fungi (FF) properties measured on Biolog® plates: activity (AUC), diversity index (H′), and number of substrates used (R). Two-way ANOVA test results (p value) were presented at the bottom of the table. Statistical significance levels are indicated: * p < 0.1; **p <0.05. Differences between sites within the soil horizon are indicated with small letters (LSD test, p < 0.05). Differences between soil horizons are not indicated in the table.

Site	AUC_{bakt}	R_{bakt}	H'_{bakt}	AUC_{fungi}	H'_{fungi}	R_{fungi}
			Litter(organic O horizons)			
SF	79.87 (34.49)**ab**	18.33 (1.15)**b**	1.09 (0.14)	218.34 (41.75)**b**	0.89 (0.30)	29.00 (9.85)
BB 12	90.32 (6.48)**ab**	24.60 (3.05)**ab**	1.08 (0.09)	307.07 (35.79)**a**	0.90 (0.16)	26.20 (11.10)

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BB 32	112.66 (17.33)**a**	26.00 (1.73)**ab**	0.93 (0.19)	216.76 (66.31)**b**	1.00 (0.04)	28.00 (7.55)
WD 9	56.52 (36.66)**b**	20.33 (5.13)**b**	1.05 (0.10)	162.78 (30.99)**bc**	1.07 (0.10)	26.00 (2.00)
WD 20	17.84 (14.09)**c**	14.00 (4.58)**b**	0.95 (0.12)	160.81 (34.60)**bc**	1.08 (0.11)	36.33 (11.37)
WD 57	107.68 (34.34)**ab**	27.40 (2.07)**a**	1.17 (0.26)	148.49 (81.49)**c**	0.98 (0.09)	21.20 (5.36)
			Humus horizons (A horizons)			
SF	45.82 (73.88)**ab**	13.33 (3.00)**b**	0.83 (0.21)	55.61 (38.36)	0.96 (0.20)	18.00 (6.08)
BB	29.59 (3.27)**b**	17.00 (3.00)**ab**	1.10 (0.01)	18.04 (5.70)	0.91 (0.03)	18.00 (6.08)
BB 32	84.82 (32.73)**a**	21.67 (2.52)**a**	0.93 (0.39)	20.24 (4.86)	0.76 (0.11)	14.00 (3.61)
WD 9	29.33 (34.63)**b**	13.67 (2.08)**b**	0.93 (0.09)	31.20 (8.76)	0.80 (0.08)	14.33 (2.89)
WD 20	11.75 (9.25)**b**	17.00 (3.00)**ab**	0.91 (0.05)	27.84 (5.55)	0.83 (0.15)	17.33 (2.31)
WD 57	38.07 (5.91)**b**	16.00 (4.47)**ab**	0.92 (0.19)	13.21 (2.29)	0.92 (0.07)	17.80 (4.21)
Vegetation	**0.0036****	**0.0087****	ns	**0.0313****	ns	ns
Soil horizon	**0.0015****	**0.0008****	ns	**0.0000****	**0.0276****	**0.0001****
Interaction	ns	ns	ns	**0.0351****	ns	ns

Among FF substrates, polyol utilisation varied significantly across plots, with the significantly lowest values in WD57 and BB12, and the highest in the forest plot (SF) in the litter. The absence of a statistically significant interaction suggests a consistent pattern of changes across both soil horizons, despite no statistically significant differences being detected within the humus horizon (Table 4). FF glycoside utilization trends among plots differed between horizons (significant interaction, $p < 0.1$). The highest utilisation was observed in the SF litter, and the lowest in WD9. In the humus horizons, utilisation was highest in WD9 and lowest in SF and BB12. However, none of these differences were statistically significant (Figure 4, Table 4).The PCA results confirmed the patterns identified by the two-way ANOVA, revealing clear differences in substrate utilisation between bark beetle-affected and windthrow-affected plots. Considering the main groups of BIOLOG® substrates utilised by bacteria and fungi, the first principal component (F1) for the litter horizons ($N = 18$) explained 25.65% of the variance and reflected a negative relationship between polymer utilisation (both ECO and FF) and the utilisation of ECO carbohydrates *vs.* the utilisation of ECO and FF amino acids, and ECO carboxylic acids. The second principal component (F2) explained 18.60% of the variance and captured a negative relationship involving FF glycosides *vs*. FF carbohydrates, and ECO miscellaneous substrates. Together, these components distinguished three groups of litter samples along F1: the oldest windthrow (WD57), the younger disturbances (WD9, WD20, BB12), and the oldest bark beetle-affected plot (BB32) (Figure 5b).

Table 6. Statistically significant correlations ($p < 0.05$) within individual soil horizons between soil properties and microbial activity indexes.

	respC-CO_2	SIR-C_{mic}	BG	NAG	XYL	SP	PH	AUC_{bact}	AUC_{fungi}	R_{bact}	R_{fungi}
						Litter (organic O horizons)					

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TN							-0.4817		-0.4845	0.8140
C/N							0.4971			
					Humus horizons (A horizons)					
TN		-0.5130			0.6436		0.5763	0.5242		
FM	0.4848		0.4805							
pHw						0.6039	-0.8414	-0.6115		
NH_4^+-N			0.5800	0.7008	0.6144		0.8950	0.8946	-0.5220	

The substrates that differed significantly among litter samples were glycogen (ECO polymer), with the highest utilization in BB32 and the lowest in WD57; D-xylose (ECO carbohydrate), again most intensively utilized in BB32 and least in WD57; and N-acetyl-D-glucosamine (ECO carbohydrate), with the highest utilization in WD9 and the lowest in WD57 (Figure 6).

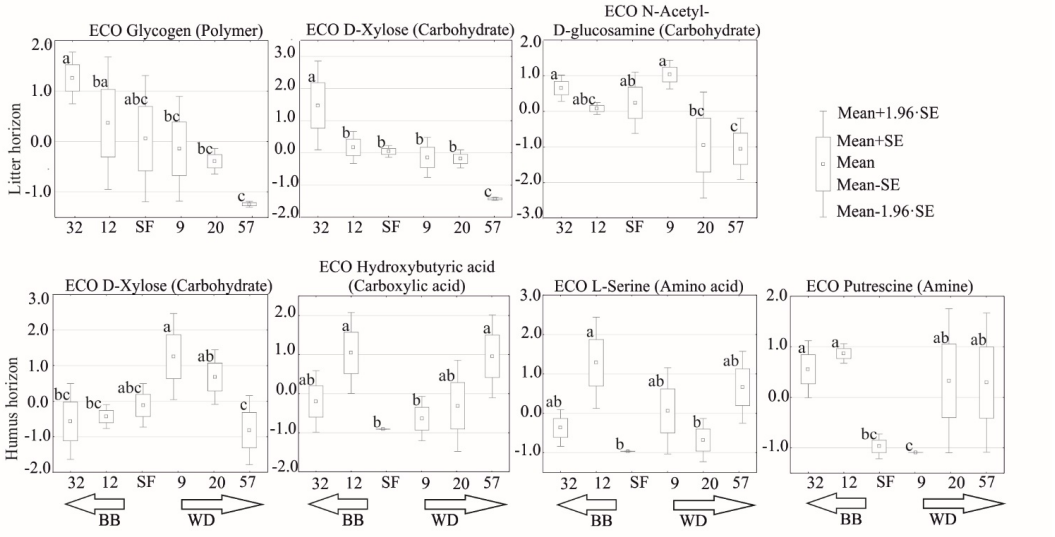

Figure 6. Differences in the utilisation of individual substrates (one-way ANOVA results). Significant differences between experimental plots are indicated by letters (a, b) (LSD test, $p < 0.05$). Only statistically significant differences ($p < 0.05$) are shown. Values on the y axis do not represent raw measurement means; data were transformed (logarithmic or square root, when required) to approximate a normal distribution and subsequently standardised.

In the humus horizons, PCA applied to the main substrate groups ($N = 18$) showed that F1 explained 27.76% of the variance and represented a negative relationship between the utilisation of ECO carbohydrates *vs.* the utilisation of ECO amino acids and ECO carboxylic acids (Figure 5b). F2 explained 21.10% of the variance and reflected a negative association between the

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utilisation of ECO amines and FF polyols *vs*. FF glycosides and ECO polymers. Based on F1, PCA distinguished clusters corresponding to young windthrows (WD9 and WD20), and BB12 with WD57.

The substrates that differed significantly among humus-horizon samples were: D-xylose (ECO carbohydrate), with significantly highest utilisation in WD9 and lowest in WD57; γ-hydroxybutyric acid (ECO carboxylic acid), utilised most intensively in BB12 and WD57 and least in SF; L-serine (ECO amino acid), with the highest utilisation in BB12 and the lowest in WD20 and SF; putrescine (ECO amine), most intensively utilised in BB12 and least in WD9 and SF (Figure 6).

3.5 Correlations between soil properties and biological activity

In the litter horizons, only a few biological parameters showed significant correlations with soil chemical properties. AUC_{bact} was positively correlated with TN and negatively correlated with the C/N ratio. Similarly, R_{bact} was negatively correlated with C/N, whereas R_{fungi} showed a positive correlation with C/N. No significant correlations were observed between respC-CO_2, SIR-Cmic, enzymatic activities, and SOC, TN, C/N, FM, pHw, NH_4^+-N, or NO_3^--N (Table 6). In the humus horizons, a greater number of biological indicators exhibited significant relationships with soil properties. RespC-CO_2 was positively correlated with FM, while SIR-C_{mic} was negatively correlated with TN. Enzymatic activities showed significant positive correlations with SOC (XYL, SP), C/N (BG), and NH_4^+-N (BG, NAG, XYL, PH). Soil pH (pHw) was positively correlated with SP and negatively correlated with PH. AUC_{fungi} was positively correlated with TN and NH_4^+-N but negatively correlated with soil pH (pHw). R_{bact} also exhibited a negative correlation with pHw (Table 6).

4 Discussion

4.1 Responses of soil biological activity to forest disturbance type across soil horizons

Our findings reveal a complex and nuanced pattern of soil responses to forest disturbance. Variations in several biological properties were related to both the type and the duration of disturbance. Furthermore, the contrasting responses observed between the litter and humus horizons indicate that distinct mechanisms regulate soil biological processes within these horizons following disturbance.

In our study, differences in soil respC-CO_2 and SIR-C_{mic} in the litter horizons were not statistically significant and therefore should be interpreted cautiously. Nevertheless, among parameters that reliably reflect overall soil biological activity, a clear temporal pattern emerged (Table 3).Soils affected by the oldest disturbances (BB32 and WD57) exhibited higher mean respC-CO_2rates and SIR-C_{mic} values than the youngest plots (WD9 and BB12) and the undisturbed forest (SF). Conversely, respC-CO_2 rates in the youngest disturbances (WD9 and BB12) were lower than in the forest plot (SF).The absence of statistical significance may be attributable to methodological limitations, such as an insufficient number of replicates. Previous studies (Gömöryová et al., 2014; Wasak et al., 2019) have demonstrated significant variation in these parameters following windthrow disturbances.In contrast to the litter horizon, mean SIR-C_{mic} and respC-CO_2 in the humus horizons

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were lower in windthrow plots than in both bark beetle–affected plots and the forest plot (Table 3). Although no statistically significant differences were observed between plots, this suggests the presence of a significant interaction between plot and soil horizon affecting SIR-C_{mic} ($p < 0.05$). Moreover, a decline in biological activity following disturbance was observed exclusively in the windthrow plots and occurred with a delay relative to the litter horizon (WD20; Table 3).

This differences between litter and humus horizons indicates that the post-disturbance conditions experienced by soil microorganisms differ markedly between those horizons. Similar differential responses have been documented previously—Geng et al. (2012) in clear-cuts and Wasak et al. (2019) in windthrow-affected soils—suggesting that environmental changes in the humus horizon are more abrupt and persistent in windthrow plots than in bark beetle–affected plots.The observed differences may be explained by contrasting microclimatic conditions and organic matter inputs associated with the different disturbance types(Schelker et al., 2013; Peplau et al., 2023; Zweigel et al., 2026).

The pattern observed in the litter horizon was partly supported by the enzyme activity results, which showed the lowest activity in the youngest disturbed plots (WD9 and BB12) and higher activity in WD20 (Figures 4, 5a). This pattern may reflect post-disturbance changes in the quantity, quality, and bioavailability of organic matter inputs, resulting in a temporary lag before microbial communities fully exploit the newly available substrates. Because the litter horizon constitutes the primary reservoir of recently deposited plant residues, disturbance-induced changes in litter inputs are likely to exert a particularly strong influence on microbial activity. As noted by Berg (2000) and Klotzbücher et al. (2011), intensive DOC leaching from fresh litter typically lasts a few months and is driven by the decomposition of water-soluble compounds and cellulose. Once this initial phase subsides, microbial activity may decline unless it is sustained by continued inputs of fresh organic matter or by the progressive decomposition of more recalcitrant substrates. Windthrow-affected soils are initially enriched with coarse woody debris (Table 1; Figure 1c), an important long-term reservoir of carbon and nutrients (Nazari et al., 2023; Błońska et al., 2024), but nutrient release from decaying wood is slow. Tobin et al. (2021) reported that windrowed Sitka spruce residues lost only ~5% of their carbon after four years. The cellulolytic (CB, BG) and xylolytic (XYL) enzymes assessed in this study target relatively labile polysaccharides. However, in woody material, a substantial fraction of these substrates becomes partially inaccessible to enzymes due to lignin encapsulation until oxidative degradation of lignin increases substrate accessibility(Berg, 2000). This limited accessibility of polysaccharides may reduce microbial investment in extracellular enzyme production in recently disturbed plots (WD9, BB12) and its increase over time since disturbance (WD20). It may also indicate that extracellular enzyme production represents an important constraint on microbial activity during the early stages following disturbance; however, this hypothesis requires further investigation. In early post-disturbance stages, the decline in fresh litter inputs from the living canopy had not yet been compensated by secondary vegetation successionnor by the progressive decomposition of woody debris, as indicated by the increased utilisation of amino acids and carboxylic acids in WD57 (Figure 4). On the other hand, external factors such as increased sunlight exposure and elevated soil temperatures at disturbed plots (Zweigel et al., 2026) may partially offset reduced organic inputs to the litter, diminishing differences in overall biological activity between plots. The absence of a comparable enzymatic activity peak in the bark beetle–affected plots (BB32) (Figure 4) may be attributed to the differing quality and temporal

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dynamics of organic matter inputs in windthrow versus bark beetle disturbances. In windthrow plots, needle fall occurs gradually for up to three years after uprooting (Hroššo et al., 2020), whereas during bark beetle outbreaks, needles and bark typically fall before tree collapse (Edburg et al., 2012). Spruce needles—the dominant input in bark beetle–affected plots—decompose relatively rapidly, losing approximately 50% of their mass within two years, with the fastest decomposition occurring during the first month (10–13% mass loss) (Haňáčková et al., 2015; Hågvar, 2016). Consequently, soils affected by bark beetle infestation tend to receive a higher proportion of easily decomposable material compared to windthrow-affected soils. However, in the early stage of infestation (as in BB12), the input of easily degradable needles may be limited because many trees still retain foliage (Table 1). The substrates utilised by catabolic microorganisms, as measured by BIOLOG® bacterial and fungal assays, consist primarily of easily degradable compounds—often products of enzymatic breakdown, such as xylose and cellobiose (Figures S1, S2). Thus, the highest AUC_{bact} observed at BB32 (Figure 4), together with enhanced bacterial utilisation of carbohydrates—particularly D-xylose (Figure 6)—is consistent with the hypothesis that BB32 soils had already passed the peak of extracellular enzyme activity and entered a later stage of microbial succession.The delayed decline in biological activityin the humus horizon of the youngest windthrow (WD9) was likely due to the additional inputs of organic matter from dead fine spruce roots, which undergo relatively rapid decomposition following tree mortality. Heim and Frey (2004) reported that approximately 70% of small spruce roots decompose within three years. Dead mycorrhizal fungi may also contribute to soil organic carbon pools (Bolat, 2014; Kim et al., 2018; Settineri et al., 2018). Such decaying roots act as microbial “hotspots,” exhibiting biological activity 4–20 times higher than that of bulk soil, and remain active as long as the root material persists (Kuzyakov and Blagodatskaya, 2015). Additionally, a considerable proportion of DOC in humus horizons originates from the decomposition of organic matter within the overlying litter horizons (Michalzik et al., 2001). Soil aggregates may also adsorb soil organic matter (SOM) on ped surfaces, thereby protecting it from rapid decomposition (Wasak-Sęk et al., 2025b) andproviding protected microsites for microbial colonisation. As a result, the reduction in labile carbon supply to the humus horizon may occur later than in the litter, even though primary sources of easily decomposable carbon—root exudates and mycorrhizal secretions—decline rapidly after disturbance (Xiong et al., 2011). Together, these processes likely prolonged the availability of labile carbon in the humus horizon following windthrow, delaying the decline in microbial activity relative to the litter horizon. The decrease in biological activity observed several months after windthrow aligns with findings reported by Gömöryová et al. (2014) and Wasak et al. (2019). The pattern indicating higher biological activity in soils affected by bark beetle infestation than in windthrow-affected soils suggests that, in the case of bark beetle outbreaks, certain mitigating factors operate to buffer the impact of disturbance on soil biological functioning. These findings are consistent with the conclusions of Holden and Treseder (2013), who reported that abiotic disturbances such as windthrow exert stronger negative effects on soil biological activity than biotic disturbances like bark beetle outbreaks. One potential explanation is the downward transport of decomposition products from decomposed spruce needles from the litter into the humus horizon. As discussed earlier, spruce needles decompose far more rapidly than woody material. Moreover, external environmental changes in bark beetle–disturbed plots typically occur more gradually (Edburg et al., 2012). For example, windthrow causes immediate root

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mortality, leading to the rapid collapse of mycorrhizal associations. In contrast, during bark beetle infestation, trees die progressively, allowing the mycorrhizal hyphal network to persist for longer periods (Pec et al., 2020).

4.2 Patterns of substrate utilization across forest disturbance types

Bacterial and fungal substrate utilisation patterns clearly differentiated disturbance types (windthrow versus bark beetle), indicating distinct post-disturbance pathways of carbon cycling driven by differences in the quantity and quality of plant-derived organic matter entering the soil. Furthermore, the catabolic activity patterns observed in the humus horizon differed from those in the litter horizon, indicating that the dominant sources of microbial substrates and their utilisation changed with soil depth (Figures 4, 5b). Whereas the litter horizon showed a pronounced functional divergence between the oldest windthrow (WD57) and bark beetle–affected (BB32) plots, differences among disturbance types were less distinct in the humus horizon. Nevertheless, PCA separated WD57 along the PC1 axis, reflecting greater bacterial utilisation of amino acids and carboxylic acids together with lower utilisation of carbohydrates, in both horizons, although in humus horizons only the differences in carbohydrates utilisation were statistically significant (Figures 4, 5b). In litter horizons of BB32, microbial catabolic activity was dominated by the decomposition of carbohydrates (D-xylose, and N-acetylglucosamine) and glycogen (Figures 4, 6).The youngest bark beetle–affected plot (BB12) formed a distinct group within humus horizons, characterised by significantly greater bacterial utilisation of L-serine (amino acid), γ-hydroxybutyric acid (carboxylic acid), and putrescine (amine) than the undisturbed forest (SF), resulting in a functional profile partly resembling that of WD57 (Figure 6).

The substrate utilisation pattern observed in WD57 is consistent with an advanced stage of secondary succession, during which belowground carbon inputs become increasingly important. Dense grass cover likely contributed substantial dissolved organic carbon (DOC) together with amino acid-rich root exudates, including L-arginine, L-asparagine, L-serine, and L-phenylalanine (Dakora and Phillips, 2002; Stevenson et al., 2004). Dominant grasses (e.g. *Calamagrostis* sp. and *Luzula* sp.) also contain higher protein concentrations than spruce tissues (Štýbnarová et al., 2016; Jyske et al., 2020), providing an additional source of amino acids. Similar patterns of enhanced amino acid utilisation have been reported from grass-dominated soils (Stevenson et al., 2004), whereas Osburn et al. (2022) observed increased glycine utilisation in soils affected by windthrow four decades earlier. The increased utilisation of carboxylic acids in WD57 is likewise consistent with advanced decomposition. Although compounds such as D-galactonic acid, D-galacturonic acid, and γ-hydroxybutyric acid may also originate from root exudates or humic substances (Mundie, 1976; Grayston et al., 1996; Grayston and Campbell, 1996; Zhang et al., 2011), they are also common products of lignin degradation. Because the upper soil horizons constitute a major reservoir of decomposing organic matter and dead roots, and previous studies have reported high carboxylic acid utilisation in forest soils (Stevenson et al., 2004; Wu et al., 2017), we infer that the elevated utilisation of carboxylic acids in WD57 most likely resulted from the combined influence of advanced decomposition of lignin-rich spruce woody material and roots, together with increasing inputs of grass-derived root exudates. In the humus horizon, roots provide readily

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degradable carbon-rich compounds that can stimulate microbial decomposition (Herman et al., 2006; Finzi et al., 2015). However, this effect was not clearly reflected in the overall biological activity, suggesting that its influence may become more apparent over longer periods following disturbance.

The oldest windthrow (WD57) and bark beetle–affected (BB32) plots exhibited contrasting patterns of microbial catabolic activity, indicating that these two disturbance types followed different functional trajectories where BB32 showed enhanced utilisation of carbohydrates and glycogen. In the humus horizon, the same overall contrast remained far less pronounced, suggesting that the influence of disturbance type on microbial substrate utilisation became increasingly modified by belowground carbon inputs and soil organic matter dynamics. Within the humus horizon, PCA still separated WD57 along the PC1 axis, reflecting greater bacterial utilisation of amino acids and carboxylic acids together with lower utilisation of carbohydrates, although only differences in carbohydrates utilisation were statistically significant (Figures 4, 5b).High utilization of carbohydrates and glycogen,the most pronounced in the litter horizon of oldest bark-beetle (BB32) indicates a predominance of readily degradable organic matter derived from the early stages of spruce needle decomposition together with rapid fungal turnover (Berg, 2000; Klotzbücher et al., 2011; Moore et al., 2011; Shimoi et al., 2020; Bourget et al., 2023). It further suggests that this pattern reflects both the transient input of fresh spruce litter and disturbance-induced shifts in fungal community composition.

The distinct substrate utilisation pattern observed in humus horizons of BB12 probably reflects short-term changes in belowground carbon dynamics during the early stages of bark beetle infestation. The increased bacterial utilisation of L-serine, γ-hydroxybutyric acid, and putrescine may represent metabolic responses of bark beetle–infested spruce roots to drought stress, which is known to alter root exudation and polyamine metabolism (Grayston and Campbell, 1996; Alcázar et al., 2010; Lim et al., 2020; Lin et al., 2022). In the youngest windthrow plot (WD9), enhanced bacterial utilisation of D-xylose indicates a greater contribution of readily decomposable plant-derived substrates, most likely originating from fine-root decomposition (Bourget et al., 2023).

4.3 Fungal community structure shaped by forest disturbance

Our results show substantial shifts in fungal community structure following both bark beetle and windthrow disturbances, particularly in the litter horizon. The highest utilisation of N-acetylglucosamine in litter at BB32 and WD9, together with elevated glycogen utilisation at BB32 (Figure 6), suggests intensive fungal biomass turnover. N-acetylglucosamine is a structural component of fungal cell walls as part of the chitin polymer (Shimoi et al., 2020), whereas glycogen functions as a major storage carbohydrate in fungal cells (Moore et al., 2011). These differences were particularly pronounced when compared with the oldest windthrow plot (WD57), which exhibited the lowest utilisation of both N-acetylglucosamine and glycogen (Figure 6). This pattern suggests reduced fungal biomass turnover and may indicate relatively greater bacterial activity at the WD57 plot.. The highest AUC_{fungi} values observed in the BB12 litter, combined with the lowest values at WD57 (Figure 4), further support this interpretation. Nevertheless, AUCfungi captures only one aspect of fungal activity..

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The rapid increase in enzyme production observed in the WD20 litter may reflect the proliferation of saprophytic fungi (Custer et al., 2020). Although species-level analyses would be required to confirm this interpretation, our findings are consistent with accelerated fungal biomass turnover (Custer et al., 2020). Together with the elevated NAG activity, they suggest substantial alterations in fungal community structure occurring at different stages of post-disturbance succession across the investigated plots.A rapid decline in mycorrhizal fungi accompanied by an increase in saprophytic fungi is a well-documented consequence of forest disturbances, including both windthrow and bark beetle outbreaks (Egli et al., 2002; Štursová and Baldrian, 2011; Štursová et al., 2014; Custer et al., 2020). Enhanced glycogen decomposition was observed 21 months after windthrow in the humus horizons of soils from the Lejowa Valley (Wasak et al., 2019). Similarly, Osburn et al. (2022) reported increased catabolic decomposition of autolysed yeast in windthrow-affected soils compared with undisturbed forest soils. In our study, such changes were more strongly expressed in the litter than in the humus horizon. However, the significant interaction between plot and soil horizon (Table 4; Figure 4) suggests that polymer utilisation shifts from the litter to the humus horizon over time, reaching its highest levels in the humus of the oldest windthrow plots (WD20 and WD57).

4.4. Biological activity vs. soil properties

Soil properties and their disturbance-induced changes influenced patterns of biological activity in the litter only to a limited extent (Table 6). The weak correlations observed between soil properties and biological activity in the litter horizon may reflect the relatively high nutrient availability in litter compared with the humus horizons and/or the stronger influence of temperature fluctuations, both of which are known to affect biological processes but were not directly investigated in this study.

In contrast to the litter horizon, many biological parameters in the humus horizon were significantly related to soil chemical properties, particularly nitrogen availability (TN and NH_4^+–N) and soil pH (pHw) (Table 6). However, these properties did not differ significantly among disturbance types, likely reflecting the buffering capacity of the mineral soil in these inherently fertile ecosystems. Consequently, enzymatic activities also showed only minor differences among plots, with PCA separating samples primarily according to PH and SP activity rather than enzymes involved in C and N cycling (Figure 5a).

Positive correlations of BG, XYL, NAG, and PH with NH_4^+–N, together with correlations between XYL, PH, and TN (Table 6), suggest that nitrogen availability may promote cellulolytic and phosphatase activity, consistent with previous findings (Allison and Vitousek, 2005). At the same time, the strong NAG activity observed in our study may also contribute to these relationships, in agreement with the observations of Berg (2000). NH_4^+–N was also strongly positively correlated with both AUC_{fungi} and R_{bact} (Table 6), indicating that nitrogen availability supported the activity of both fungal and bacterial communities. Nevertheless, because NH_4^+–N and TN did not differ significantly among the studied plots, these relationships were not reflected in significant differences in enzyme activities between disturbance types (Table 2).

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Our results further identify soil pH as an important factor controlling SP and PH activity in the humus horizon (Table 6). This agrees with established enzyme kinetics, whereby sulfatases generally exhibit higher activity at elevated pH, whereas acid phosphatases are most active under acidic conditions (Pettit et al., 1977; Dick and Tabatabai, 1984; Hui et al., 2013). The differences in pHw observed in the humus horizon, which were not mirrored in the litter (Table 2), likely reflect local variation in soil substrate properties rather than direct effects of post-disturbance vegetation changes. Overall, these findings highlight the greater physicochemical stability of the humus and underlying mineral horizons relative to the more dynamic litter horizon.

4.5 Biological indicators of soil activity under forest disturbance

By combining multiple complementary methods to assess soil microorganisms, this study provides new insights into the strengths and limitations of biological indicators. Under stable conditions, enzymatic activity is generally well correlated with microbial biomass and respiration (Moorhead et al., 2013). Enzymatic activity is also positively associated with metabolic profiles obtained using BIOLOG® ECO plates (Winding and Hendriksen, 2007). However, under disturbance conditions, the balance between microbial abundance and activity may become disrupted. For example, Moorhead et al. (2013) showed that following fresh organic matter inputs, microbial biomass increased rapidly whereas enzymatic activity temporarily declined because microorganisms preferentially exploited readily available substrates that required little extracellular enzymatic degradation.Discrepancies between enzymatic activity and BIOLOG® ECO responses have also been reported under conditions of soil contamination (Floch et al., 2011; Liu et al., 2015). The weak or absent correlations among several biological indicators observed in our study are consistent with these findings and suggest that forest disturbances temporarily decouple microbial abundance, enzymatic functioning, and metabolic activity.Nevertheless, our results demonstrate that combining complementary biological indicators provides a more comprehensive assessment of disturbance-induced changes in soil elemental cycling than any single method alone.

5 Conclusions

The effects of forest disturbance on soil microbial activity followed a non-linear temporal trajectory. The litter and humus horizons exhibited distinct patterns of change in overall biological activity, enzymatic functioning, and substrate utilisation, indicating that these horizons respond differently to external drivers and substrate availability.

Natural forest disturbances—windthrow and bark beetle infestation—generated markedly different soil biological responses, particularly in relation to the decomposition dynamics of various substrate types. These contrasting trajectories were most likely driven by differences in the type of organic material entering the soil. In bark beetle–affected areas, a large proportion of inputs consisted of decomposing spruce needles, whereas windthrow plots were characterised by inputs of root exudates and lignin-rich woody debris. Consequently, bark beetle infestation produced a more gradual biological response than

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windthrow, particularly during the initial post-disturbance period. This likely reflects the continuous input of organic material and the absence of an abrupt interruption in carbon supply.

The litter horizon was generally more responsive to functional changes—such as enzymatic activity and substrate utilisation—than the humus horizon, most likely because its biological activity depends more directlyon fresh inputs of organic material. Variability within the humus horizon was smaller but more strongly linked to soil chemical properties, particularly nitrogen availability and soil pH.

Under disturbed conditions, different indicators captured different aspects on soil biological activity, reflecting disruptions in elemental cycling—especially carbon cycling—and deeper alterations within the soil biota. Therefore, applying multiple complementary indicators is valuable for accurately assessing the extent and nature of ecosystem disturbance.

Together, these findings contribute to a better understanding of nutrient cycling and microbial recovery following natural disturbances in mountain forest ecosystems.

Code and data availability

The datasets supporting this study, including soilproperties, soil respiration, SIR-C_{mic},enzymatic activity and catabolic activity (BIOLOG ECO and FF plates) measurements, and the Supplement containingthe mean proportion of utilisation of individual bacterial and fungal (ECO and FF) substrates by substrate group (Tables S1, S2) are openly availablein the figshare repository at https://doi.org/10.6084/m9.figshare.32157468.

Author contributions

The experimental conceptualization and investigation was a collaboration between all co-authors. Funding acquisition, project management and supervision: KWS, JH, MD; Methodology, validation, formal analysis and data curation: KWS, BK, EB; Resources: BK, EB, MD; Visualization: KWS, MD; Writing: First draft: KWS; Rewriting and editing: MD, BK, EB, JH.

Competing interests

The contact author has declared that none of the authors has any competing interests.

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