The effects of soil copper contamination and invasive plants on soil properties, microbial communities and plant growth in riparian habitats

by

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Abstract

Riparian areas are an important transition zone in freshwater systems, connecting and regulating both aquatic and terrestrial systems. They are characterized by a high diversity and are important for conservation. However, riparian areas are frequently under stress from human activities. Two of these stressors are agricultural activity and introduction of invasive plant species. One example of the impact of agriculture is pollution with heavy metals such as copper. Copper is commonly used as a fungicide in agriculture and can be introduced into riparian areas via flooding events via streams in areas previously unaffected by pollution. There, copper is toxic to animals, plants and microorganisms at high concentrations, damaging DNA, enzymes, cell membranes and chloroplasts. This leads to a reduction in growth and reproduction of the affected organisms, potentially disrupting the ecosystems. Invasive alien plants are another major cause of biodiversity loss in animals, plants and microorganisms. This can negatively affect entire ecosystems above as well as belowground, leading to alterations of resources and ecosystem functions. Especially soil fungi are important for ecosystem functions. For example by forming symbiotic interactions with plants, which can be disrupted by plant invasion and copper pollution. Two common invasive plant species in riparian areas are *Fallopia japonica* and *Impatiens glandulifera*. They frequently invade stands of the native *Urtica dioica*. The aim of this project was to investigate the impact of these two plant invaders, especially of *F. japonica*, on native soil communities further modified by copper pollution. This was done in two parts: a field study, investigating the impact of the invasive plants on soil properties, invertebrates, fungi and activity and a mesocosm experiment under the influence of copper pollution, comparing the impact of copper on plants, soil invertebrates, microorganisms and activity depending on the presence of a native or invasive plant species. Under field conditions, plant invasion mainly reduced the diversity of fungi directly associated with the plants but not the biomass of fungi. Direct impacts on soil invertebrates were also observed. In the mesocosms, microbial biomass was reduced under the invasive plant and no impact on invertebrates was observed. Similarly, the soil activity was not affected in the field but was strongly reduced by the presence of *F. japonica* in the mesocosms. These results align with the enemy release hypothesis, indicating that these invasive species, especially *F. japonica*, may be less associated with fungal parasites in the invasive range, allowing them to perform better than native species. These findings also indicate that these invaders have various and contrasting impacts on belowground systems, making their effects highly context-dependent and site-specific. Copper pollution inhibited growth in both *F. japonica* and *U. dioica*. *Urtica dioica* seemed to be more sensitive to copper pollution compared to the invasive plant. In the soil, copper pollution further amplified the reduction of soil activity by the invasive plant and had variable effects on invertebrates and microbial biomass. This indicates that *F. japonica* may gain an advantage against the commonly occurring *U. dioica*, especially in polluted areas. The negative impact of copper pollution on soil functions could therefore be amplified by facilitating invasion by *F. japonica*, which also negatively impacts soil functions. Therefore, disturbances by agricultural activity, one major source of copper pollution, could have an even stronger impact across much wider distances and in previously undisturbed areas.

Zusammenfassung

Ufergebiete sind eine wichtige Übergangszone in Süßwassersystemen und verbinden und regulieren sowohl aquatische als auch terrestrische Lebensräume. Sie zeichnen sich durch eine hohe Artenvielfalt aus und sind wichtig für den Naturschutz. Allerdings sind Ufergebiete auch häufig durch menschliche Aktivitäten belastet. Zwei dieser Belastungen sind Landwirtschaftliche Aktivitäten und die Einführung von invasiven Pflanzen. Ein beispiel für den Einfluss von Landwirtschaft ist die Verschmutzung durch Schwermetalle wie zum beispiel Kupfer. Kupfer ist ein häufig angewandtes Fungizid in der Landwirtschaft. Durch Überflutungen kann es über Flüsse und Bäche in Ufergebite eingebracht werden, die ursrpünglich nicht durch Umweltverschmutzung beeinflusst waren. Dort kann Kupfer in hohen Konzentrationen schädlich für Tiere, Pflanzen und Mikroorgansimen wirken. Es beschädigt DNA, Enzyme, Zellmembranen und Chloroplasten. Dies führt zu einer Reduktion im Waschstum und der Fortpflanzung der beeinflussten Organismen, was potentiell das ganze Ökosystem beeinflussen kann. Invasive Pflanzen sind eine andere wichtige Ursache für den Verlust von Biodiversität von Tieren, Pflanzen und Mikroorganismen. Dieser Verlust kann gesamte überirdische und unterirdische Ökosysteme negativ beeinflussen, was zu Veränderungen in der Nährstoffverfügbarkeit und Ökosystemfunktionen führen kann. Insbesondere Pilze im Boden sind wichtig für Ökosystemfunktionen, zum Beispiel indem sie symbiotische Interaktionen mit Pflanzen eingehen. Diese können durch invasive Pflanzen und Kupfer gestört werden. Zwei weit verbreitete invasive Pflanzen in Ufergebiten sind *Fallopia japonica* und *Impatiens glandulifera*. Diese Arten dringen häufig in Bestände der einheimischen *Urtica dioica* ein. Das Ziel dieses Projekts ist es den Einfluss der zwei invasiven Pflanzen (insbesondere den von *F. Japonica*) auf heimische Bodengemeinschaften zu untersuchen und wie dieser Einfluss weiter durch Kupferverschmutzung beeinflusst wird. Dies wurde in zwei Schritten gemacht. In einer Feldstudie wurde der Einfluss der invasiven Pflanzen auf Bodeneigenschaften, Invertebraten, Pilze und Aktivität untersucht. In einem Mesokosmos Experiment unter dem Einfluss von Kupferbelastung wurde anschließend der Effekt von Kupfer auf Pflanzen, Bodeninvertebraten, Mikroorganismen und Aktivität abhängig von der Präsenz einer invasiven oder heimischen Pflanze untersucht. Im Freiland reduzierten invasive Pflanzen hauptsächlich die Diversität von Pilzen die direkt mit der Pflanze assoziiert sind und Invertebraten im Boden aber nicht die Pilzbiomasse. In den Mesokosmen war die mirkobielle Biomasse durch *F. japonica* reduziert aber es wurde kein Einfluss auf Invertebraten festgestellt. Ähnlicherweise war die Bodenaktivität im Freiland nicht beeinflusst aber in den Mesokosmen durch *F. japonica* stark reduziert. Diese Ergebnisse stimmen mit der enemy release Hypothese überein, die besagt, dass invasive Arten wie *F. japonica* im invasiven Verbreitungsgebiet weniger von parasitischen Pilzen belastet sind und daher besser als heimische Arten wachsen können. Außerdem scheinen diese invasiven Arten verschiedene und auch gegensätzliche Einflüsse auf unterirdische Gemeinschaften zu haben, was deren Effekte abhängig vom Kontext und Standort macht. Kupferverschmutzung reduziert das Wachstum von *F. japonica* und *U. dioica* wobei letztere stärker von Kupfer beeinflusst zu werden scheint als die invasive Art. Im Boden verstärkt Kupfer die Reduktion der Bodenaktivität durch *F. japonica* und hat verschiedene Einflüsse auf Invertebraten und mikrobielle Biomasse. Dies weist darauf hin, dass F. japonica einen Vorteil gegenüber *U. dioica* in verschmutzen Gebieten gewinnen kann. Der negative Einfluss von Kupfer auf die Bodenaktivität könnte demnach weiter verstärkt werden, indem es die Invasion durch *F. japonica* erleichtert. Der negative Einfluss von Landwirtschaft (eine der wichtigsten Quellen von Kupfer) könnte daher einen stärkeren und weitreichenderen Einfluss auf Uferbereiche auch in bisher unbelasteten Gebieten haben.

1 Introduction

Riparian areas are the interface between the aquatic and the terrestrial ecosystems, making them an important transition zone in freshwater systems (González et al. 2017; Naiman and Decamps 1997; Swanson et al. 1982). By forming a buffer between land and water, they connect and are involved in regulation of ecological functions of both systems (Gregory et al. 1991; Naiman and Décamps 1997). Riparian areas are often characterized by a high diversity of species and fulfill important ecological functions and provide key services such as soil fertility and water purification (González et al. 2017; Naiman and Décamps 1997; Schulz et al. 2015). For example, Zhang et al. (2023) found that riparian areas may become climate refugia for threatened woody species. The diverse riparian plant communities help increase the overall diversity by providing habitats and food to animal populations. Therefore, riparian corridors are important for conservation of both aquatic and terrestrial ecosystems (Casotti 2015; Pusey and Arthington, 2003; Decamps 2004). Riparian habitats are threatened by many different stressors. Among these, two of the most widespread and important ones are plant invasion and, especially in the study region, pollution by heavy metals and especially copper due to agricultural activity.

1.1 Copper in the environment

1.1.1 Copper in agriculture and transfer to non-target ecosystems

Copper as a fungicide has been a part of agriculture since the 19th century (Johnson 1935). One major use of copper is the protection against pathogenic fungi such as *Plasmopara viticola* in vineyards. *Plasmopara viticola* is the cause of grapevine downy mildew and can drastically reduce yield in vineyards (Dick 2002; Kühne et al. 2009). Through precipitation, the fungicides can enter the soil and since copper does not degrade, it accumulates in soils. This leads to elevated concentrations in the soil after long-term use in these areas (Wightwick et al. 2008). In European vineyards, for example, soil copper levels of up to 600 mg kg^{-1} have been observed, while corresponding uncontaminated sites ranged between 20 and 100 mg kg^{-1} (Ruyters et al. 2013). Similarly, vineyards established for over 100 years in France also had elevated soil copper levels of up to 300 mg kg^{-1} (Parat et al. 2002). The long-term application of copper can however also result in much higher levels - reaching up to 3000 mg kg^{-1} - in agricultural areas (Brunetto et al. 2016). Copper can then be transported to adjacent non-target aquatic ecosystems from agricultural soil through spray drift, groundwater drainage, and surface run-off (Ripke et al. 2001) and accumulate in sediments (Han et al. 2001). where it can be remobilized and transferred to riparian ecosystems during flooding with potential effects on local plant species, affecting ecosystem structure and functioning (Schulz et al. 2015).

1.1.2 Effects of copper on organisms and functions

Copper is an essential micronutrient for virtually all organisms, for example supporting enzyme activity and chlorophyll production in plants (Gilbert 1952). For example, the protein Plastocyanin, which is involved in the electron transport chain contains copper (Gross 1993). It is also involved in many other enzymes as well as electron transport and redox reactions (Cervantes & Gutierrez-Corona 1994, Husak 2015). However higher concentrations are detrimental or even toxic for organisms such as plants (Kuhns & Sydnor 1976; Lombardi & Sebastiani 2005; Schmitz et al. 2023, see Amendment II) or microorganisms. The main pathways of toxicity are damages to DNA, enzymes and membranes by creating radicals in the cell (Cervantes & Gutierrez-Corona 1994, Husak 2015; Shabbir et al. 2020).

Moreover, copper can induce cell membrane ruptures and abnormalities in leaf chloroplasts, which impacts photosynthesis and nutrient transport (Reichman 2002; Tsay et al. 1995). Protective processes, which vary between species, have evolved including detoxification using enzymatic and non-enzymatic antioxidants as well as sequestration of excess copper (Shabbir et al. 2020). Nonetheless, plants often respond to heavy metal exposure by growth impairments. For example, $CuSO_4$ concentrations of 100 µM caused reduced growth rates and necrosis in *Prunus cerasifera* (Lombardi and Sebastiani 2005). Copper toxicity manifesting as chlorosis and subsequent desiccation and death of plant parts as well as stunted growth has been observed in woody ornamentals (Kuhns and Sydnor 1976), which is similar to symptoms produced by other heavy metals. Bouazizi et al. (2010) also reported a reduction in biomass and leaf number in Phaseolus vulgaris.

In general, bacteria are reported to be less impacted by copper pollution than fungi (Flemming & Trevors 1989). However, Wang et al. (2018) found that soil copper pollution of 1.60 mmol kg^{-1} similarly reduced the abundance of both bacteria and fungi. Huysman et al. (1994) even reported a higher sensitivity of bacteria to copper pollution, where copper concentrations of 10 µg g^{-1} caused a 1000-fold increase in the number of Cu-resistant bacteria while not affecting total microbial biomass.

Soil invertebrates are also impacted by copper pollution. For example, the earthworm species *Octolasium cyaneum* was found to be highly sensitive to copper pollutions starting at 40 ppm, while oribatid mites were not significantly impacted by copper conditions of 200 ppm in the same experiment (Streit 1984). Further, for three other invertebrate species (the earthworm *Eisenia andrei*, the potworm *Enchytraeus crypticus* and the springtail *Folsomia candida*), the EC_{50} for reproduction were between 130 and 190 mg Cu kg^{-1} (Caetano et al. 2016). However, other soil properties such as pH value also impact the toxicity of copper to invertebrate soil organisms (Criel et al. 2008).

1.2 Invasive riparian plants

1.2.1 Plant invasions

Due to human activity, many plant species have been introduced in habitats outside their native range (Fuentes-Lillo et al. 2021, van Kleunen et al. 2015). Some of those species exhibit traits which enable them to become invasive, resulting in multiple effects on the structure and functions of affected ecosystems (Blackburn et al. 2011; Hulme 2009; Schirmel et al. 2016; van Kleunen et al. 2015). Invasive alien plants are widely accepted as a major cause of biodiversity loss in native communities, which, in turn, can negatively affect the entire ecosystem (Hooper et al. 2012).

1.2.2 Impact of invasive plants

Plant invasion often occurs in riparian ecosystems (Schirmel et al. 2016). Plant invasion impacts native plant communities, reducing diversity and abundance of native plants (Powell et al. 2011). The effect on the native plants can be caused by different alterations caused by plant invasion. Some of these effects are disruption of the pollination of native species (Parra-Tabla and Arceo-Gómez 2021). For example, the invasive plant *Impatiens glandulifera* is highly attractive to some pollinators (Bartomeus et al. 2010) and many invasive species are highly integrated into pollinator networks (Vilà et al. 2009). Further, allelopathic effects which are often found in invasive plants (Kalisz et al. 2021) can negatively impact native vegetation. The phytotoxins from *Centaurea maculosa* for instance displace native western US plants by inhibiting native species' growth and germination (Bais et al. 2003).

Another factor is competition for resources. Especially competition for nutrients and light is an important factor in plant communities (Lekberg et al. 2018). An example for invasive plants displacing native vegetation is *Agropyron cristatum*, which can prevent other species from establishing via strong competitive effects (Bakker and Wilson 2001). Under nutrient poor conditions, Sardans et al. (2017) found higher nutrient uptake efficiency in invasive species under nutrient poor conditions. However, no species can maximize its competitive ability across all environments, so the success of invasive species is habitat dependent (Funk and Vitousek 2007). In general, nutrient-rich habitats are more commonly invaded than resource-poor habitats, because traits generally associated with invasion success such as high growth rates and early reproduction are favoured under these conditions (Funk and Vitousek 2007). Another factor favouring invasive plants over native species are resource fluctuations (Davis et al. 2000) but these effects are not generalized across invasive plant species (Radford 2013).

Besides effects on aboveground compartments, invasive plants also impact belowground compartments (Liao et al. 2008; Abgrall et al. 2019), altering resources and ecosystem functions and processes (Crooks 2002; Ehrenfeld 2003; Waller et al. 2020). Invasive plants can impact soil physicochemical properties, e.g. by increasing pH and water content (*Leucanthemum vulgare,* Ahmad et al. 2020) or lowering pH and increasing water content (*Carpobrotus edulis*, Novoa et al. 2014). Invasive plants also have various effects on the soil nutrient status. For example, invasion by *Spartina alterniflora* can increase nitrogen and organic carbon levels (Liao et al. 2007) or increase phosphorus storage while having no effect on C and N (Wang et al. 2019). As a result, soil microbial communities, including fungi, can be affected by plant invasion. These communities are influenced by plant invasion with respect to biomass (e.g. increasing biomass with woody plant invasion; Liao and Boutton 2008) or community structure of the soil microorganisms (Ravit et al. 2003; Si et al. 2013; Sapsford et al. 2022). Soil functions can be affected in terms of decreased litter decomposition and increased microbial activity (Vilà et al. 2011) or reduced soil biological activity (Pehle and Schirmel 2015). Soil fungi have important roles in ecosystem functioning (Öpik et al. 2006). The community of mycorrhizal, endophytic as well as parasitic fungi depends on the plant species and may thus be modified by invasion (Knogge 1996; Christian et al. 2016). In contrast to mycorrhizal and endophytic fungal groups, saprobic fungi are involved in the decomposition of particulate organic matter (Duguay and Klironomos 2000) and, hence, may be less affected by plant invasion.

Plant invasion can also affect soil functions such as soil activity either directly or indirectly by influencing soil microorganisms. Inderjit and van der Putten (2010) propose three pathways in which soil communities and invasive plants interact. One, invasive plants are less affected by plant soil feedback, two, they can alter soil biota (specifically decreasing the number of symbionts) and three, they can produce toxic chemicals that cannot be neutralized by soil biota. Plant invasion can cause a shift in soil functions compared to uninvaded soils and if these shifts cause long term changes in the soil, they are likely to promote invasion by other exotic plants (Kourtev et al. 2003). Another example is *Bromus tectorum* invasion, which increased the number of active bacteria in newly invaded sites while lowering species richness and absolute numbers of fungi and invertebrates (Belnap and Phillips 2001). *Carpobrotus edulis* and *Mikania micrantha* invasion also cause changes in enzymatic activities and microbial biomass (Novoa et al. 2014; Yu et al. 2021).

Furthermore, like for the microbial communities, plant invasions can influence the soil invertebrate fauna (Abgrall et al. 2019; Belnap et al. 2005; Biederman and Boutton 2009), which are necessary for soil functions like decomposition (Deyn et al. 2003; Tresch et al. 2019). For example, Collembola and Acari, often the dominant

arthropod groups in soil (Petersen and Luxton 1982; Deharveng 1996; Behan-Pelletier 2003) respond to changes in the vegetation, including plant invasion (Salamon et al. 2004; Wissuwa et al. 2012). Altering the vegetation composition by replacing native plant species, could thus negativly affect soil arthropod abundance and diversity (Salamon et al. 2004; Wissuwa et al. 2012; Rusterholz et al. 2014). However, impacts of invasive plants on soil fauna are highly variable and depend on the habitat type (Abgrall et al. 2019), since many soil arthropod species are also dependent on abiotic soil properties (Cassagne et al. 2003; Bedano 2004).

1.3 Study species

1.3.1 *Fallopia japonica*

The Asian knotweed, *Fallopia japonica*, is a perennial, rhizome-forming species and one of the most invasive plants in Europe due to its ability to quickly produce high high above and below ground biomass (Beerling et al. 1994). Fallopia japonica is native to East Asia and was introduced to Europe as a garden plant. It subsequently became widespread in many parts of Europe (Beerling et al. 1994). In Japan it occurs as a pioneer species on volcanic gravel, while it is mostly found in habitats influenced by human activity in the invasive range but is also often present in riparian areas (Beerling et al. 1994).

Fallopia japonica outcompetes native plants for resources such as light and nutrients by forming dense stands and reducing biodiversity (Aguilera et al. 2010) and altering soil characteristics, like modifying soil nutrient stocks (Dassonville et al. 2007; Stefanowicz et al. 2017). *Fallopia japonica* shows allelopathic effects (Murrell et al. 2011; Parepa and Bossdorf 2016) with significant impacts on the soil food web structure (Abgrall et al. 2018), decreasing abundance and diversity of soil and root fungi, such as mycorrhizal fungi (Schmitz et al. accepted, see Amendment I; Zubek et al. 2016). The success of invasive plants can be related to a higher tolerance toward heavy metals, relative to native species (Li et al. 2022). The genus *Fallopia* has been shown to be tolerant to heavy metal contamination including copper (Barberis et al. 2020), which is accumulated mainly in roots (*F. convolvulus*: Pedersen et al. 2000, *F. japonica*: Berchová-Bímová et al. 2014; Sołtysiak 2020). While copper at concentrations up to 200 mg kg^{-1} did not affect the ability of *F. japonica* to establish from rhizomes, at concentrations equal to or above 300 mg kg^{-1} it delayed plant growth and impacted morphology, which could give the species a competitive advantage in heavy metal contaminated soils (Sołtysiak 2020). The tolerance to heavy metals in *F. japonica* is thought to be connected to the plant's ability to sequester copper into the cell wall (Nishizono et al. 1989) and produce specific binding proteins even at high metal concentrations (Kubota et al. 1988).

1.3.2 *Impatiens glandulifera*

Another highly invasive plant in Europe is the Himalayan balsam, *Impatiens glandulifera* (Beerling and Perrins 1993). This annual herb native to the Himalayans has strong dispersal capabilities with masses of floatable seeds. The plant was first introduced as an ornamental plant in the 19th century and quickly became a naturalized alien found in the majority of Europe (Pyšek and Prach 2000). In the native range it is found at altitudes of more than 2000 m above sea level, while it is commonly found along riparian areas in Europe (Beerling and Perrins 1993).

Impatiens glandulifera is known for its impact on native communities with positive effects on soil fauna abundance (Tanner et al. 2013; Rusterholz et al. 2014), modifying soil fungal community composition by increasing fungal diversity and lowering arbuscular mycorrhizal fungal root colonization (Gaggini et al. 2018, 2019).

1.3.3 *Urtica dioica*

In European riparian ecosystems, an abundant, naturally occurring species is the Common nettle (*Urtica dioica* L., Urticaceae), a rhizomatous, perennial herb often found in nitrogen-rich soils (Olsen 1921; Taylor 2009). It forms compact stands excluding other vegetation which are often formed via vegetative reproduction (Taylor 2009). *Urtica dioica* is tolerant to heavy metals such as copper (Balabanova et al. 2015; Grubor 2008; Štrba et al. 2015), which is often accumulated in aboveground parts (Bislimi et al. 2021).

1.4 Author Contributions

This is a cumulative dissertation containing two published articles in peer-reviewed international journals and one manuscript in preparation for the Journal of Plant Nutrition and Soil Science. The contributions of each author for the publications included in this thesis are outlined below.

1.4.1 Amendment I

Daniel Schmitz: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing - original draft, Writing - review & editing.

Johanna Girardi: Data curation, Investigation.

Eva Ulrich: Data curation, Investigation.

Katherine Muñoz-Sepulveda: Investigation, Methodology, Writing - review & editing.

Mirco Bundschuh: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing - review & editing.

Kai Riess: Conceptualization, Supervision, Writing - review & editing.

Jens Schirmel: Conceptualization, Supervision, Writing - review & editing.

1.4.2 Amendment II

Daniel Schmitz: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing - original draft, Writing - review & editing.

Johanna Girardi: Methodology, Investigation, Writing - review and editing.

Jellian Jamin: Methodology, Investigation, Writing - review and editing.

Mirco Bundschuh: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing - review & editing.

Benedict Geng: Investigation, Writing - review and editing.

Rico Feldmann: Investigation, Writing - review and editing.

Verena Rösch: Methodology, Writing - review & editing.

Kai Riess: Conceptualization, Methodology, Supervision, Validation, Writing - review & editing.

Jens Schirmel: Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing - review & editing.

1.4.3 Amendment III

Daniel Schmitz: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing - original draft, Writing - review & editing.

Mirco Bundschuh: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing - review & editing.

Benedict Geng: Investigation, Writing - review and editing.

Elyssa Dubois: Methodology, Investigation, Writing - review and editing.

Jens Schirmel: Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing - review & editing.

Kai Riess: Conceptualization, Investigation, Methodology, Supervision, Validation, Writing - review & editing.

2 Research objectives

Aquatic micropollutants and invasive plant species are factors that strongly impact riparian ecosystems. The aim of this project is to investigate the effect of that factors exhibit on multiple trophic levels. The project is split into two experiments (Fig. 1): a field study investigating the impact of plant invasion on riparian soil microbial and invertebrate community structure under field conditions and a semi-controlled experiment (Joint pot experiment) testing the causal effects of micropollutants on the soil microbial community and subsequent effects on the focal plant species.

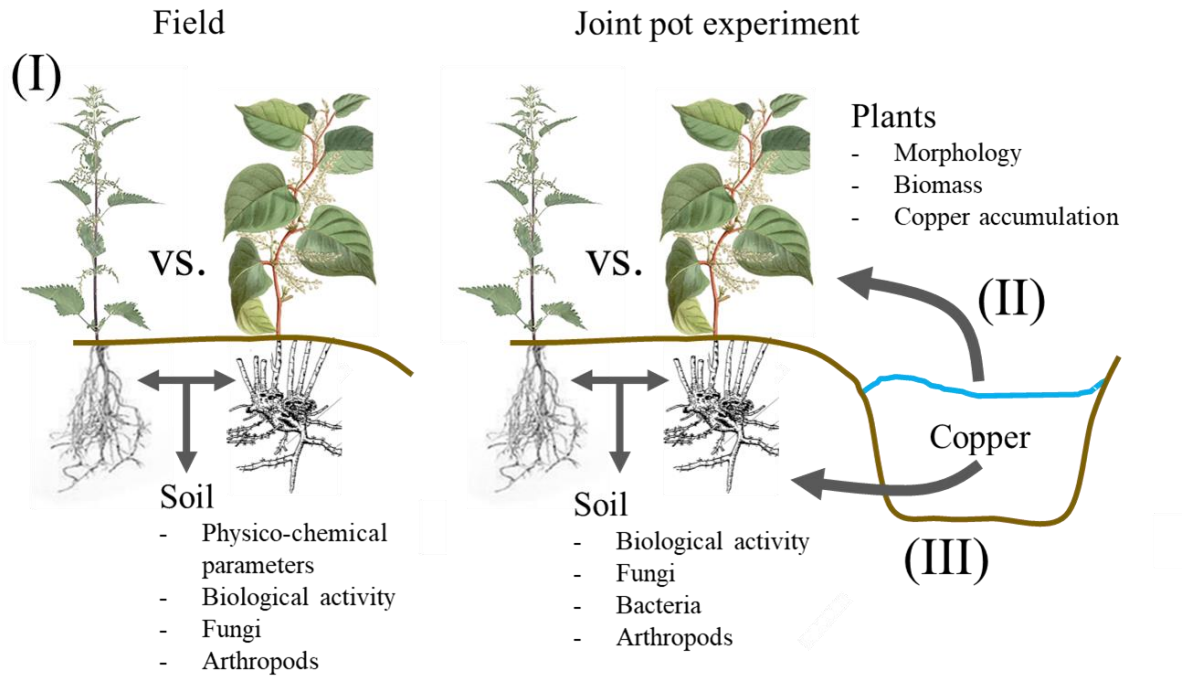

Fig. 1 Overview of the project. The first study on the left (I) investigates the effect of invasive plant species on the soil and soil biota. The second study (Joint pot experiment) is split into two parts, (II) investigates the effect of copper contamination on an invasive and native plant and (III) the effect of copper on the soil biota present under an invasive or native plant.

2.1 Field study

For the field study (I), I investigated 16 riparian sites located along small streams in forests around Landau, Rhineland-Palatinate, Germany invaded by both species (*F. japonica* or *I. glandulifera*) and corresponding control sites dominated by the native common nettle, *Urtica dioica* L., Urticaceae.

The aim was to investigate the chemistry of soil and effects on soil activity, soil arthropod abundance as well as soil fungal biomass, diversity and composition in both soil and root samples. For the fungi, we used a metabarcoding approach where fungi were assigned to Operational taxonomic units (OTU). We assessed the following research questions: (i) Are soil physicochemical parameters (organic carbon content, pH value, water content) different between riparian sites invaded by *F. japonica* or *I. glandulifera* compared to native sites with *U. dioica*? (ii) How does plant invasion by both species and soil physicochemical parameters affect soil microbial activity and soil arthropod abundance (Acari and Collembola)? (iii) How is the biomass, OTU richness, and composition of fungi in the bulk soil affected by *F. japonica* and *I. glandulifera* invasion and related to soil physicochemical parameters? (iv) How does the richness and composition of root-associated fungal OTUs differ in the presence of invasive and native plant species?

2.2 Joint pot experiment

The joint pot experiment was set up on an open field located in Siebeldingen at the Julius Kühn-Institut (Federal Research Centre for Cultivated Plants), Rhineland-Palatinate, Germany (49.219643, 8.049299) from April 2020 until September 2021 (Fig. 2). A total of 100 pots were filled with a mixture of sand and natural riparian soil collected from a nearby unpolluted riparian area within the biosphere reserve Palatinate Forest (Dürrentalbach, 49.255048, 7.939021). Five levels of $CuSO_4$ (Centrum Metal Odczynniki Chemiczne, Falenty, Poland) were applied (0, 90, 270, 810, and 2430 mg Cu kg^{-1} soil) and pots were planted with rhizomes and shoots collected from a riparian zone at the river Queich in Landau (49.198357, 8.096627). Four rhizomes of the invasive (*F. japonica*) or the native (*U. dioica*) species were planted in each pot.

2.2.1 Effect of copper on plants

The objective of study (II) was to investigate if the native plant (*U. dioica*) shows a different response to copper contamination than the invasive plant (*F. japonica*). Such a difference could impact the invasive success of *F. japonica* which is a major factor in riparian ecosystems. To test if there is such a difference in response, we conducted a pot experiment under field conditions. In a randomized block design, the plants were grown in soils with increasing soil copper concentrations (0 to 2430 mg kg−1 soil). We expected that (i) higher copper concentrations reduce plant growth of both species. However, since F. japonica is considered to be more tolerant, this invasive species should only respond at higher copper concentrations compared to *U. dioica*. We further hypothesized, based on the known ability of *F. japonica* to accumulate heavy metals, that (ii) the invasive species exhibits higher copper concentrations and takes up copper more effectively in all plant parts compared to *U. dioica*.

Fig. 2 Experimental setup of the experiment. General view of the pots arranged in a randomized block design and sample pictures of *Fallopia japonica* (left) and *Urtica dioica* (right) growing in the pots in spring 2021. From Schmitz et al. 2023, see Amendment II.

2.2.2 Effect of copper on soil organisms

In study (III) we wanted to investigate the impact of copper pollution in combination with plant invasion by *F. japonica* on soil communities. Especially we investigated the abundance of soil microbes, the soil microbial activity and the soil invertebrate community. To test these parameters, we conducted a pot experiment with both *F. japonica* and *U. dioica* under field conditions in a randomized block design with five soil copper concentrations (0 to 2,430 mg kg^{-1} soil). We expect that (i) abundance of soil arthropods, soil microbial activity and the microbial abundance is lower in the invasive *Fallopia japonica* than in the native *Urtica dioica*. We further expect that (ii) higher copper concentrations reduce the abundance of arthropods, soil biological activity and microbes for both plant species. For the microbial abundance we expect (iii) fungi to be more affected than bacteria due to use of copper as a fungicide.

3 Conclusion

3.1 Effect of invasive plant species on belowground systems

In the field, both invasive species (*Fallopia japonica* and *Impatiens glandulifera*) had only minor effects on soil physicochemical properties (organic carbon content, pH, water content), soil microbial activity as well as soil fungal biomass, diversity, and composition compared to *Urtica dioica*. This contrasts with earlier studies, where plant invasion induced changes especially in fungal biomass, diversity and function (Wolfe and Klironomos 2005; Si et al. 2013). For example, Liao and Boutton (2008) found higher microbial biomass under woody plant invasion. Stefanowicz et al. (2021) found a decrease in both bacterial and fungal biomass under *F. japonica*. One possible way in which *F. japonica* affects microbial communities is by producing secondary metabolites such as phenolic compounds (Stefanowicz et al. 2021, 2022). The lack of effect on soil fungi might be because neither invasive plant altered physicochemical properties, which strongly influence soil fungi abundances and diversity (Lauber et al. 2008; Wakelin et al. 2016). This is also unexpected since previous studies have found that micronutrient levels can increase in presence of *F. japonica*, especially under poor nutrient conditions (Dassonville et al. 2007). Only the saprobic fungi richness in soils from *F. japonica* sites compared to those from *U. dioica* increased, which might be because they are promoted by increased amounts of litter introduced by *F. japonica* (Lebreton et al. 2021). This indicates that plant invasion may only affect the soil fungi community if it alters the physicochemical properties of the habitat. However, contrary to the field conditions, in the mesocosms, *F. japonica* presence reduced the fungal as well as the bacterial biomass in the bulk soil. This could mean that in the field, the negative impact of plant invasion could be mitigated by factors that were excluded in the mesocosm study, like recruitment of different fungi from the surrounding environment that are capable to tolerate the presence of the invasive plant since the microbial community present in the mesocosm is likely only a snapshot of the actual diversity in the field.

Contrary to the soil fungi, the root-associated fungal communities in the field were composed of low phylogenetic OTU richness in the invasive species (especially in *F. japonica*) and were remarkably distinct to the native counterpart *U. dioica*. Especially the lower endophyte and mycorrhizal OTU richness in *F. japonica* and *I. glandulifera* roots might have been caused by changes in host-symbiont interactions. While symbionts are often not host specific, functional variability between different plant-mycorrhiza combinations have been observed (Klironomos 2000). This could lead to less efficient associations with the invasive plants and reduce fungal diversity since those incompatible species are lost (Čuda et al. 2020). For example Callaway et al. (2011) who reported that arbuscular mycorrhizal fungi (AMF) from a native range stimulated stronger positive feedbacks in invasive *Robinia pseudoacacia* than AMF from an invasive range. Further, as for the fungi in the soil, secondary metabolites can inhibit the growth of mycorrhizal fungi (Pinzone et al. 2018), which might also explain the even lower number of root-associated fungi found in *F. japonica* compared to *I. glandulifera*. The root associated fungi in the invasive species also include no AMF, which indicates that these species may not be able to associate well with symbiotic fungi in the invasive range. The number of fungal pathogens was also reduced in the invasive species, which might contribute to the invasion success. This would be in line with the enemy release hypothesis, which states that invasive species have increased success because of lower numbers of natural enemies (Mitchell and Power 2003).

In the field, soil arthropods (Acari and Collembola) were strongly affected by the presence of the invasive plants in contrasting ways. Collembola increased under *I. glandulifera* and Acari decreased under *F. japonica*. Collembola might benefit from more available food under the annual *I. glandulifera* (Tanner et al. 2013), detrivores in general are often less affected by invasive plants than other trophic groups, which might be explained by often increased plant productivity in invaded habitats (Ehrenfeld, 2010; Schirmel et al. 2016). Further, soil invertebrates depend strongly on the habitat type (Abgrall et al. 2019) because they are also influenced by abiotic soil properties (Cassagne et al. 2003; Bedano 2004), which were not affected in the field. These might be reasons for the lack of effect of *F. japonica* in this study despite its strong allelopathic effects. Salamon et al. (2004) and Wissuwa et al. (2012) on the other hand found that Collembola respond to changes in the vegetation, including plant invasion. The same secondary metabolites might cause the reduction in Acari abundance (Abgrall et al. 2018; Skubala and Mierny 2009). In the mesocosms, *F. japonica* also did not affect Collembola abundances or other soil invertebrates.

Root traits such as root exudation can influence soil functions (Bardgett 2017), the lack of an effect on soil microbial activity measured by soil respiration in the field was unexpected. Both invasive plants have been shown to negatively impact soil microbial activity as well (Stefanowicz et al. 2016). However, *Fallopia* spp. may mainly affect anaerobic respiration through root exudates (for example procyanidins), while aerobic respiration is less impacted (Bardon et al. 2017). This observation might be directly linked to the lack of effects in the fungal community and biomass among sites, as fungi are considered a main driver of microbial activity in soils (Žifčáková et al. 2016; Xu et al. 2019). Contrary to the field, in the mesocosms, soil biological activity was measured by bait lamina sticks and was lower under *F. japonica* compared to *U. dioica*. Since microbial abundance was reduced in the bulk soil of the invasive species, the soil activity which largely depends on especially fungi is also expected to be reduced. This impact on soil fungi was absent in the field, which would explain these differing results. Stefanowicz et al. (2016) also showed a negative effect of *F. japonica* on soil microbial activity.

3.2 Effect of copper on plants

Higher soil copper concentrations negatively impacted the growth of both the invasive plant species *Fallopia japonica* and the native *Urtica dioica*. This reduction in plant growth is in line with the previous studies on various species, for example *Buxus sempervirens*, *Cotoneaster divaricatus* and *Rhododendron obtusum* (Kuhns and Sydnor 1976). A key finding is that the reduction in growth occurred at different threshold concentrations: For *F. japonica*, growth was only lower at the highest analyzed concentration (810 mg kg^{-1}), while *U. dioica* already showed significant loss of growth at 270 mg kg^{-1}. In other studies, the negative impact of copper on *F. japonica* started at lower concentrations. For example, Sołtysiak (2020) found impact on F. japonica at 300 mg copper kg^{-1}. This pattern is likely due to *F. japonicas* higher tolerance to copper (Barberis et al. 2020). This supports the hypothesis that invasive plants benefit from disturbances by heavy metal contamination due to their higher tolerance to stressors such as copper (Li et al. 2022).

Uptake of copper by plant tissue increased with soil copper content for both plants. Copper content was highest in the belowground plant parts, especially rhizomes. This observation contrasts with literature highlighting higher heavy metal concentrations in aboveground plant parts (mainly leaves) for *F. japonica* (Sołtysiak 2020) and *U. dioica* (Balabanova et al. 2015). Contrary to the hypothesis that accumulation would be higher in *U. dioica*, it

showed higher absolute content in nearly all copper treatments and plant parts. *Fallopia japonica* might therefore be able to regulate the copper uptake better.

3.3 Effect of copper on soil organisms and soil activity

Contrary to expectations, bulk soil fungal biomass increased under higher copper treatment levels and only decreased at lower concentrations in *F. japonica.* Since copper at such concentrations is highly toxic to fungi this is unexpected. Heavy metal resistant fungi (Iram et al. 2015; Tam 1995) could be a possible reason for this result, as they would benefit from the lower competition in conditions that are unfavourable to most fungi species and can therefore increase in biomass. Additionally, copper contamination affected the fungal biomass in both soil types differently for *F. japonica* and *U. dioica*. The decrease in biomass in the lowest treatment (90 mg kg^{-1}) was higher in *F. japonica* compared to *U. dioica* for both bulk and rhizosphere soil. This indicates that the fungal community associated with *U. dioica* is more resistant to disturbance compared to *F. japonica*, since the plant by itself already has a negative impact on the microbial communities (see Chapter 3.1). As hypothesized, biological activity decreased under copper contamination in both plants but decreased more strongly under *F. japonica*. Since soil fungi are the main driver of soil activity, a loss in abundance of fungi and bacteria in the bulk in the *F. japonica* pots (see Chapter 3.1) will also cause a stronger reduction in the soil activity. Surprisingly, the increase in abundance of soil fungi under higher copper contamination did not increase the soil activity. This could mean that the fungal species present at high copper concentrations are not able to fulfil the same function as those at low concentrations.

Copper contamination affected Collembola abundance positively at lower concentrations and negatively at higher concentrations in *F. japonica*, which is in line with previous findings. For example, Filser et al. (2000) found that many collembolan species tended to be more abundant in the Cu-enriched soil of up to 240 mg kg^{-1} while at higher concentrations of 1000 mg kg^{-1} or more the abundance of Collembola was reduced in forests impacted by a copper smelter (Haimi and Siira-Pietikäinen 1996).

3.4 Outlook

Overall, the soil microbial activity in the mesocosm study was lower under *F. japonica* which is further intensified by copper contamination even when microbial biomass itself seems to not always be affected negatively by copper. This indicates that while the abundance of fungi may not decrease, ecosystem functions are lost under higher copper concentrations, which indicates a shift in the community composition. Thus, it would be important to investigate the fungal species composition under copper stress. In the field, neither soil fungi nor soil activity were strongly impacted by plant invasion. Since there was a lack of additional stressors such as copper contamination present in the field, the negative impact of plant invasion might only become damaging to soil microbes when they are already under stress. This could mean that if pollution of natural habitats increases further, a major loss of soil functions, such as litter degradation, could occur in riparian areas. The existence of a negative effect of plant invasion especially by *F. japonica* on soil microorganisms even in the field can be seen in the diversity of tissue-entering fungi. *Fallopia japonica* clearly had lower fungi OTU richness and *I. glandulifera* was also characterized by fewer mycorrhizal and saprobic OTUs.

Both plant invasion and copper contamination had direct but contrasting effects on soil invertebrates. Both Acari and Collembola were impacted by plant invasion in the field and copper contamination also altered the abundance

of Collembola. This indicates that the riparian soil food web may be altered by the presence of invasive plants which might even intensify if pollution increases. Copper contamination also impacts the native plant *U. dioica* more strongly than *F. japonica*. In this way, *F. japonica* may gain a selective advantage against this commonly occurring native species in its invasive range and especially in polluted areas because of its higher tolerance to copper.

Overall, this project indicates that disturbances by agricultural activity, one major source of copper pollution, could have an impact across much wider distances than expected by facilitating plant invasion success of *F. japonica* in previously undisturbed areas by impacting native plants and belowground communities of fungi, bacteria, and invertebrates. This invasion in turn might alter the riparian soil microbial and invertebrate communities even further. However, my findings indicate that both stressors may have impacts that are partly contrasting to previous studies on belowground ecosystem compartments. Therefore, the effects seem to be highly context-dependent and site-specific.

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Amendment I

Fallopia japonica and *Impatiens glandulifera* are colonized by species-poor root-associated fungal communities but have minor impacts on soil properties in riparian habitats

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Abstract

Fallopia japonica and *Impatiens glandulifera* are major plant invaders on a global scale that often become dominant in riparian areas. However, little is known about how these species affect interactions in soil-plant systems. The aim of this study was to investigate the impact of both species on abiotic and biotic soil properties, with a special focus on fungi. We investigated eight sites along small streams invaded by *F. japonica* and *I. glandulifera* respectively, and compared each with nearby sites dominated by the native species *Urtica dioica*. Three different types of samples were collected: bulk soil, rhizosphere soil and roots from invasive and native stands at each site. Bulk soil samples were analysed for soil physicochemical, microbial properties (soil microbial respiration and ergosterol) and soil arthropod abundance (i.e., Acari and Collembola). Soil respiration was also evaluated in rhizosphere samples. The fungal community composition of both bulk soil and roots were analysed using a metabarcoding approach. Soil physicochemical properties as well as soil microbial activity, fungal biomass and soil fungal diversity did not differ between invaded and native riparian habitats, indicating only minor belowground impacts of the two invasive plants. Soil microbial activity, fungal biomass and soil fungal diversity were rather related to the soil physicochemical properties. In contrast, Acari abundance decreased by 68 % in the presence of *F. japonica*, while Collembola abundance increased by 11 % in *I. glandulifera* sites. Moreover, root-associated fungal communities differed between the invasive and native plants. In *F. japonica* roots, fungal diversity of all investigated ecological groups (mycorrhiza, endophytes, parasites, saprobes) were lower compared to *U. dioica*. However, in *I. glandulifera* roots only the diversity of mycorrhiza and saprobic fungi was lower. Overall, our findings show that *F. japonica* and *I. glandulifera* can influence the abundance of soil arthropods and are characterized by lower diversity of root-associated fungi.

Keywords: Acari; Collembola; *Fallopia japonica; Impatiens glandulifera*; metabarcoding; *Urtica dioica*

1 Introduction

Invasive alien plants are widely accepted as a major cause of biodiversity loss in native communities, which, in turn, can negatively affect the entire ecosystem (Hooper et al. 2012). Besides effects on aboveground compartments, invasive plants also impact belowground compartments (Liao et al. 2007; Abgrall et al. 2019) altering resources and ecosystem functions and processes (Crooks 2002; Ehrenfeld 2003; Liao et al. 2008; Waller et al. 2020).

Invasive plants can impact soil physicochemical properties, e.g. by increasing pH and water content (*Leucanthemum vulgare,* Ahmad et al. 2020) or lowering pH and increasing water content (*Carpobrotus edulis*, Novoa et al. 2014). Invasive plants also have various effects on the soil nutrient status. For example, invasion by *Spartina alterniflora* can increase nitrogen and organic carbon levels (Liao et al. 2007) or increase phosphorus storage while having no effect on C and N (Wang et al. 2019). Soil functions can be affected in terms of decreased litter decomposition and increased microbial activity (Liao et al. 2007; Vilà et al. 2011) or reduced soil biological activity (Pehle and Schirmel 2015). Soil microbial communities, including fungi, are influenced by plant invasion with respect to biomass (e.g. increasing biomass with woody plant invasion; Liao and Boutton 2008) and shifts in the community composition and diversity (Ravit et al. 2003; Wolfe and Klironomos 2005; Si et al. 2013; Sapsford et al. 2022). Plant invasions can further influence the soil fauna (Belnap et al. 2005; Biederman and Boutton 2009; Abgrall et al. 2019), which support important soil functions such as decomposition (de Deyn et al. 2003; Tresch et al. 2019). For example, Collembola and Acari, often the dominant arthropod groups in soil (Petersen and Luxton 1982; Deharveng 1996; Behan-Pelletier 2003) respond to changes in the vegetation, including plant invasion (Salamon et al. 2004; Wissuwa et al. 2012). Altering the vegetation composition by replacing native plant species, could thus negativly affect soil arthropod abundance and diversity (Salamon et al. 2004; Wissuwa et al. 2012; Rusterholz et al. 2014). However, impacts of invasive plants on soil fauna are highly variable and depend on the habitat type (Abgrall et al. 2019), since many soil arthropod species are also dependent on abiotic soil properties (Cassagne et al. 2003; Bedano 2004).

Fallopia japonica (Houtt.) Ronse Decr., Polygonaceae and *Impatiens glandulifera* Royle, Balsaminaceae are highly abundant invasive plant species in many areas worldwide (Beerling and Perrins 1993; Beerling et al. 1994). Although their effects on soil physicochemical and biological properties are well studied, their impacts on fungal communities under field conditions are not well understood, especially due to the lack of studies using modern molecular analyses (metabarcoding). Soil fungi have important roles in ecosystem functioning (Öpik et al. 2006). Symbionts such as mycorrhizal species interact with plants through their roots by providing water and nutrients, and are essential for survival and health of most plant species (Smith and Read 2008; Kivlin et al. 2011; Tedersoo et al. 2014). In contrast to mycorrhizal fungi, root endophytes only interact with plant roots during a part of their life cycle. Mycorrhizal and endophytic fungi are especially beneficial under stressful environmental conditions and can enhance the performance, stress tolerance and disease resistance of plants (Smith and Read 2008; Rodriguez et al. 2009; Kivlin et al. 2011; Redman et al. 2011; Riess et al. 2014; Tedersoo et al. 2014). The community of mycorrhizal, endophytic as well as parasitic fungi (also occurring on living tissue) depends on the plant species and may thus be modified by invasion (Knogge 1996; Christian et al. 2016). In contrast to mycorrhizal and endophytic fungal groups, saprobic fungi are involved in the decomposition of particulate organic matter (Duguay and Klironomos 2000) and, hence, may be less affected by plant invasion.

The Asian knotweed, *Fallopia japonica*, is a perennial, rhizome-forming species and one of the most invasive plants in Europe due to its ability to quickly produce high biomass (Beerling et al. 1994). It can affect soil characteristics (e.g., increasing mineral modifying soil nutrient stocks; Dassonville et al. 2007; Stefanowicz et al. 2017), C and N dynamics in soil (Koutika et al. 2007) or increase soil pH compared to adjacent non-invaded sites (Čerevková et al. 2019). *Fallopia japonica* shows allelopathic effects (Murrell et al. 2011; Parepa and Bossdorf 2016) with significant impacts on the soil food web structure (Abgrall et al. 2018), decreasing abundance and diversity of soil fungi, such as mycorrhizal fungi (Zubek et al. 2016). Another highly invasive plant in Europe is the Himalayan balsam, *Impatiens glandulifera* (Beerling and Perrins 1993). This annual herb has strong dispersal capabilities with masses of floatable seeds. *Impatiens glandulifera* is known for its impact on native communities with positive effects on soil fauna abundance (Tanner et al. 2013; Rusterholz et al. 2014), modifying soil fungal community composition by increasing fungal diversity and lowering arbuscular mycorrhizal fungal root colonization (Pattison et al. 2016; Gaggini et al. 2018, 2019).

In this study, we investigated riparian sites invaded by both species (*F. japonica* or *I. glandulifera*) and corresponding control sites dominated by the native common nettle, *Urtica dioica* L., Urticaceae. We aimed to investigate the chemistry of soil and effects on soil bacterial activity, soil arthropod abundance as well as soil fungal biomass, diversity and composition in both soil and root samples. For the fungi, we used a metabarcoding approach where fungi were assigned to Operational taxonomic units (OTU). We assessed the following research questions: (i) Are soil physicochemical parameters (organic carbon content, pH value, water content) different between riparian sites invaded by *F. japonica* or *I. glandulifera* compared to native sites with *U. dioica*? (ii) How does plant invasion by both species and soil physicochemical parameters affect soil microbial activity and soil arthropod abundance (Acari and Collembola)? (iii) How is the biomass, OTU richness, and composition of fungi in the bulk soil affected by *F. japonica* and *I. glandulifera* invasion and related to soil physicochemical parameters? (iv) How does the richness and composition of root-associated fungal OTUs differ in the presence of invasive and native plant species?

2 Materials and methods

2.1 Study area and target plant species

Study sites were located along small streams in forests around Landau (study area ~ 1000 km²), Rhineland-Palatinate, Germany (Online Resource 1). Sixteen riparian sites containing either sites dominated by *Fallopia japonica* and *Urtica dioica* (N = 8 site pairs) or *Impatiens glandulifera* and *U. dioica* (N = 8 site pairs) were selected (Online Resource 2). The perennial herb *Urtica dioica* was selected as a reference species because it is a highly abundant, naturally occurring species at riparian floodplains in Central Europe (Edwards et al. 1998). Stands between the invasive and native plant species of each site pair were in direct vicinity (maximum distance up to 10 meters), to minimize differences in soil conditions. All sites were at least 10 m² and characterized by homogenous vegetation and a cover of >80 % by their respective target species. Other common plants within the sites were *Glechoma hederacea* and *Rubus* sect. *rubus*. In the sites dominated by *F. japonica*, an average of 3.4 plant species were identified, while in corresponding *U. dioica* sites, the average was 3.9. In sites dominated by *I. glandulifera*, the average number of species was 4.8 and in corresponding *U. dioica* sites, the average was 3.3. The forests around the target sites can be assigned to the phytocoenology classes Alnetea glutinosae and Quercetea

robori-petraea according to Schubert. (2010), with *Alnus glutinosa* as the dominant tree. Details of the vegetation at each site can be found in Online Resource 1.

2.2 Sampling

In each site, plant-specific samples were collected, namely bulk soil, rhizosphere soil and plant roots. Sampling was performed during September to October 2019. Bulk soil samples were randomly collected at the topsoil (0 to -10 cm) using a bulb planter from three plant-specific stands and subsequently pooled and homogenized to one composite sample. Roots were taken from four randomly chosen individuals of each species using a shovel and homogenized to a composite sample by species and site. The soil attached to the roots after shaking the collected plant was separated from the plants by hand and collected as rhizosphere soil, in total 4 replicates, which were subsequently homogenized to a composite sample per species and site. Roots were further rinsed first with tap water, and finally with autoclaved water. A part of each soil type was frozen at -20 °C and stored for analysis of soil microbial activity. The remaining bulk soil samples, rhizosphere soil samples and individual root samples were subsequently dried at 50 °C for 48 h.

2.3 Soil physicochemical properties

The bulk soil samples were used for analyses of soil physicochemical properties. Soil pH was measured using a pH meter (pH 3110 SET 2, Xylem Analytics, Weilheim, Germany) in a 0.01 molar $CaCl_2$ solution (DIN EN 15933:2012-11) with field moist soil. Water content in bulk and rhizosphere soil was determined gravimetrically according to ISO 11465:1993. Samples were further analysed for organic carbon (DIN ISO 10694:1996-08) by dry burning and total nitrogen (VDLUFA I,A2.2.5:2012) via thermal conductometry. These analyses were performed by Speyer Agricultural Research Institute (Speyer, Germany).

2.4 Soil microbial activity

Soil microbial activity was investigated separately for the bulk soil and the rhizosphere soil. Basal and glucose-induced soil microbial activity were assessed by the MicroResp™ method (Campbell et al. 2003). The evolved CO_2 after incubation is indicative for soil respiration, which is an expression of soil microbial activity (Creamer et al. 2016a, 2016b). The frozen samples were thawed at 6 °C and sieved to 2 mm. Then, they were adjusted to 45 % of water holding capacity, following the recommendations of the producer. The substrates (water or glucose) were added to the soil samples followed by an incubation of 6 hours at 21 °C. CO_2 was measured by a shift of the cresol red colour in the deep well plate system at 572 nm (Nanophotometer Infinite® M200, Tecan, Switzerland). Quantitative analysis was done using known concentrations of sodium bicarbonate and an excess of hydrochloric acid (Campbell et al. 2003) by interpolation of data in the calibration curve.

2.5 Soil arthropod abundance

We focussed on the highly abundant and functionally important soil arthropod taxa Acari and Collembola. We combined two methods, pitfall traps and Berlese funnels, for sampling. Four pitfall traps (plastic cups with an opening diameter of 6.5 cm) were placed one meter apart in each sampling site. Traps were set between 24th August and 15th September 2019 and were emptied and refilled every ten days. A 1:4 propylene glycol:water solution with some drops of detergent was used as a trapping liquid. Captured individuals were stored in 70 % ethanol. Soil organisms were additionally extracted from the soil bulk samples using Berlese funnels. 200 ml of fresh soil per site from the collected bulk soil was filled into a Berlese funnel for one week (4-11th September). A

constant light source was attached to the top of the funnel to induce downward movement for the soil organisms. Organisms were collected at the bottom of the funnel in vials containing 20 ml of 70 % ethanol and the collected invertebrates were identified to class level. The abundance of Acari and Collembola individuals from pitfall traps and Berlese funnels were combined for the analyses.

2.6 Fungi

2.6.1 Soil fungal biomass

Ergosterol was used as a proxy of soil fungal biomass in bulk soil samples, based on the method of Gong et al. (2001). 4 g of air-dried and milled soil (Planetary Micro Mill PULVERISETTE 7 premium line, Fritsch, Idar-Oberstein, Germany) was extracted with 12 ml of methanol for 60 min on a horizontal shaker (Kreisschüttler 3015, GFL, Burgwedel, Germany). The mixture was sonicated for 10 min (DT 514H, Bandelin electronics, Berlin, Germany) and centrifuged for 10 min at 2000g (Universal 320, Hettich Lab Technology, Tuttlingen, Germany). The supernatant was ultra-centrifuged for 3 min at 7270g (Micro centaur, MSE, London, UK) prior to high-performance liquid chromatography (HPLC) analysis. An aliquot of 20 µL of the supernatant was analysed via HPLC with UV detection at 282 nm (HPLC 1200 series, Agilent technologies, Santa Clara, CA, USA), equipped with a C18 LiChrospher® column (LiChrospher RP-18e, 5 µm, 100 Å, 250 × 4.6 mm, Merck, Darmstadt, Germany). Methanol (flow rate 1.7 ml min^{-1}) was used as a mobile phase under isochratic condition. The temperature of the column was set at 38 °C. The limit of detection (LOD) of the method was 0.06 mg kg^{-1} (Meyer et al. 2021).

2.6.2 Soil and root fungal OTU richness and ecological groups

Fungal OTU richness was analysed separately for bulk soil and root samples of the investigated plant species. Next-generation sequencing was carried out by the company Advanced Identification Methods (AIM) (Munich, Germany). For detailed processing of sequences, see Online Resource 3. In short, the internal transcribed spacer 2 region of the nuc-rDNA (ITS2) was amplified using the primers ITS3tagmix/ITS4ngs (Tedersoo et al. 2014; Tedersoo et al. 2015). Quality filtering reduced the initial 3,287,492 full sequences to a final dataset with 241,258 unique, non-singleton sequences. Clustering with a threshold of 2 % and *de novo* chimera detection resulted in 6,468 operational taxonomic units (OTUs) (Online Resource 4). OTUs were matched against NCBI GenBank database (ncbi.nlm.nih.gov; October 2019; see Online Resource 5). Only 5,012 OTUs matched with sequences assigned to Fungi with more than 95 % identity and were used for further analysis. In general, there is a large discrepancy between the described and the estimated number of species (Hawksworth and Rossman 1997). Hence, some OTUs cannot be assigned to any taxon or only at a higher taxonomic level. To increase the quality of database labelling, ambiguously attributed OTUs (e.g., "uncultured eukaryote" or “fungal endophyte") were checked manually and assigned to phyla using MycoBank (mycobank.org; Robert et al. 2013) as a reference database.

Prior to the statistical analysis, four main fungal ecological groups were identified: (i) Mycorrhiza is a widespread well-known symbiotic formation supporting both fungal and plant partner (Smith and Read 2008). In our study area, two main mycorrhizal groups are common: the arbuscular mycorrhizal fungi (AMF) comprising only Glomeromycota (Parniske 2008) and the ectomycorrhizal fungi (ECM) including mainly Basidiomycota, e.g. the large genus *Cortinarius* (Garnica et al. 2016), and Ascomycota, e.g. the species-rich order Helotiales (Tedersoo et al. 2009). In Europe, AMF typically occurs in herbaceous plants whereas ECM is usually found in woody species.

(ii) Root endophytes include phylogenetically diverse fungi and occurs ubiquitously in terrestrial plants across a variety of ecosystems. At some stage of their complex life cycle, they inhabit plant tissues without causing any obvious disease symptoms (Rodriguez et al. 2009). Species-rich endophytic fungal taxa are for example *Trichoderma* (Harman et al. 2004) and many members of the Sebacinales (Weiß et al. 2011). (iii) Saprobic fungi break down energy-rich organic substances from tissue of dead organisms or soil, where they play a key role beside bacteria (Lebreton et al. 2021). Typical saprobic fungi are representatives of the genera *Peziza* (Hansen et al. 2011) and *Mortierella* (Spatafora et al. 2016). (iv) Parasitic fungi occur on living tissue and harm their hosts. Typical examples are *Taphrina* (Ascomycota; Rodrigues et al. 2003) and *Ustilago* (Bauer et al. 1997). Annotation of fungal OTUs to the ecological groups was realized in a two-step process: First, a literature search for taxa with well-known ecology was carried out. This is reasonable on genus or higher taxonomical range (Põlme et al. 2020). In a subsequent second step, a screening for OTU ecology was carried out using information deposited in metadata in the NCBI database (Online Resource 6). This approach allowed us to assess the fungal OTU richness of soil and root-associated fungi as well as the OTU richness in the respective ecological group (Zanne et al. 2020).

2.7 Data analysis

The data was analysed using R version 4.0.5 (R Core Team 2021). The package glmmTMB version 1.0.2.1 was used to create generalized linear mixed models (Brooks et al. 2017). The models were run separately for the site pairs of *F. japonica* / *U. dioica* and *I. glandulifera* / *U. dioica*, respectively. For the comparison of soil properties (organic carbon, total nitrogen, pH value, water content) between invaded and native sites, only plant species (factor with the two levels "invasive" [either *F. japonica* or *I. glandulifera*] and "native" [*U. dioica*]) was included as an explanatory variable and a Gaussian distribution was used. The study site was included as a random effect to account for our paired study design. The models for questions ii-iv incorporated plant species (factor with two levels), pH value (continuous), organic carbon content (continuous), and water content (continuous) as explanatory variables, and study site as a random effect. We decided on these variables by investigating the intercorrelation of the different explanatory variables (Online Resource 7). For example, organic carbon and total nitrogen content ($r = 0.89$) were highly correlated. This allowed us to simplify the model by excluding nitrogen content. The response variables used were the soil microbial activity in bulk and rhizosphere soil, soil organism abundance, fungal biomass, total fungal OTU richness, and the OTU richness of the different fungal ecological groups (analysed separately for soil fungi and plant-associated fungi). Depending on the analysed variables, different family functions for the models were used. For the fungal OTU richness and the abundance of soil organisms, a negative binomial model for over-dispersed count data was applied (Lindén & Mäntyniemi 2011). For the fungal biomass and both soil microbial activities, a gaussian model was used. The model results are presented by showing the estimate (Est.), z-value and P-value.

To reveal differences in the fungal OTU composition between invaded (either *F. japonica* or *I. glandulifera*) and native (*U. dioica*) sites and between fungi associated with invasive and native plants, permutational multivariate analyses of variances (PERMANOVA) were performed (command adonis2 in the package 'vegan', Oksanen et al. 2019). The parameters organic carbon content, pH value and water content were used as further environmental variables to correspond to the previous models. Because of the paired study design, the study sites were used as strata in the formula. The PERMANOVA were based on presence-absence data for fungal OTUs occurring in more than one site or plant and the Jaccard index was used as a similarity measure. PERMANOVA were performed

for soil fungal OTU composition (question iii) and root associated fungal OTU composition (question iv). Variation of fungal OTU compositions were visualized using nonmetric multidimensional scaling (NMDS) with the command 'metaMDS' in R package 'vegan'.

3 Results

3.1 Soil physicochemical properties between invaded and native riparian vegetation

Soil properties (organic carbon content, nitrogen content, pH value and water content) did not significantly differ between sites invaded by *Fallopia japonica* or *Impatiens glandulifera* and the paired sites dominated by native *Urtica dioica* (Table S1).

3.2 Relations of soil microbial activity to riparian plant invasion and soil physicochemical properties

Water content and soil organic carbon were significantly related to bulk soil microbial activity in *F. japonica* site pairs. The basal soil respiration decreased with increasing water content (Est. = -0.04, z = -2.91, P = 0.004) and increased with increasing soil organic carbon (Est. 0.59, z = 2.86, P = 0.004). However, the glucose-induced respiration was not affected by soil physicochemical parameters (Table S2). We did not find significant effects in basal and glucose-induced soil respiration for bulk soils from the *I. glandulifera* site pairs. Moreover, no significant effects were found for rhizosphere soils from both *F. japonica* or *I. glandulifera* site pairs (Table S2).

3.3 Soil invertebrates in relation to riparian plant invasion and soil physicochemical properties

Acari abundance was over three times higher in *U. dioica* (96 ± 99.7) than *F. japonica* (30 ± 21.6) soils (Est. = 1.33, z = 3.77, P < 0.001). Moreover, Acari abundance decreased with increasing pH (Est. = -0.55, z = -2.91, P = 0.004). When comparing *I. glandulifera* with *U. dioica* sites, Acari abundance was not different between invaded and native sites (Table S2). However, abundances slightly increased with increasing pH (Est. = 0.54, z = 2.82, P = 0.005). Collembola abundance was not affected by the presence of *F. japonica* compared to *U. dioica* (Table S2) but increased with elevated pH (Est. = 0.43, z = 2.85, P = 0.004) and organic carbon content (Est. = 0.17, z = 2.19, P = 0.029). However, when comparing *I. glandulifera* with *U. dioica*, Collembola abundance increased under presence of the invasive species (Est. = -0.33, z = -2.61, p = 0.009). Additionally, Collembola were less abundant in soils with higher organic carbon content (Est. = -0.32, z = -3.70, P < 0.001), whereas an increase in their abundance was still associated with higher pH (Est. = 0.43, z = 2.52, P = 0.012).

3.4 Soil fungi in relation to plant invasion and soil physicochemical properties

Analysis of bulk soil samples from the site pairs of *F. japonica* / *U. dioica* revealed no differences in total fungal biomass measured as ergosterol. Fungal biomass was positively correlated with organic carbon content (Est. = 0.88, z = 2.56, P = 0.010) while pH and water content had no influence (Table S3).

The general fungal OTU richness and OTU richness of three out of the four ecological fungal groups (endophytes, mycorrhiza, parasites) did not differ between *F. japonica* and *U. dioica* sites (Table S3, Fig. 1). The only exception was the OTU richness of saprobes, which was 20 % higher in *F. japonica* (103 ± 35.3) relative to *U. dioica* (82 ± 33.5) (Est. = -0.27, z = -3.71, P < 0.001). In our model, all soil parameters were significantly related to the OTU richness of fungi: Overall diversity decreased with increasing organic carbon (Est. = -0.11, z = -3.01, P = 0.003) and increased with increasing pH (Est. = 0.06, z = 2.60, P = 0.009). The latter was also observed with OTU richness of endophytes (Est. = 0.23, z = 2.72, P = 0.007), saprobes (Est. = 0.15, z = 2.15 p = 0.032), and parasites

(Est. = 0.18, z = 1.98, P = 0.048). OTU richness of mycorrhizal fungi decreased with increasing organic carbon (Est. = -0.18, z = -2.06, P = 0.039) and water content (Est. = -0.04, z = -3.73, P < 0.001). In contrast to OTU richness, the composition of the soil fungal community did not differ between *F. japonica* and *U. dioica* sites and was not affected by any of the other soil characteristics (Table 1, Fig. 2a).

Regarding the site pairs of *I. glandulifera* and *U. dioica*, neither fungal biomass nor fungal OTU richness were different between the invaded and uninvaded sites (Table S3). However, fungal biomass increased with increasing organic carbon content (Est. = 1.59, z = 6.15, P < 0.001) but decreased with higher soil water content (Est. = -0.11, z = -2.52, P = 0.012). In terms of the fungal OTU richness, only soil water content was found to have effects (Table S3). Specifically, the OTU richness of endophytes (Est. = -0.02, z = -2.06, P = 0.039), saprobes (Est. = -0.02, z = -2.66, P = 0.008) and parasites (Est. = -0.02, z = -2.17, P = 0.030) decreased with higher water content. The soil fungal community did not differ between *I. glandulifera* and *U. dioica* sites and none of the soil parameters had a significant effect (Table 1, Fig. 2b). For details of the most common fungi taxa found in soil samples of both *F. japonica* and *I. glandulifera* site pairs, see Online Resource 8.

3.5 Root-associated fungi in relation to plant invasion and soil physicochemical properties

The overall root-associated fungal OTU richness was almost six times higher in *U. dioica* (63 ± 31.3) than in *F. japonica* (11 ± 4.9) (Est. = 1.75, z = 7.57, P < 0.001). Similar patterns were found for mycorrhizal OTU richness (*U. dioica*: 8 ± 5.4, *F. japonica*: 1 ± 1.4) (Est. = 3.57, z = 4.99, P < 0.001), endophytes (*U. dioica*: 7 ± 3.3, *F. japonica* 0 ± 0.5) (Est. = 2.74, z = 4.53, P < 0.001), saprobes (*U. dioica*: 14 ± 11.6, *F. japonica* 4 ± 3.6) (Est. = 1.24, z = 3.22, P = 0.001) and parasites (*U. dioica*: 7 ± 4.5, *F. japonica* 1 ± 0.9) (Est. = 2.46, z = 4.72, P < 0.001) (Fig. 3). Higher soil water content was related to a decrease in OTU richness of mycorrhiza (Est. = -0.01, z = -2.07, P = 0.038) and an increase in OTU richness of endophytes (Est. = 0.02, z = 2.15, P = 0.031) and parasites (Est. = 0.02, z = 2.50, P = 0.013). Additionally, the OTU richness of mycorrhiza decreased with increasing organic carbon content (Est. = -0.30, z = -2.01, P = 0.045) and pH (Est. = -1.01, z = -3.07, P = 0.002). The root associated fungal community differed between *F. japonica* and *U. dioica* (R^2 = 0.21, P = 0.008) (Fig. 4a). In contrast, soil parameters did not affect root colonizing fungal communities (Table 1).

Plant invasion effects were much less obvious for *I. glandulifera* compared to *F. japonica*. Only OTU richness of mycorrhiza (*U. dioica*: 6 ± 3.8, *I. glandulifera*: 3 ± 1.2) (Est. = 0.60, z = 2.29, P = 0.022) and saprobes (*U. dioica*: 21 ± 15.9 *I. glandulifera* 9 ± 3.6) (Est. = 0.64, z = 2.04, P = 0.042) were lower in *I. glandulifera* stands compared to *U. dioica* (Fig. 3). Moreover, the overall plant associated fungal OTU richness (pH: Est. = -0.10, z = -2.66, P = 0.008; water content: Est. = -0.03, z = -2.19, P = 0.028) and the richness of mycorrhizal OTU (pH: Est. = -0.36, z = -2.26, P = 0.024; water content: Est. = -0.03, z = -2.30, P = 0.021) decreased with increasing pH and water content. OTU richness of parasites decreased with increasing water content (Est. = -0.03, z = -2.05, P = 0.041). While *I. glandulifera* and *U. dioica* showed distinct root associated fungal OTU compositions (R^2 = 0.091, P = 0.016), the soil parameters were not related to the fungal community composition (Table 1, Fig. 4b). For an overview over the most common fungi taxa found in root samples of both *F. japonica* and *I. glandulifera* site pairs, see Online Resource 8.

4 Discussion

In this study we analysed the impact of riparian plant invasions on soil, arthropods, and fungi by comparing sites invaded by *Fallopia japonica* or *Impatiens glandulifera* to corresponding sites inhabited by the native *Urtica dioica*. We found that both invasive species had only minor effects on soil physicochemical properties, soil microbial activity, soil arthropod abundance and soil fungal biomass, diversity, and composition. We further investigated root-associated fungal communities of the three plant species. As a key finding, we observed strong reductions in fungal diversity in roots of *F. japonica* and, to a lesser extent, in roots of *I. glandulifera* compared to *U. dioica*.

4.1 Riparian plant invasion has low impact on soil physicochemical properties and soil microbial activity

None of the investigated soil physicochemical parameters (organic carbon content, pH, water content) nor soil microbial activity were significantly affected by the presence of an invasive plant invader investigated here. While soil organic carbon content seems to be less influenced (Aguilera et al. 2010), previous studies have found that micronutrient levels can increase in presence of *F. japonica*, especially under poor nutrient conditions (Dassonville et al. 2007). We studied riparian and relatively nutrient-rich habitats along streams, which could be one reason why we did not detect an impact of *F. japonica* on soil physicochemical parameters. Moreover, impacts of plant invasions often change over time (Strayer et al. 2006; Dostál et al. 2013), but information about the age of our studied plant stands was not available. For *I. glandulifera*, our results conform with findings of Čuda et al. (2017) who also found only minor impact of this species on soil physiochemical properties. Since root traits such as root exudation can influence soil functions (Bardgett 2017), the lack of an effect on soil microbial activity (measured by soil respiration) was unexpected. Both *F. japonica* and *I. glandulifera* have also been shown to negatively impact soil microbial activity (Stefanowicz et al. 2016). However, *Fallopia* spp. may mainly affect anaerobic respiration through root exudates (for example procyanidins), while aerobic respiration is less impacted (Bardon et al. 2016, 2017). This observation might be directly linked to the lack of effects in the fungal community and biomass among sites (see below), as fungi are considered a main driver of microbial activity in soils (Žifčáková et al. 2016; Xu et al. 2019).

4.2 *Fallopia japonica* and *I. glandulifera* affect soil arthropod abundance

The analysed soil arthropods Acari and Collembola were strongly affected by the presence of the invasive plants, though in contrasting ways. The abundance of Collembola significantly increased in *I. glandulifera* stands but was not affected by *F. japonica*. While the impact of *I. glandulifera* was previously linked to its annual life cycle, which leads to a higher availability of food for collembolan herbivores (Tanner et al. 2013), the lack of an effect by *F. japonica* was unexpected due to its known allelopathic impacts (Kato-Noguchi 2021) on soil microfauna (Abgrall et al. 2018). However, detritivores are often less affected by invasive plants than other trophic groups, which might be explained by often increased plant productivity in invaded habitats (Liao et al., 2008; Ehrenfeld, 2010; Schirmel et al. 2016).

In our study, invasion by *F. japonica* negatively impacted Acari abundance while *I. glandulifera* had no significant effect. For *I. glandulifera*, this is in accordance with earlier reports, where its presence had differential effects on different groups of Acari, which lead to no effect in total abundances (Rusterholz et al. 2014). The lower Acari abundances in *F. japonica* sites may be explained by its phenolic compounds in the leaf litter, which have negative

impacts on saprobic species (Skubala and Mierny 2009; Abgrall et al. 2018). Moreover, reduced Acari abundances under *F. japonica* might be a result of changes in the soil food web (Abgrall et al. 2018), although this was not demonstrated in our study.

In the case of *F. japonica*, we uncovered a positive and negative relationship between Collembola abundances and organic carbon contents and pH, respectively. For *I. glandulifera* sites, the opposite patterns for both variables were observed. This supports previous studies that have documented the importance of soil organic matter for shaping soil fauna communities (Rendoš et al. 2016). The influence of pH might also be indirect, by altering the soil microbial community (Fierer et al. 2009). Acari abundances were also affected by soil pH. The abundances increased with pH value in *F. japonica* site pairs but decreased in *I. glandulifera* site pairs. The effect of pH value on Acari abundance may be explained by the fact that pH differences are associated with different microhabitats, which influence Acari abundance and communities (Nielsen et al. 2010; Wissuwa et al. 2013).

4.3 Soil physicochemical properties are more influential for soil fungi than the presence of invasive plants

The presence of invasive plant species had little effect on the soil fungal biomass, diversity and composition in the context of this study. We only found an increase in saprobic fungi richness in soils from *F. japonica* sites compared to those from *U. dioica*. Saprobic fungi may be promoted by increased amounts of litter introduced by *F. japonica* which is degraded by saprobes (Lebreton et al. 2021). This finding is unexpected and contrasts with earlier studies, where plant invasion induced changes in fungal diversity and function (Wolfe and Klironomos 2005; Si et al. 2013). One explanation might be that the plant invasions did not significantly alter physicochemical properties compared to the uninvaded sites, which are known to strongly influence soil fungi abundances and diversity (for example Lauber et al. 2008; Wakelin et al. 2016; Yang et al. 2017). This indicates that plant invasion may only affect the soil fungi community if it alters the physicochemical properties of the habitat. Consequently, the soil physicochemical properties were much more important in explaining soil fungi biomass and diversity. It is well known that water availability, organic carbon and pH have major effects on the fungal species composition (Li et al. 2015; Xiao et al. 2018). Here, the fungal biomass was affected by the soil organic carbon content providing more resources for saprobic fungal species supporting their development (Bossio and Scow 1998; Yao et al. 2000; Drenovsky et al. 2004). However, increased organic carbon also leads to a reduction in overall as well as mycorrhizal OTU numbers in the *F. japonica* site pairs in our study. Contrary to this observation, Zhu et al. (2020) showed that mycorrhizal species richness increases with organic carbon content. Other studies observed a decrease in fungal diversity along a gradient of the same variable (Liu et al. 2015). This might be because the interaction of mycorrhizal taxa and organic carbon in the soil is dependent on the local conditions (Hanson et al. 2008; Wei et al. 2019).

At *F. japonica* site pairs, pH value was associated with an increase of fungal diversity in all ecological groups except mycorrhiza (endophytes, parasites, and saprobes). In contrast, at *I. glandulifera* site pairs, pH had no significant influence on fungal diversity. Moreover, we did not observe an effect of pH on fungal community composition. Our findings are thus surprising, because pH value is known to affect especially mycorrhizal species richness and composition in various habitats (Porter et al. 1987; An et al. 2008, Garnica et al. 2013; Davison et al. 2015; Řezáčová et al. 2019). One possible explanation for this contradictory result might be that the differences in the pH values across our study sites were too small (on average 5.7–6.2; Table S1) to impact the fungal diversity

and composition. In other studies, these pH ranges were much wider, for example between 4.8 and 7.5 (Porter et al. 1987) or 4.0 and 7.6 (Řezáčová et al. 2019). Increasing soil water content negatively affected mycorrhizal fungal diversity in the *F. japonica* site pairs and fungal biomass as well as fungal diversity of endophytes, parasites, and saprobes in the *I. glandulifera* site pairs. Fungi have a lower moisture optimum than other soil microbes and have a lower competitive ability under high soil moisture conditions (Kouyeas 1964). This has also been shown by Cavagnaro (2016), where higher soil moisture negatively impacted mycorrhizal growth and colonization of plants, and Deepika and Kothamasi (2015) who found lower levels of arbuscular mycorrhizal fungi (AMF) diversity in flooded soils.

4.4 Invasive plant species are characterised by low diversity of root fungal communities

The root-associated fungal communities were composed of low phylogenetic OTU richness in the invasive species, and were remarkably distinct to the native counterpart *U. dioica*. Irrespective of the fungal ecological group, significantly fewer root-associated OTUs were found for *F. japonica* compared to *U. dioica*. The second species investigated, *I. glandulifera*, was also characterized by reduced root-associated fungal OTUs, however significant reductions compared to *U. dioica* were observed only for mycorrhiza and saprobic taxa. The lower endophyte and mycorrhizal OTU richness in *F. japonica* and *I. glandulifera* roots were likely caused by changes in host-symbiont interactions. Symbionts are often not host specific but functional variability between different plant-mycorrhiza combinations have been observed (Klironomos 2000). Therefore, an invasive plant species may not be able to benefit from local soil fungi which might reduce mycorrhizal and root endophytic diversity (Čuda et al. 2020). This is in line with Callaway et al. (2011) who reported that AMF from a native range stimulated stronger positive feedbacks in invasive *Robinia pseudoacacia* than AMF from an invasive range. On the other hand, root endophytes of *Centaurea stoebe* were reported to directly increase the competitive effects of the introduced plant species (Aschehoug et al. 2012). Root endophytes of two *Centaurea* species (one invasive and one native) were also found to be different, indicating that endophytes may not be able to form associations with a variety of plants (Geisen et al. 2017). Garnica et al. (2022) reported a strong colonization rate for the root endophytic species *Serendipita herbamans* in *F. japonica* in a lab study, especially in low-nutrient conditions. Secondary metabolites inhibit the growth of especially mycorrhizal fungi (Pinzone et al. 2018), which might also explain the lower number of root-associated fungi found in *F. japonica* compared to *I. glandulifera*. Moreover, *I. glandulifera* is able to associate to some degree with AMF (Tanner et al. 2014) and according to Pattison et al. (2016), *I. glandulifera* can affect the AMF and fungal endophyte diversity.

The lower number of mycorrhizal OTUs in roots of both *F. japonica* and *I. glandulifera* compared to native *U. dioica* indicates reduced symbiotic interactions. The root associated fungi in the invasive species include no AMF, which indicates that these species may not be able to associate well with symbiotic fungi in the invasive range. Some Glomeromycota were however detected in *U. dioica* roots (*Rhizophagus* spp., see Online Resource 8). Root endophytic OTUs were lower in *F. japonica*, which might be caused by the absence of the most common and diverse genus *Trichoderma* (Harman et al. 2004). One reason might be due to the secondary metabolites utilized by *F. japonica*. We also found reduced numbers of parasitic fungal OTUs in root samples of *F. japonica* compared to *U. dioica*. The most common genus in *U. dioica*, *Fusarium*, was absent in *F. japonica*. The absence of directly associated major fungal parasites inhibiting its growth in the invasive range might contribute to the invasion success of *F. japonica*. This is in accordance with the enemy release hypothesis, which states that invasive species

have increased success because of lower numbers of natural enemies (Mitchell and Power 2003). Root associated OTUs classified as saprobic were lower in both invasive species compared to the native species. Saprobic fungi may enter tissue to break down dead matter (Lebreton et al. 2021). The reduced number of OTUs in the invasive species might indicate that a smaller subset of species is able to metabolize the tissue of the two invasive plant species. Mincheva et al. (2014) showed that *F. japonica* is a low-quality food source and had a different fungal composition compared to litter from native sites.

Like the soil fungal OTU abundances, root associated fungi were affected by some soil parameters as well. The most important factor was soil water content, having a predominantly negative effect on the overall fungal OTU richness as well as the ecological groups mycorrhiza and parasites in the *I. glandulifera* site pairs. In *F. japonica* site pairs, only the number of mycorrhizal OTUs was negatively associated with increasing water content, while higher water content was positively correlated with OTU richness in endophytes and parasites. The negative effect of higher moisture on mycorrhiza has previously been shown by Cavagnaro (2016) and Deepika and Kothamasi (2015). Tree root associated saprotrophic and parasitic community structure has also previously been shown to correlate with soil moisture, where higher soil moisture benefited only some species (Burke et al. 2009).

5 Conclusion

We observed only minor effects of the investigated plant invasive species *Fallopia japonica* and *Impatiens glandulifera* on soil physicochemical properties, soil microbial activity, soil fungal biomass and diversity. In contrast, the abundances of Acari and Collembola were impacted, indicating that both species can have direct impacts on soil biota. However, these impacts were not uniform between *F. japonica* and *I. glandulifera*, which implies that these effects are species-specific and may depend on the local soil biota composition. Looking at tissue-entering fungi, *F. japonica* clearly had lower fungi OTU richness while *I. glandulifera* was characterized only by fewer mycorrhizal and saprobic OTUs. This was further related to soil characteristics, especially water content and pH decreased fungal diversity. Overall, our findings indicate that *F. japonica* and *I. glandulifera* invasions may have various and partly contrasting impacts to previous studies on belowground ecosystem compartments such as soil invertebrates and fungi. Therefore, effects of these plant invaders seem to be highly context-dependent and site-specific. Furthermore, our results are in line with the enemy release hypothesis, indicating that these invasive species, especially *F. japonica*, may be less associated with fungal parasites in the invasive range, allowing them to perform better than native species.

Data availability statement

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Table 1 Bulk soil and root fungal operational taxonomic units (OTU) composition in relation to plant species (invasive *Fallopia japonica* or *Impatiens glandulifera* vs. native *Urtica dioica*), soil organic carbon, pH value, and water content. Shown are R^2, pseudo F-ratios and P-values based on permutational multivariate analyses of variance for presence-absence data. Significant values at the 0.05 threshold are given in bold are marked with asterisks as (** $P \leq 0.01$, P * ≤ 0.05).

	R^2	F	P
Bulk soil samples			
Fallopia japonica / *Urtica dioica* site pairs			
Plant species	0.05	0.83	0.109
Organic carbon	0.06	0.86	0.539
pH value	0.10	1.56	0.215
Water content	0.09	1.41	0.336
Impatiens glandulifera / *Urtica dioica* site pairs			
Plant species	0.05	0.74	0.563
Organic carbon	0.09	1.41	0.563
pH value	0.09	1.50	0.395
Water content	0.08	1.30	0.785
Root samples			
Fallopia japonica / *Urtica dioica* site pairs			
Plant species	0.21	3.77	**0.009 ****
Organic carbon	0.05	0.93	0.734
pH value	0.07	1.27	0.148
Water content	0.07	1.36	0.063
Impatiens glandulifera / *Urtica dioica* site pairs			
Plant species	0.09	1.43	**0.016 ***
Organic carbon	0.07	1.05	0.555
pH value	0.07	1.17	0.320
Water content	0.07	1.03	0.563

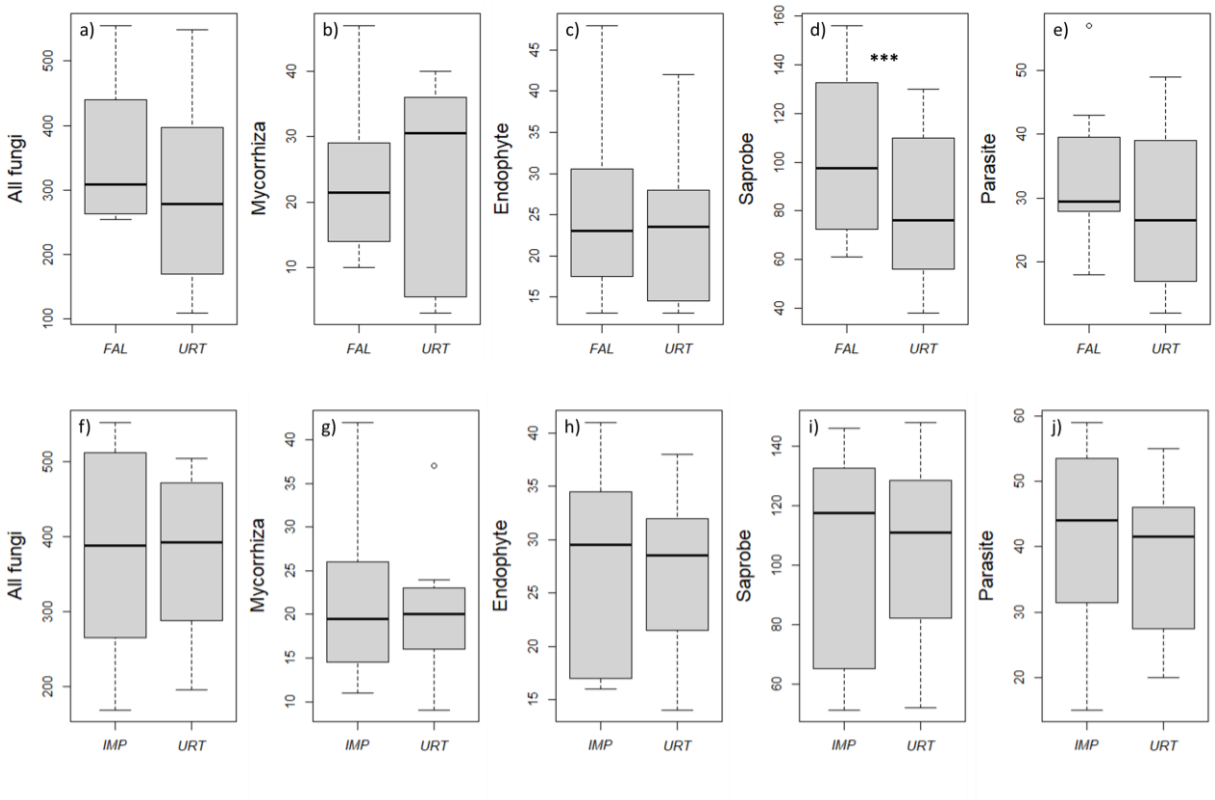

Fig. 1 Comparisons of bulk soil fungal ecological group OTU richness in riparian habitats invaded by *Fallopia japonica* (FAL, a-e) or *Impatiens glandulifera* (IMP, f-j) compared to native *Urtica dioica* (URT) sites. OTU richness for all fungi and ecological groups (mycorrhiza, endophytes, saprobes, and parasitic fungi) are compared. Boxplots show the median (thick black line), interquartile range (grey box), whiskers and outliers (circles). Significances are indicated by asterisks (*** $P \leq 0.001$).

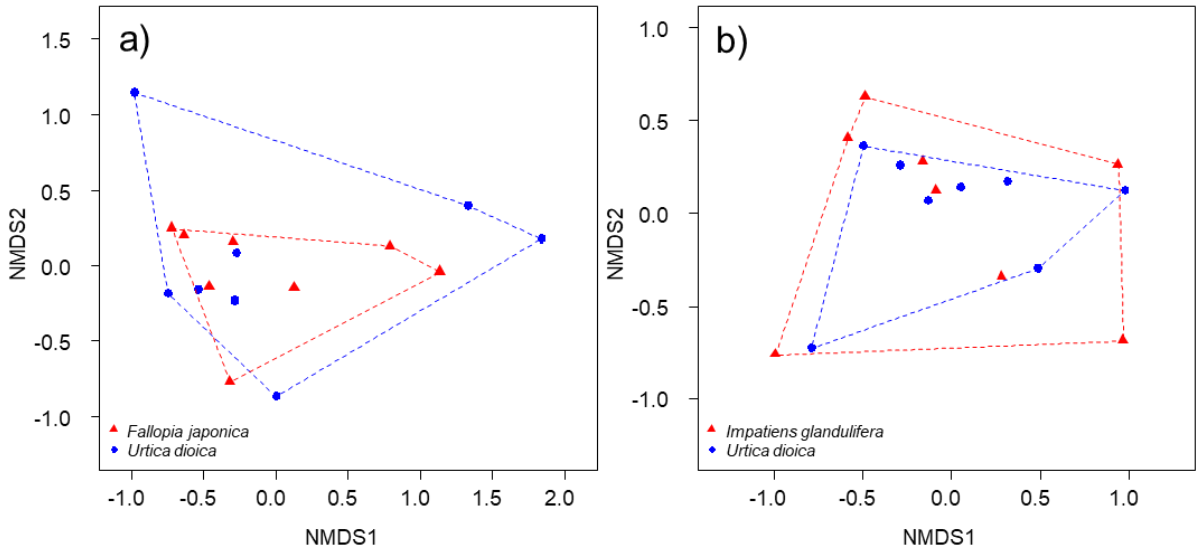

Fig. 2 Nonmetric multidimensional scaling (NMDS) ordination for the bulk soil fungal operational taxonomic units (OTU) composition of riparian site pairs of (a) *Fallopia japonica* / *Urtica dioica* and (b) *Impatiens glandulifera* / *Urtica dioica*. Boundaries indicate the OTU composition of native (blue) and invaded (red) sites. Relations between soil fungal OTU composition and environmental variables were not significant (tested with permutational multivariate analyses of variances, see Table 1).

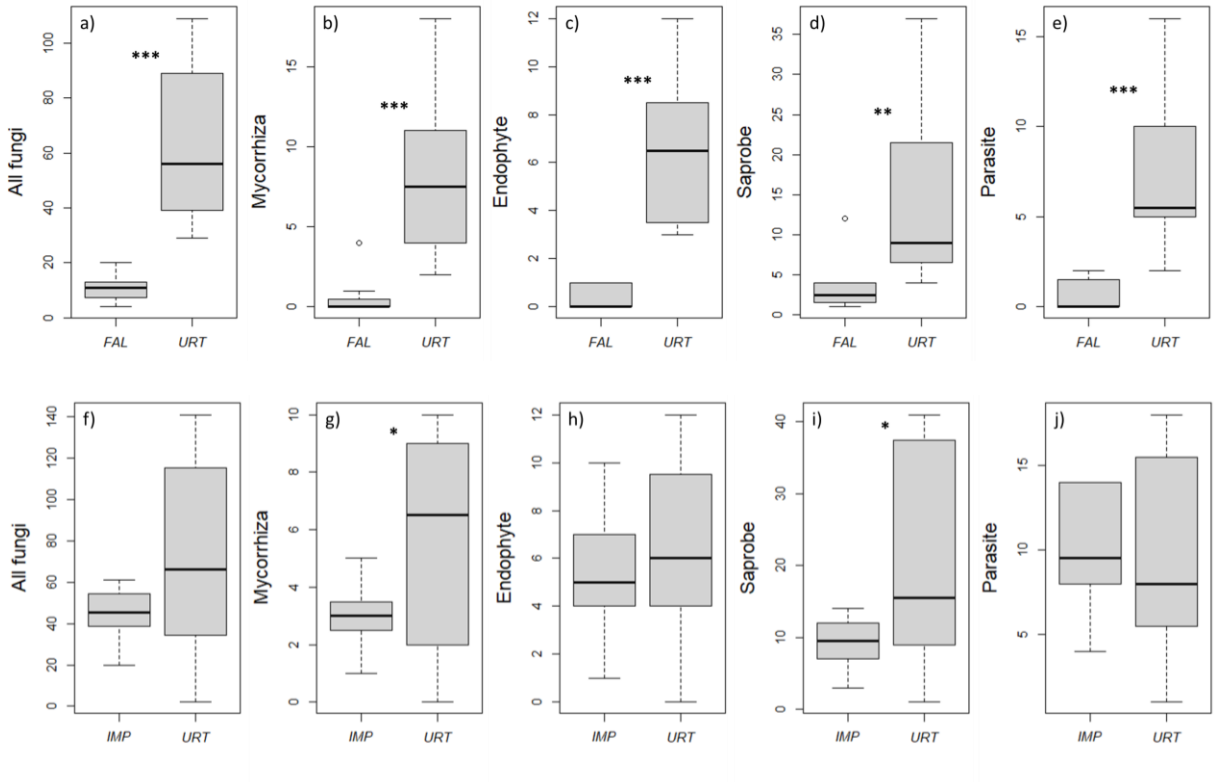

Fig. 3 Comparisons of root fungal ecological group OTU richness in riparian habitats invaded by *Fallopia japonica* (FAL, a-e) or *Impatiens glandulifera* (IMP, f-j) compared to native *Urtica dioica* (URT) sites. OTU richness for all fungi and ecological groups (mycorrhiza, endophytes, saprobes, and parasitic fungi) are compared. Boxplots show the median (thick black line), the interquartile range (grey box), whiskers and outliers (circles).. Significances are indicated by asterisks (*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$).

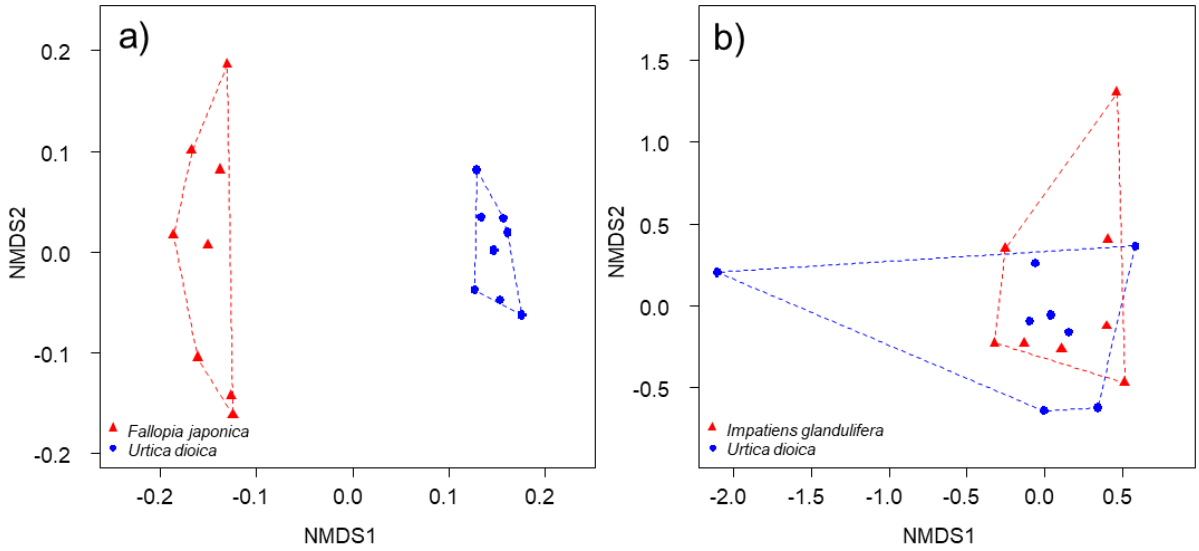

Fig. 4 Nonmetric multidimensional scaling (NMDS) ordination showing significant differences in the root fungal Operational taxonomic units (OTU) composition between riparian site pairs of (a) *Fallopia japonica* / *Urtica dioica* and (b) *Impatiens glandulifera* / *Urtica dioica*. Boundaries indicate the OTU composition of native (blue) and invaded (red) sites. Relations between soil fungal OTU composition and environmental variables were tested with permutational multivariate analyses of variances, see Table 1.

Supplemental Tables

Table S1 Comparison of soil parameters (mean and standard error) between corresponding *Fallopia japonica* / *Urtica dioica* sites and *Impatiens glandulifera* / *U. dioica* sites. The Estimate and P-values were derived from generalized linear mixed models. Parameters used as explanatory variables in the models for explaining soil microbial activity and soil organisms, soil fungi and plant associated fungi are marked with an asterisk (*).

Soil parameter	***Fallopia japonica***	***Urtica dioica***	**Estimate**	**P**	***Impatiens glandulifera***	***Urtica dioica***	**Estimate**	**P**
Water content* [% dry matter]	29.60 ± 7.74	34.80 ± 20.77	5.20	0.393	38.57 ± 12.76	34.70 ± 11.76	-3.87	0.449
pH value*	5.66 ± 1.32	5.77 ± 1.25	0.10	0.769	5.74 ± 1.13	6.16 ± 0.72	0.41	0.065
Organic carbon* [% dry matter]	3.01 ± 1.12	4.03 ± 2.52	1.01	0.298	4.12 ± 2.51	3.36 ± 1.29	-0.76	0.254
Total nitrogen [mg / kg dry matter]	26.35 ± 8.22	31.51 ± 10.02	51.62	0.260	34.61 ± 11.39	29.03 ± 8.92	-55.88	0.170

Table S2 Model results for microbial soil activity, measured as soil respiration (base) and soil respiration (glucose), and soil organisms (Acari and Collembola abundances) in relation to plant species (invasive *Fallopia japonica* or *Impatiens glandulifera* vs. native *Urtica dioica*), soil organic carbon, pH value, and water content. Shown are model estimates and the significance as *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. Significant values are given in bold.

Category	**Plant species (invasive / native)**	**Organic carbon**	**pH value**	**Water content**
Fallopia japonica / *Urtica dioica* site pairs				
Bulk soil respiration (base)	-0.01	**0.59 ****	-0.15	**-0.04 ****
Bulk soil respiration (glucose)	0.17	1.02	6.64	-0.01
Rhizosphere soil respiration (base)	-1.43	0.74	2.29	-0.03
Rhizosphere soil respiration (glucose)	11.39	5.27	0.33	-0.38
Acari	**1.33 *****	-0.10	**-0.56 ****	0.004
Collembola	0.24	**0.17 ***	**0.43 ****	0.01
Impatiens glandulifera / *Urtica dioica* site pairs				
Bulk soil respiration (base)	1.63	0.26	-0.38	-0.11
Bulk soil respiration (glucose)	0.19	-2.57	-2.28	-0.11
Rhizosphere soil respiration (base)	5.05	-0.19	-3.75	-0.14
Rhizosphere soil respiration (glucose)	-12.38	3.70	12.78	-1.26
Acari	-0.02	-0.15	**0.54 ****	0.03
Collembola	**-0.33 ****	**-0.32 *****	**0.43 ***	0.00

Table S3 Model results for fungal biomass (ergosterol) and OTU richness of ecological groups found in the bulk soil samples in relation to plant species (invasive *Fallopia japonica* or *Impatiens glandulifera* vs. native *Urtica dioica*), soil organic carbon, pH value, and water content. Shown are model estimates and the significance as *** P ≤ 0.001, ** P ≤ 0.01, P * ≤ 0.05. Significant values are shown in bold.

Category	**Plant species (invasive / native)**	**Organic carbon**	**pH value**	**Water content**
Fallopia japonica / *Urtica dioica* site pairs				
Fungal biomass	-1.00	**0.88 ***	-0.69	-0.06
Fungal OTU richness	-0.09	**-0.11 ****	**0.06 ****	-0.01
Mycorrhiza	0.26	**-0.18 ***	-0.19	**-0.04 *****
Endophytes	-0.09	0.01	**0.23 ****	-0.00
Saprobes	**-0.27 *****	0.03	**0.15 ***	-0.00
Parasites	-0.20	-0.02	**0.18 ***	-0.00
Impatiens glandulifera / *Urtica dioica* site pairs				
Fungal biomass	1.59	**1.59 *****	-0.57	**-0.11 ***
Fungal OTU richness	-0.06	0.00	0.05	-0.01
Mycorrhiza	-0.01	-0.00	-0.22	-0.01
Endophytes	-0.08	-0.04	-0.02	**-0.02 ***
Saprobes	-0.02	-0.07	-0.11	**-0.02 ****
Parasites	-0.10	0.01	-0.08	**-0.02 ***

Table S4 Model results for OTU diversity of fungal ecological groups found in the root samples in relation to plant species (invasive *Fallopia japonica* or *Impatiens glandulifera* vs. native *Urtica dioica*), soil organic carbon, pH value, and water content. Shown are model estimates and the significance as *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. Significant values are shown in bold.

Category	Plant species (invasive / native)	Organic carbon	pH value	Water content
Fallopia japonica / Urtica dioica site pairs				
All fungi	**1.75 *** **	-0.02	0.03	0.01
Mycorrhiza	**3.57 *** **	**-0.3 ***	**-1.02 ** **	**-0.01 ***
Endophytes	**2.74 *** **	-0.01	0.24	**0.02 ***
Saprobes	**1.24 ** **	0.07	0.37	0.02
Parasites	**2.46 *** **	-0.16	-0.02	**0.02 ***
Impatiens glandulifera / Urtica dioica site pairs				
All fungi	0.13	-0.06	**-0.10 ** **	**-0.03 ***
Mycorrhiza	**0.60 ***	0.00	**-0.36 ***	**-0.03 ***
Endophytes	0.11	-0.04	-0.20	-0.02
Saprobes	**0.64 ***	-0.03	-0.11	-0.03
Parasites	-0.13	-0.02	-0.17	**-0.03 ***

Digital resources

The digital resources 1 to 8 are provided in the attached CD disk.

Digital Resource 1 List of the field sites used for sample collection. Location is the name of the associated stream used to identify the field site. GPS coordinates of the field sites are given. Target plant species allows differentiation between the native vs. the invaded subplot. Herbaceous and woody vegetation characterize the dominant vegetation present at the subplots (after the target species). Species number indicate the number of herbaceous plant species per site.

Digital Resource 2 Example pictures of field sites with a representative population of the target species. From top to bottom: *Fallopia japonica*, *Impatiens glandulifera* and *Urtica dioica*.

Digital Resource 3 Detailed explanation of the processing of sequences applied in this study.

Digital Resource 4 List of all sequences identified in the metabarcoding analysis and the assigned operational taxonomic units (OTU) used in subsequent analyses, given in FASTA format.

Digital Resource 5 Information obtained from matching operational taxonomic units (OTU) against NCBI GenBank database. Species assignments above 95% surety are highlighted in yellow and above 97% in green. Seq_length identifies the length of the given sequence. The columns "Michelsbach 1 root Urt" to "Rottenbach 2 soil Fal" show the number of occurrences (reads) of each OTU sequence in each of the field sites. The corresponding codes for the field sites are explained in Online Resource 1. "root" and "soil" identify root and soil samples respectively. "Fal", "Imp" and "Urt" identify the species present in the corresponding plot. The abbreviations refer to *Fallopia japonica*, *Impatiens glandulifera* and *Urtica dioica*, respectively. High numbers of occurrences in these columns as well as for the total number of occurrences are indicated by deeper blue coloring of the value. The columns "NCBI_taxon_01" to "NCBI_taxon_31" give the taxonomic information obtained from NCBI GenBank in descending order of rank starting from Domain. Fungal OTUs with over 95% surety were used for further analysis.

Digital Resource 6 Taxonomic information and nutrition strategy of fungal operational taxonomic units (OTU). Taxonomy based on NCBI GenBank, updated by manual BLAST and Mycobank search for unclear OTUs with unclear phylogenetic position. For ecology, available literature was screened for OTUs and added if possible. MYC = mycorrhiza, END = endophytes, SAP = saprobes, PAR = parasites. NA stands for sequences where no ecological group could be assigned, that were subsequently disregarded for analysis of specific ecological groups.

Digital Resource 7 Correlation matrix of soil properties. In the lower part of the figure, a graph representing the correlation between the two variables is displayed and the numeric representation of the correlation (Pearson correlation coefficient) can be found in the upper part. Values closer to 1 indicate a high correlation, which is also signified by increasing font sizes. Significant correlations are indicated by asterisks (*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$).

Digital Resource 8 Fungi genera with the three highest OTU richness. OTU richness is given as a number after each genus. The data is separated by site pair (*Fallopia japonica* & *Urtica dioica* and *Impatiens glandulifera* & *Urtica dioica*). Within each site pair, the functional groups are displayed separately (MYC = mycorrhiza, END = endophytes, SAP = saprobes, PAR = parasites). Genera with the same OTU richness are displayed in the same line.

Amendment II

Copper uptake and its effects on two riparian plant species, the native *Urtica dioica,* and the invasive *Fallopia japonica*

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Abstract

Copper accumulating in stream sediments can be transported to adjacent riparian habitats by flooding. Although being an essential element for plants, copper is toxic at high concentrations and restricts, among other things, plant growth. Besides copper, invasive plants, such as *Fallopia japonica*, which are known to be tolerant toward heavy metals, modify riparian habitats. If the tolerance of *F. japonica* is higher compared to native plants, this could accelerate invasion under high heavy metal stress. Therefore, we aimed to compare the effect of copper on two common riparian plants, the invasive *F. japonica* and the native *Urtica dioica*. We performed a pot experiment with a gradient from 0 to 2430 mg kg^{-1} of soil copper. We hypothesized that (i) negative effects on plant growth increase with increasing soil copper concentrations with *F. japonica* being less affected and (ii) accumulating higher amounts of copper in plant tissues compared to *U. dioica*. In support of our first hypothesis, growth (height, leaf number) and biomass (above- and belowground) of *F. japonica* were impacted at the 810 mg kg^{-1} treatment, while the growth of *U. dioica* was already impacted at 270 mg kg^{-1}. Due to 100% mortality of plants, the 2430 mg kg^{-1} treatment was omitted from the analysis. In contrast, chlorophyll content slightly increased with increasing copper treatment for both species. While *U. dioica* accumulated more copper in total, the copper uptake by *F. japonica* increased more strongly after exposure compared to the control. In the 810 mg kg^{-1} treatment, copper concentrations in *F. japonica* were up to 2238% higher than in the control but only up to 634% higher in *U. dioica*. Our results indicate that *F. japonica* might be able to more efficiently detoxify internal copper concentrations controlling heavy metal effects compared to the native species. This could give *F. japonica* a competitive advantage particularly in polluted areas, facilitating its invasion success.

Keywords: inorganic pollution; bioaccumulation; contamination; copper; copper sulphate; heavy metals; plant growth

1 Introduction

Since the 19th century, the use of copper as a fungicide has been an inherent part of agriculture [1]. Copper does not degrade but accumulates in soils leading to elevated concentrations [2]. In fact, the long-term application of copper resulted in high levels—reaching up to 3000 mg kg^{-1}—in agricultural areas [3]. In European vineyards, for example, soil copper levels of up to 600 mg kg^{-1} have been observed, while corresponding uncontaminated sites ranged between 20 and 100 mg kg^{-1} [4]. From agricultural soils, copper can be transported to adjacent non-target aquatic ecosystems through spray drift, groundwater drainage, and surface run-off [5]. In rivers and streams, copper tends to accumulate in sediments [6] where it can be remobilized and transferred to riparian ecosystems during flooding with potential effects on local plant species, affecting ecosystem structure and functioning [7].

Although copper is an essential element supporting enzyme activity and chlorophyll production [8], at high concentrations it is toxic, causing oxidative stress that can lead to DNA damage [9]. Moreover, copper can induce cell membrane ruptures and abnormalities in leaf chloroplasts, which impacts photosynthesis and nutrient transport [10,11]. Protective processes, which vary between species, have evolved including detoxification using enzymatic and non-enzymatic antioxidants as well as sequestration of excess copper [9]. Nonetheless, plants often respond to heavy metal exposure—such as copper—by growth impairments. For example, $CuSO_4$ concentrations of 100 µM caused reduced growth rates and necrosis in *Prunus cerasifera* [12]. Copper toxicity manifesting as chlorosis and subsequent desiccation and death of plant parts as well as stunted growth has been observed in woody ornamentals [13], which is similar to symptoms produced by other heavy metals. Bouazizi et al. [14] also reported a reduction in biomass and leaf number in *Phaseolus vulgaris*.

Besides copper, plant invasion can be an additional pressure in riparian ecosystems. Common nettle (*Urtica dioica* L., Urticaceae) is a native, rhizomatous, perennial herb that often dominates in nitrogen-rich soils [15]. Its stands are frequently invaded by the Asian knotweed (*Fallopia japonica* (Houtt.) Ronse Decr., Polygonaceae), one of the most widespread invasive plants in central Europe. It is a perennial, rhizome-forming species which quickly produces high above and below ground biomass [16]. Consequently, *F. japonica* outcompetes native plants for resources such as light and nutrients by forming dense stands and altering the soil nitrogen composition, finally reducing biodiversity [17]. The success of invasive plants is often related to a higher tolerance toward heavy metals, relative to native species [18]. In support of this general statement, members of the genus *Fallopia* have been shown to be tolerant to heavy metal contamination including copper [19], which is accumulated mainly in roots (*F. convolvulus* [20], *F. japonica* [21–23]). While copper at concentrations up to 200 mg kg^{-1} did not affect the ability of *F. japonica* to establish from rhizomes, at concentrations equal to or above 300 mg kg^{-1} it delayed plant growth and impacted morphology [23]. This high tolerance supports the species' competitive advantage in soils contaminated with heavy metals [24]. The co-occurring native species *U. dioica* is also considered to be tolerant to heavy metals [25], including copper [26,27], which is often accumulated in aboveground parts such as leaves [28]. However, information on the tolerance to higher soil copper concentrations is scarce. Therefore, the objective of our study was to investigate if the native plant (*U. dioica*) shows a different response to copper contamination than the invasive plant (*F. japonica*). Such a difference could impact the invasive success of *F. japonica* which is a major factor in riparian ecosystems.

To test if there is such a difference in response, we conducted a pot experiment under field conditions. In a randomized block design, the plants were grown in soils with increasing soil copper concentrations (0 to 2430 mg

kg^{-1} soil). We expected that (i) higher copper concentrations reduce plant growth of both species. However, since *F. japonica* is considered to be more tolerant, this invasive species should only respond at higher copper concentrations compared to *U. dioica*. We further hypothesized, based on the known ability of *F. japonica* to accumulate heavy metals, that (ii) the invasive species exhibits higher copper concentrations and takes up copper more effectively in all plant parts compared to *U. dioica*.

2 Results

Since no plants survived in the highest copper treatment (2430 mg copper kg^{-1} soil) (see Table A1), this treatment was disregarded for further analysis. In the text, all mentioned values will be mean and standard deviation (see Section 4.4 for more details).

2.1 Seasonal plant growth

Plant height and leaf number significantly decreased with increasing copper concentrations in both *Fallopia japonica* and *Urtica dioica*. In the control treatment, *F. japonica* (26.8 ± 12.9 cm) was about 25% higher than in the treatment with 810 mg kg^{-1} (21.4 ± 12.6 cm), which was statistically significant (Table 1). Similarly, in *U. dioica* the average height was significantly higher in the control (35.7 ± 22.9 cm) than at 810 mg copper kg^{-1} (30.0 ± 22.3), a reduction by about 20%. Regarding leaf number, *F. japonica* had an average of 56.2 ± 37.4 leaves in the control, a value significantly lower by 25% (43.0 ± 26.0) in the 810 mg copper kg^{-1} treatment. For *U. dioica*, a significant reduction in leaf number from 140.0 ± 131.9 to 73.6 ± 72.2 (by approximately 50%) in the same treatments was also observed (Table 1). Overall, the decrease in plant height and leaf number became apparent at 810 mg copper kg^{-1} for *F. japonica* while *U. dioica* showed significant responses already at 270 mg copper kg^{-1} (Figure 1).

In contrast, the chlorophyll content increased statistically significantly with increasing copper concentration (Table 1). For *F. japonica*, chlorophyll increased by about 10% from 21.1 ± 5.7 in the control to 23.1 ± 6.0 in the 810 mg copper kg^{-1} treatment. With an increase in chlorophyll from 27.3 ± 7.2 to 30.6 ± 13.1, the effect was comparable for *U. dioica*.

Apart from treatment effects, the factor time also significantly influenced all plant parameters. Height and leaf number of both species increased during spring but remained rather constant thereafter (Figure 1). In contrast, the chlorophyll content significantly decreased over time independent of the plant species (Table 1). Both copper treatment and time significantly interacted for several variables in both species (Table 1). At lower copper concentrations, leaf number of *F. japonica* increased more strongly over time while higher treatment levels showed a decrease in the later part of the season. Height, a parameter describing growth over time, of *U. dioica* was lower at higher copper concentrations while leaf numbers decreased more strongly later in the growing season (Figure 1).

2.2 Biomass after a two-year experiment

The final biomass of *F. japonica* for both above- and belowground plant parts significantly decreased with higher copper concentrations, which became statistically significant at 810 mg copper kg^{-1} (Table 2). Above- and belowground biomass significantly decreased by a factor of two from 72.1 ± 24 g to 32.5 ± 28 g and 228.2 ± 98.5

g to 114.6 ± 126 g, respectively. At lower copper concentrations, biomass remained largely constant relative to the control (Figure 2a,c).

For *U. dioica*, there was a tendency for a decrease in above- and belowground biomass with increasing copper concentrations but no significant effect overall (Table 2). Above- and belowground biomass decreased by a factor of two and three respectively from 29.9 ± 19.1 g to 12.6 ± 11.3 g and 54.3 ± 30.1 g to 17 ± 18.8 g. The statistical non-significance of the copper treatments in *U. dioica* may be explained by a rather low biomass in the control, with the lowest copper treatment of 90 mg kg^{-1} having higher biomass than the control (Figure 2b,d).

2.3 Copper content of plant tissues

The copper content of almost all plant tissues was higher in *U. dioica* compared to *F. japonica* across the experimental soil copper gradient. The only exception was the root copper content, which was 46% higher in *F. japonica* at the 810 mg kg^{-1} level (17.1 ± 9.5 mg g^{-1} in *F. japonica* and 11.7 ± 2.1 mg g^{-1} in *U. dioica*). Especially for the aboveground and rhizome material, the samples from *U. dioica* showed much higher copper contents compared to *F. japonica*. In the 810 mg kg^{-1} treatment, *U. dioica* aboveground material contained about 2.5 times more copper compared to *F. japonica* (9.7 ± 5.9 mg g^{-1} in *F. japonica* and 24 ± 11.9 mg g^{-1} in *U. dioica*) but aboveground copper content was higher in *U. dioica* across all treatment levels (Figure 3a,b).

Copper content of the three sample types (aboveground, roots, and rhizome) significantly increased with increasing soil copper concentration in both *F. japonica* and *U. dioica* (Table 3). Like the absolute concentrations, the strongest increase was found in the rhizome where the mean copper content increased from 1.3 ± 1.0 mg g^{-1} in the control to 30.4 ± 31.9 mg g^{-1} in the 810 mg kg^{-1} level for *F. japonica* and from 9.4 ± 13.5 mg g^{-1} to 69.0 ± 33.9 mg g^{-1} in *U. dioica* (Figure 3e,f). Unlike the absolute concentrations, *F. japonica* showed a much higher accumulation compared to the control, increasing copper concentrations in rhizomes by more than 23.3 times compared to 7.3 times in *U. dioica*. Accumulation in the 810 mg kg^{-1} treatment compared to the control for the two other plant parts was also higher in *F. japonica* compared to *U. dioica*. For aboveground samples, copper content increased by 19 times in *F. japonica* vs. 17 times in *U. dioica* and for the roots, the difference was 3.5 times compared to 2 times.

In *F. japonica*, the highest copper content in tissues was found in the rhizome, with a two-fold higher copper concentration at the highest treatment level compared to root samples (30.4 ± 31.9 mg g^{-1} compared to 17.1 ± 9.5 mg g^{-1}) and a three-fold higher concentration compared to aboveground samples (30.4 ± 31.9 mg g^{-1} compared to 9.7 ± 5.9 mg g^{-1}). The rhizome copper concentration increased by 2238% from the control (1.3 ± 1 mg g^{-1}) to the 810 mg kg^{-1} treatment. For *U. dioica*, the average copper content was also highest in rhizomes (six times higher than roots and three times higher than aboveground material at the highest treatment; Figure 3). For *U. dioica*, the increase from control (9.4 ± 13.5 mg g^{-1}) to the highest treatment was 634%.

3 Discussion

We investigated the effect of copper contamination (treatments with 0, 90, 270, and 810 mg kg^{-1}) on the invasive plant *Fallopia japonica* and the native *Urtica dioica*. Higher soil copper concentrations negatively impacted the growth of both the invasive plant species *Fallopia japonica* and the native *Urtica dioica*. However, the native species was more sensitive relative to the invasive species. Uptake of copper by plant tissue increased with soil

copper content for both plants. Unexpectedly, *U. dioica* showed higher absolute content of copper in plant material compared to *F. japonica*. However, the accumulation of copper compared to the control was higher in *F. japonica* than in *U. dioica*, which corresponds to the hypothesis that *F. japonica* can accumulate copper more effectively.

3.1 Copper contamination negatively impacts plant growth, but more strongly in *U. dioica* than *F. japonica*

As expected, increasing copper concentrations impacted all observed plant parameters (above- and belowground biomass, plant height, leaf number and chlorophyll content) in both plant species. We also found that the growth of *F. japonica* and *U. dioica* depended on the time of measurement. Plant height and leaf number increased during the growing season due to seasonal progression in plant growth. Regarding the copper treatment, we found that biomass, plant height, and leaf number decreased with increasing copper concentration. This reduction in plant growth is in line with the previous studies on various species (*Buxus sempervirens*, *Cotoneaster divaricatus* and *Rhododendron obtusum* [13]; *Phaseolus vulgaris* [14]; *Prunus cerasifera* [12]) and reflects the general known responses of plants to copper [9]. The impact of copper on plant growth can be explained by oxidative damages caused by excess copper in cells, which damage cell organelles and DNA [9–11]. This leads to reduced growth and necrosis in affected plants [12–14].

Importantly, we found that the reduction in growth occurred at different threshold concentrations: For *F. japonica*, growth was only lower at the highest analyzed concentration (810 mg kg^{-1}), while *U. dioica* already showed significant loss of growth at 270 mg kg^{-1}. Our results therefore only partly confirm the few existing studies on *Fallopia* species, where negative effects of copper started at lower concentrations than in our study. For example, Sołtysiak [23] found impact on *F. japonica* at 300 mg copper kg^{-1} and Pedersen et al. [20] estimated a threshold for the onset of adverse effects on *Fallopia convolvulus* growth at slightly lower concentrations (i.e., 200 mg kg^{-1}). In our study, plants grown at 270 mg copper kg^{-1} had similar biomass compared to the lower treatments. Although we observed the highest above- and belowground biomass in *U. dioica* at 90 mg copper kg^{-1} and a subsequent decrease toward the higher concentrations, the overall effect of copper was not significant, likely due to the high variability in our data. The stimulation of above- and belowground biomass in *U. dioica* corresponds well to the higher leaf numbers and height in the same treatment. In earlier studies, *U. dioica* has shown the ability to tolerate higher concentrations of copper with phytostabilisation of contaminated soil, meaning a reduction in the bioavailability of copper in the soil [26]. The pattern observed in our study might be due to hormesis, where a mild stress, here induced by copper, can stimulate plant growth, which might be linked to the production of antioxidants in combination with a stimulation of chlorophyll levels [29]. This effect is often observed in hyperaccumulator plants [30]. A possible reason for the stronger growth at high copper concentrations in *F. japonica* might be its higher tolerance to copper [19], which is thought to be connected to the plant's ability to sequester copper into the cell wall [31] and produce specific binding proteins even at high metal concentrations [32]. This also allows *F. japonica* to hyperaccumulate metals [33,34]. Our findings therefore support the hypothesis that invasive plants benefit from disturbances by heavy metal contamination due to their higher tolerance to stressors such as copper [18].

In contrast, the chlorophyll content increased with copper concentration in both species and decreased along the time of measurement, since chlorophyll production is reduced in order to focus on production of flowers and seeds later in the year. According to Kalaji et al. [35], the effect of heavy metal contamination on the number of

photosynthetic pigments is not uniform. For example, lead caused a decrease in the amount of pigment in *Pisum sativum* [36]. Similarly, chromium contamination decreased total chlorophyll by up to 61% in *Brassica juncea* [37] and by 54% in *Solanum lycopersicum* [38]. On the other hand, cadmium had no impact on the amount of photosynthetic pigments in *Brassica napus* but still disrupted photosynthetic processes [35]. However, in mosses (*Ptychanthus striatus*, *Thuidium delicatulum*, and *T. sparsifolium*) as well as in *Empetrum nigrum* and *Camellia sinensis*, copper showed a stronger inhibitory effect on total chlorophyll than other heavy metals, due to the disruption of chlorophyll production [39–41]. The contrary pattern observed in our study could be explained by substantial differences in plant traits. Both *F. japonica* and *U. dioica* are known to accumulate heavy metals [20,42] and might therefore be efficient in detoxifying copper before it can damage chlorophyll production. This might be due to chelating agents such as oxalic acid which can bind heavy metals and reduce toxicity. This has been shown for chromium toxicity [37,43]. Another factor might be a higher concentration of the plant hormone salicylic acid in these species, which can increase the stress tolerance of plants. Application of salicylic acid can decrease the toxicity of heavy metals such as chromium and aluminum [38,44]. A further explanation might be that the observed loss of leaves in higher copper treatments caused an increase in chlorophyll production in the remaining leaves to keep the photosynthetic activity of the organism stable. This form of compensation has previously been found as a response to herbivory [45,46].

3.2 Copper content in plant tissue is higher in *U. dioica* than in *F. japonica*

It could be verified that both *F. japonica* and *U. dioica* accumulate copper, which is in line with the studies dealing with roots and rhizomes [21,28,31,42]. We also found that the copper content was highest in the belowground plant parts, especially rhizomes. This observation contrasts with literature highlighting higher heavy metal concentrations in aboveground plant parts (mainly leaves) for *F. japonica* [23] and *U. dioica* [26,28]. Moreover, our study points to a lower copper concentration in the plant relative to the surrounding environment (i.e., soil) for both species, suggesting they are not hyperaccumulators of copper, which was postulated for *F. japonica* [47] and *Fallopia* × *bohemica* [48]. However, copper hyper accumulation was reported for roots of *F. convolvulus* with a factor 2 : 1 for root: soil [20].

Unexpectedly, *the* absolute copper concentrations in *F. japonica* were lower in nearly all copper treatments and plant parts than in *U. dioica*. This difference might be explained by *F. japonica* being able to regulate the copper uptake compared to *U. dioica*. Lerch et al. [49] showed in a pot experiment that the transfer of copper from soil to plant does not increase strongly under copper contamination. While copper in roots of *F. convolvulus* increased linearly with increasing soil copper concentration, a saturation in aboveground plant parts was reported for 200 to 300 mg kg^{-1} [20]. Only in our treatment with a copper concentration of 810 mg kg^{-1}, less copper was detectable in roots of *U. dioica* compared to *F. japonica* (Figure 3). Since saturation in copper uptake was observed for *F. convolvulus* only for aboveground plant parts [20], this could also provide an explanation for the observation of increased copper content in roots of *F. japonica*. Furthermore Lu et al. [50] demonstrated a higher concentration of Fe, Al, and Cu in roots compared to rhizomes, stems, and leaves in *F. sachalinensis*. This accumulation in roots of *Fallopia* spp. is consistent with both our hypothesis and literature where *F. japonica* was found to have a high potential to accumulate heavy metals under field conditions and elevated heavy metal concentrations [21–23,50]. Metal accumulation is linked to metal tolerance and is common in plants found in metalliferous habitats [51]. The higher metal tolerance in *F. japonica* found in this study might therefore be linked to this ability.

4 Materials and methods

4.1 Experimental Setup

The mesocosm experiment was set up on an open field located in Siebeldingen at the Julius Kühn-Institut (Federal Research Centre for Cultivated Plants), Rhineland-Palatinate, Germany (49.219643, 8.049299). Pots were established in April 2020 and the experiment lasted until September 2021 for two full growing seasons. In 2020, the average temperature was 11.9 °C and the total precipitation was 630 mm, while in 2021, the average temperature was 10.4 °C and the total precipitation was 814 mm [52].

In April 2020, 100 pots with a diameter of 63 cm were filled with 5 cm of gravel at the bottom as a root drainage and a mixture of 70 liters of sand originating from locally sourced Triassic sandstone (BVG, Albersweiler, Germany) and 20 liters of natural riparian soil. The riparian soil (topsoil 0–25 cm) including its natural microbial community was collected in March 2020 from a nearby unpolluted riparian area within the biosphere reserve Palatinate Forest (Dürrentalbach, 49.255048, 7.939021). Before mixing, the soil was sieved and visible plant parts were removed. While filling the pots with soil, 10 g of nitrogen fertilizer, N-P-K: 6-6-9 (ORGASAN, Cuxin DCM, Telgte, Germany) and 9 g of trace element fertilizer (Micromax Premium, Everris International, de Heerlen, The Netherlands) were added to each pot. The fertilization was repeated in the second growing season in April 2021. The final average soil pH was 7.5.

The pots were arranged in a two-factorial randomized block design (Figure 4). Plant species (*F. japonica* and *U. dioica*) and soil copper concentration were used as treatment factors. Five levels of $CuSO_4$ (Centrum Metal Odczynniki Chemiczne, Falenty, Poland) in 5 liters of tap water solution were applied and mixed with the soil. This compound was chosen since it is a common fungicide utilized in agriculture. The four nominal concentrations were 0, 90, 270, 810, and 2430 mg Cu kg^{-1} soil. Each treatment combination was replicated ten times.

Nominal copper concentrations were verified using inductively coupled plasma—optical emission spectrometry (ICP-OES) (Agilent 720 ICP OES, Agilent Technologies, Santa Clara, United States), following digestions with aqua regia [53,54] directly after test initiation (April 2020) and at its termination (August 2021). Measurements suggest adequate spiking with deviations relative to nominal concentrations not exceeding 15%, justifying the use of the latter throughout the present document (see Table 4). The full results of the ICP-OES measurements can be found in Table A2.

Plants were introduced into the pots in June 2020 as rhizomes with one aboveground shoot. Rhizomes of both species originated from a riparian zone at the river Queich in Landau (49.198357, 8.096627). This stand of *F. japonica* was selected since it is genetically pure without the hybrid influence of *F. sachalinensis* (F. Schmidt ex Maxim.) [55]. Rhizomes were washed and cut to approximately 5 cm, making sure that at least one node was included for regeneration. Four rhizomes of the invasive or the native species, respectively, were evenly distributed in each pot.

During the growing season, the plants were watered depending on the weather conditions. Under hot and dry conditions, each pot received four liters of tap water at a maximum rate of twice a week. After precipitation events no water was provided. Randomly growing other plant species were weeded every week to minimize their impact. During the first season, the test system equilibrated and plant-related measurements started in spring of the second

season. Pots without plant growth were excluded from analyses (see Table A1 for an overview of plant mortality in the experiment).

4.2 Plant parameter measurements

Plant parameters were measured bi-weekly covering ten timepoints between April and August 2021 (Table A3). These parameters included plant height, leaf number, and chlorophyll content. Plant height (cm) was defined as size from the soil surface to the extended tip of the highest stem using a measuring stick. The number of leaves were counted. Chlorophyll content, a proxy for photosynthetic activity [56], was measured using a SPAD-502 device (Konica Minolta, Tokyo, Japan) on three randomly chosen healthy leaves per plant. Measurements with the SPAD-502 produce relative values that are proportional to the amount of chlorophyll present in the leaf [57]. The average of these measurements for each pot entered further statistical analyses. In September 2021, plants were harvested individually, separating above- and belowground biomass (Table A4). All plant material was dried for 4 days at 60 °C. The dry biomass was then quantified using a KERN ADJ 200-4 scale (linearity ± 0.4 mg, Kern & Sohn, Balingen, Germany). In the subsequent statistical analyses, total biomass per pot separated into above- and belowground biomass was used.

4.3 Plant Copper Content

Dried plant material of aboveground parts (stems and leaves), roots, and rhizomes was ground into a powder separately. For each plant species and the three plant parts selected, five samples of each copper treatment were prepared, resulting in a total of 120 samples. Plant copper content was measured using ICP-OES (Agilent 720 ICP OES, Agilent Technologies, Santa Clara, United States). Measurement of copper was done at a wavelength of 327.395 nm using the same method as the soil copper measurement [53,54]. Prior to analyses, samples were digested in 10 mL aqua regia overnight and afterwards heated to 175 °C for 15 min in a microwave (Mars 5 System, CEM Corporation, Matthews, North Carolina, USA). The samples remained in the microwave at 175 °C for another hour. Aliquots were subsequently diluted to 1:10 or 1:20 with MiliQ water depending on the limits of quantification supporting a reliable quantification of copper. The measured copper content in the plant parts can be found in Table A5.

4.4 Data Analysis

R version 4.0.5 [58] was used for statistical analyses. Linear mixed-effects models (lmer) were created separately for the two plant species (*F. japonica* and *U. dioica*) using the package "lme4" [59]. The highest copper treatment (2430 mg kg^{-1} soil) was disregarded for further analysis because no plants survived (Table A1). The models for each dependent variable (above- and belowground final biomass, plant height, leaf number, chlorophyll content, plant copper content) used the copper levels (factor with the four levels "0", "90", "270", "810" mg kg^{-1}) as a fixed effect. In the models for plant height, leaf number, and chlorophyll content, time (factor with 10 time points over the growing season) and the interaction of time and copper treatment were included as additional fixed effects. In all models, the random effect "block" was used to account for the block design and possible variability introduced by the position on the experimental site. Additionally, the random effect "stem number", representing the total number of plant individuals found in each pot, was used to account for differences in growth caused by

the uneven number of plant individuals that survived until the start of measurement in 2021. Significance levels were calculated using the ANOVA command from the "car" package [60].

5 Conclusions

Overall, we found that soil copper contamination inhibited the growth in terms of leaf number, height, and biomass in both *Fallopia japonica* and *Urtica dioica* pointing to a higher sensitivity of *U. dioica* (significant effects starting with 270 mg copper kg^{-1}) compared to *F. japonica* (significant effects starting at 810 mg copper kg^{-1}). On the other hand, *F. japonica* contained far lower absolute concentrations of copper in its above- and belowground parts compared to *U. dioica* but was able to accumulate copper more effectively when exposed to it. In this way, *F. japonica* may gain a selective advantage against this commonly occurring native species in its invasive range and especially in polluted areas because of its higher tolerance to copper. Our study demonstrated that disturbances by agricultural activity, one major source of copper pollution, could have an impact across much wider distances than expected by facilitating plant invasion success of *F. japonica* in previously undisturbed areas.

Data Availability Statement

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files.

Conflicts of Interest

The authors declare no conflict of interest.

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Author Contributions

Conceptualization, D.S., M.B., K.R., and J.S.; methodology, D.S., M.B., J.G., J.J., K.R., V.R., J.S.; software, D.S.; validation, J.S., K.R., and M.B.; formal analysis, D.S. and J.S.; investigation, D.S., B.G., J.J., J.G., and R.F.; data curation, D.S.; writing—original draft preparation, D.S.; writing—review and editing, D.S., J.S., M.B., K.R., V.R., J.J., J.G., B.G., and R.F.; visualization, D.S.; supervision, J.S., K.R., and M.B.; funding acquisition, M.B. All authors have read and agreed to the published version of the manuscript.

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Table 1 Results of the lmer models for both *Fallopia japonica* and *Urtica dioica* performance parameters across the growing season of 2021. Chi-squared and corresponding *p*-values for all analyzed parameters are given. Treatment represents the effect of the copper treatment; time represents the effect of the time over the season when the parameter was recorded; and treatment * time represents the interaction between both parameters. Significant values below the level of 0.05 are printed in bold.

	Treatment		**Time**		**Treatment * Time**	
	Chi²	***p***	**Chi²**	***p***	**Chi²**	***p***
Fallopia japonica						
Plant height	77.37	**<0.001**	209.69	**<0.001**	3.89	0.273
Leaf number	48.82	**<0.001**	286.16	**<0.001**	21.35	**<0.001**
Chlorophyll content	40.49	**<0.001**	220.73	**<0.001**	8.65	**0.034**
Urtica dioica						
Plant height	147.29	**<0.001**	383.30	**<0.001**	21.14	**<0.001**
Leaf number	35.88	**<0.001**	354.69	**<0.001**	30.85	**<0.001**
Chlorophyll content	14.73	**0.002**	232.31	**<0.001**	7.01	0.072

Table 2 Results of the lmer models for both *Fallopia japonica* and *Urtica dioica* above- and belowground plant biomass collected at the end of the second growing season. Chi-squared and corresponding *p*-value for each analyzed parameter are given. Treatment represents the effect of the copper treatment. Significant values below the level of 0.05 are printed in bold.

	Treatment	
	Chi²	***p***
Fallopia japonica		
Aboveground biomass	34.92	**<0.001**
Belowground biomass	19.00	**<0.001**
Urtica dioica		
Aboveground biomass	4.17	0.244
Belowground biomass	6.59	0.086

Table 3 Results of the lmer models for both *Fallopia japonica* and *Urtica dioica* plant copper content separated by aboveground, root and rhizome samples collected at the end of the second growing season. Chi-squared and corresponding *p*-value for each analyzed parameter are given. Treatment represents the effect of the copper treatment. Significant values below the level of 0.05 are printed in bold.

	Treatment	
	Chi²	***p***
Fallopia japonica		
Aboveground samples	168.89	**<0.001**
Root samples	47.16	**<0.001**
Rhizome samples	44.57	**<0.001**
Urtica dioica		
Aboveground samples	143.79	**<0.001**
Root samples	41.56	**<0.001**
Rhizome samples	65.84	**<0.001**

Table 4 Total copper found in the soil in mg kg^{-1} in relation to the desired treatment. See Table A2 for the corresponding data.

Treatment [mg kg^{-1}] (April 2020)	**Initial Soil Copper Content [mg kg^{-1}] (April 2020)**	**Deviation from Treatment**	**Final Soil Copper Content [mg kg^{-1}] (August 2021)**	**Deviation from Treatment**
0	8.18 ± 1.35	-	4.99 ± 1.32	-
90	87.63 ± 11.62	−2.6%	93.63 ± 9.58	4.0%
270	238.99 ± 38.50	−11.5%	252.83 ± 35.89	−6.4%
810	781.64 ± 154.91	−3.5%	714.48 ± 85.42	−11.8%

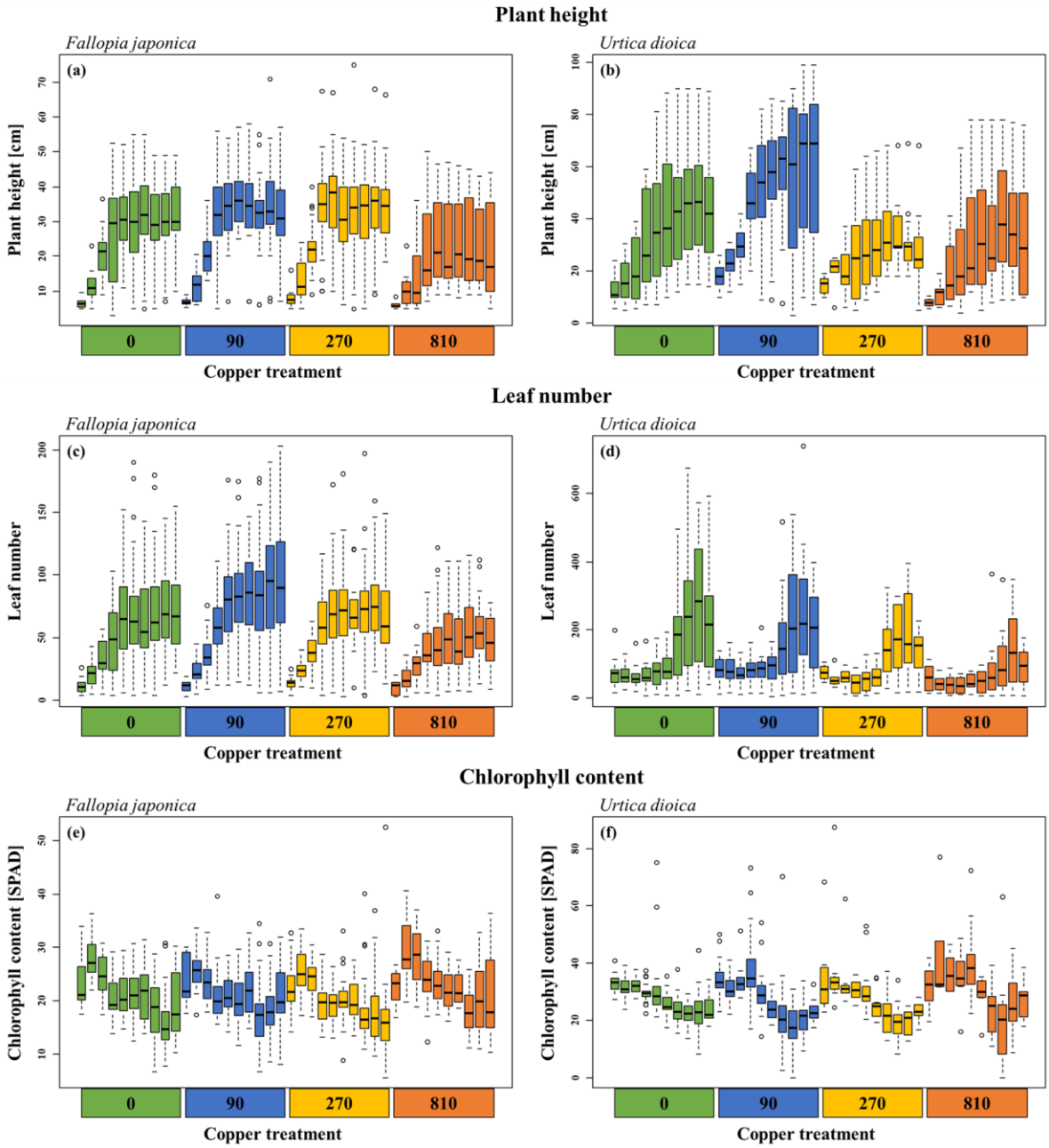

Fig. 1 Plant growth parameters of *Fallopia japonica* and *Urtica dioica* recorded bi-weekly across the growing season (n = 10 time points). Shown are boxplots with median values represented by the black line. Each color represents one copper treatment from control to 810 mg kg^{-1}. Within each treatment, the time in the season is shown from left to right. (a) Plant height of *Fallopia japonica*, (b) plant height of *Urtica dioica*, (c) leaf number of *Fallopia japonica*, (d) leaf number of *Urtica dioica*, (e) chlorophyll content of *Fallopia japonica*, (f) chlorophyll content of *Urtica dioica*.

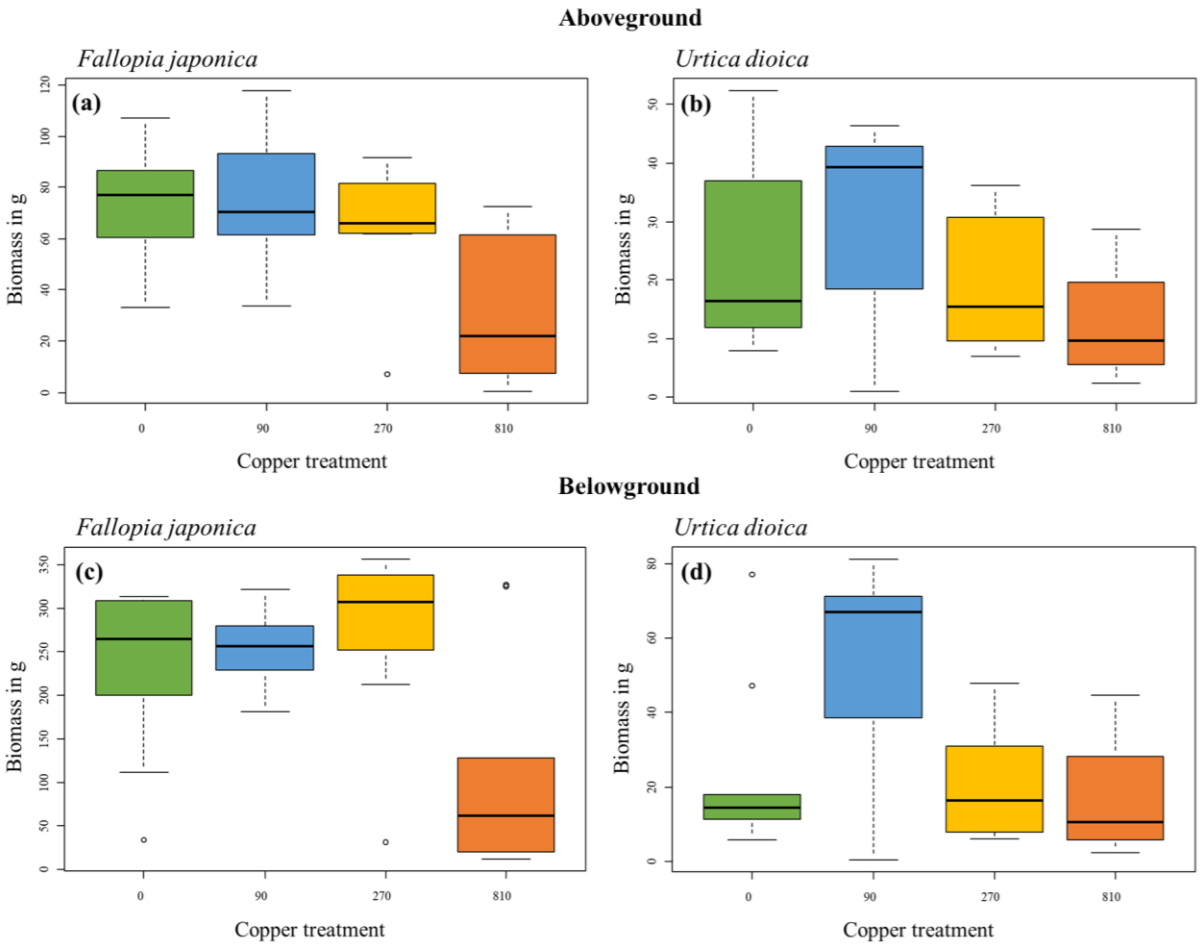

Fig. 2 Biomass of above- and belowground plant material of *Fallopia japonica* and *Urtica dioica* collected at the end of the growing season in each copper treatment (n = 10 replicates). Shown are boxplots with median values represented by the black line. Each color corresponds to one copper treatment from control to 810 mg kg^{-1} copper. (a) Aboveground biomass of *Fallopia japonica*, (b) aboveground biomass of *Urtica dioica*, (c) belowground biomass of *Fallopia japonica*, (d) belowground biomass of *Urtica dioica*.

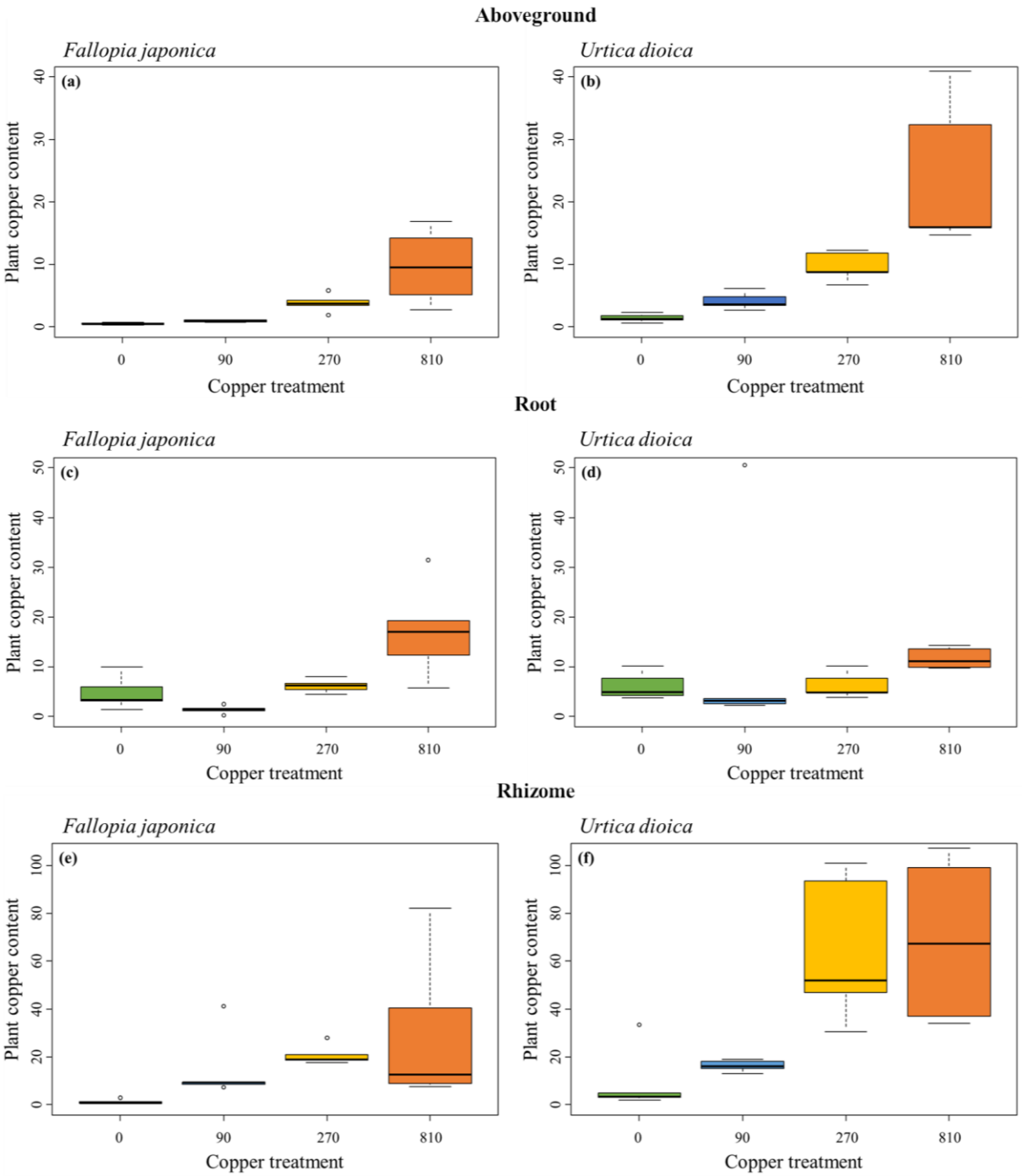

Fig. 3 Plant copper content in mg g^{-1} for each copper treatment (0 mg kg^{-1} in green, 90 mg kg^{-1} in blue, 270 mg kg^{-1} in yellow and 810 mg kg^{-1} in orange, n = 5) in *Fallopia japonica* and *Urtica dioica*. The y-axis is scaled to the same values for aboveground, root, and rhizome samples, respectively, but differs between the three sample types. Shown are boxplots with median values represented by the black line. (a) Aboveground copper content in *Fallopia japonica*, (b) aboveground copper content in *Urtica dioica*, (c) root copper content in *Fallopia japonica*, (d) root copper content in *Urtica dioica*, (e) rhizome copper content in *Fallopia japonica*, (f) rhizome copper content in *Urtica dioica*.

Fig. 4 Experimental setup of the experiment. General view of the pots arranged in a randomized block design and sample pictures of *Fallopia japonica* (left) and *Urtica dioica* (right) growing in the pots in spring 2021.

Appendix A

Table A1 Plant mortality of *Fallopia japonica* and *Urtica dioica* in each copper sulphate treatment. The percentage of pots without surviving plant individuals out of ten is noted for each treatment combination.

	Control	**90 mg kg^{-1}**	**270 mg kg^{-1}**	**810 mg kg^{-1}**	**2430 mg kg^{-1}**
Fallopia japonica	10%	0%	0%	10%	100%
Urtica dioica	10%	30%	50%	60%	100%

Supplementary Materials

The supplemental tables A2 to A5 are provided as digital resources in the attached CD disk. Table A1: Plant mortality of *Fallopia japonica* and *Urtica dioica*. Table A2: Copper content of soil. Table A3: Plant growth measurements. Table A4: Plant biomass measurement. Table A5: Copper content of plant parts.

Amendment III

Copper effects on riparian soil communities and biological activity influenced by a native and invasive plant species

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Abstract

Riparian ecosystems are highly affected by anthropogenic stressors such as heavy metals and invasive species. Although copper is an essential micronutrient, higher concentrations – as a result of its intensive use as a fungicide in agriculture - are toxic for plants, soil microorganisms and soil invertebrates. Invasive alien plants are a major cause of biodiversity decline in native communities. By modifying resources and changing ecosystem functions they can also affect belowground systems such as microbial communities and soil invertebrates, altering soil functions such as litter degradation. In a mesocosm experiment, we investigated the impact of copper pollution in combination with plant invasion by *Fallopia japonica* on riparian soil communities. We a focused on the microbial biomass, the abundance of invertebrates, biological activity and fungal diversity and used the native *Urtica dioica* as a native reference plant. We performed the mesocosm experiment with a gradient from 0 to 2430 mg kg^{-1} of soil copper. We hypothesized that (i) the soil microbial biomass, the abundance of soil invertebrates, the soil biological activity and the fungal diversity are lower in *F. japonica* than in the native *U. dioica*. We further expect that (ii) higher copper concentrations reduce the soil microbial biomass, the abundance of soil invertebrates, soil biological activity and fungal diversity for both plant species. Finally, we expect (iii) fungi to be more affected than bacteria due to use of copper as a fungicide. We found that the fungal and bacterial biomass as well as the soil biological activity was lower under the invasive *F. japonica*, while no impact on the abundance of soil invertebrates was found. Increasing copper concentrations had variable effects depending on the plant species. In *F. japonica* it reduced Collembola abundance but increased fungal biomass. In *U. dioica* it shifted the fungi:bacteria ratio towards a higher proportion of bacteria. While fungal diversity was negatively impacted by the presence of *F. japonica,* copper contamination led to an increase in fungal diversity at intermediate levels under both plants instead. Despite this, the soil biological activity was negatively affected by increasing copper contamination in both plant species. Overall, both stressors lower especially the soil biological activity. This indicates that while the abundance of fungi may not be affected, ecosystem functioning may be reduced under higher copper concentrations by shifting the community composition. This may have important implications for restoration efforts in often highly impacted riparian ecosystems.

Keywords: Acari; bacteria, bait lamina sticks, Collembola; *Fallopia japonica*; fungi, *Urtica dioica*

1 Introduction

Copper is used as a fungicide in agriculture since the 19th century (Johnson 1935) and accumulates in soils (Wightwick et al. 2008), possibly reaching concentrations of up to 3000 mg kg^{-1} (Brunetto et al. 2016). From these contaminated soils copper can be transported to aquatic ecosystems (Ripke et al. 2001) and accumulate in sediments (Han et al. 2001). This copper contamination can then again be transferred to riparian ecosystems, for example during flooding, impacting the ecosystem (Schulz et al. 2015).

Copper is an essential micronutrient for virtually all organisms, involved in many enzymes as well as electron transport and redox reactions (Cervantes & Gutierrez-Corona 1994, Husak 2015). However higher concentrations are detrimental or even toxic for plants (Kuhns & Sydnor 1976; Lombardi & Sebastiani 2005; Schmitz et al. 2023) and microorganisms. The main pathways of microorganism toxicity are changes to DNA, enzymes and membranes by creating radicals in the cell (Cervantes & Gutierrez-Corona 1994, Husak 2015). In general, bacteria are reported to be less impacted by copper pollution than fungi (Flemming & Trevors 1989). However, Wang et al. (2018) found that soil copper pollution of 1.60 mmol kg^{-1} similarly reduced the abundance of both bacteria and fungi. Huysman et al. (1994) even reported a higher sensitivity of bacteria to copper pollution, where copper concentrations of 10 µg g^{-1} caused a 1000-fold increase in the number of Cu-resistant bacteria while not affecting total microbial biomass. Besides microorganisms, soil invertebrates are also impacted by copper pollution. For example, the earthworm species *Octolasium cyaneum* was found to be highly sensitive to copper pollutions starting at 40 ppm, while oribatid mites were not significantly impacted by copper conditions of 200 ppm in the same experiment (Streit 1984). Further, for three other invertebrate species (the earthworm *Eisenia andrei*, the potworm *Enchytraeus crypticus* and the springtail *Folsomia candida*), the EC_{50} for reproduction were between 130 and 190 mg Cu kg^{-1} (Caetano et al. 2016). However, other soil properties such as pH value also impact the toxicity of copper to invertebrate soil organisms (Criel et al. 2008).

Another stressor that is often found in riparian ecosystems is plant invasion (Schirmel et al. 2016). Invasive alien plants are a major cause of biodiversity decline in native communities, which may even negatively impact the entire ecosystem (Hooper et al. 2012). Invasive plants can also affect belowground ecosystems by modifying resources (Crooks 2002; Fogarty and Facelli 1999; Standish et al. 2001) and changing ecosystem functions (Ehrenfeld 2003, Liao et al. 2008). As a result, soil microbial communities, including archaea, bacteria, fungi, can be affected by plant invasion. This can be changes in biomass (Liao and Boutton 2008) or community structure of soil microorganisms (Si et al. 2013; Wolfe & Klironomos 2005). This can in turn impact major soil functions such as litter degradation as well as the soil microbial activity in general (Liao et al. 2007; Vilà et al. 2011; Pehle & Schirmel 2015). Furthermore, like for the microbial communities, plant invasions can influence the soil invertebrate fauna (Abgrall et al. 2019; Belnap et al. 2005; Biederman and Boutton 2009), which are necessary for soil functions like decomposition (Deyn et al. 2003; Tresch et al. 2019). For example, Collembola and Acari, often regarded as the most important and abundant arthropod groups in soil (Behan-Pelletier 2003; Deharveng 1996; Petersen and Luxton 1982) are known to respond to vegetation changes. Thus, these groups can be affected by plant invasions (Rusterholz et al. 2014; Salamon et al. 2004; Wissuwa et al. 2012). However, the impact of plant invasion depend on the habitat type (Abgrall et al. 2019) since these invertebrates also depend on abiotic soil properties (Bedano 2004; Cassagne et al. 2003).

In European riparian ecosystems, a common native species is the Common nettle (*Urtica dioica* L., Urticaceae), a rhizomatous, perennial herb often found to be dominating in nitrogen-rich soils (Taylor 2009). Its stands are often invaded by the Asian knotweed (*Fallopia japonica* (Houtt.) Ronse Decr., Polygonaceae), one of the most wide-spread invasive plants in Central Europe. It is a perennial, rhizome-forming species which can produce high above and below ground biomass very quickly (Beerling et al. 1994). *Fallopia japonica* can outcompete native plants for light and nutrients by creating dense populations and the plant can also alter soil nitrogen (Aguilera 2010), giving it another advantage over native species. *Fallopia japonica* can also impact other parts of the ecosystem. For example, via allelopathic effects (Murrell et al. 2011; Parepa and Bossdorf 2016) it can impact the soil food web structure (Abgrall et al. 2018) and decrease the abundance and diversity of soil fungi, such as mycorrhizal fungi (Schmitz et al. 2023b; Zubek et al. 2016).

In this study we wanted to investigate the impact of copper pollution in combination with plant invasion by *F. japonica* on soil communities. Especially we investigated the biomass of soil microbes, the abundance of soil invertebrates, the soil biological activity and the fungal diversity. Therefore, we conducted a mesocosm experiment with both *F. japonica* and *U. dioica* under field conditions in a randomized block design with five soil copper concentrations (0 to 2,430 mg kg^{-1} soil). We expect that (i) the soil microbial biomass, the abundance of soil invertebrates, soil biological activity and the fungal diversity is lower in the invasive *Fallopia japonica* than in the native *Urtica dioica*. We further hypothesised that (ii) higher copper concentrations reduce the soil microbial biomass, the abundance of soil invertebrates, soil biological activity and fungal diversity for both plant species. For the microbial biomass we expect (iii) fungi to be more affected than bacteria due to use of copper as a fungicide.

2 Material and Methods

2.1 Experimental setup

The mesocosm experiment was set up on an open field located in Siebeldingen at the Julius Kühn-Institut (Federal Research Centre for Cultivated Plants), Rhineland-Palatinate, Germany (49.219643, 8.049299). The experiment ran from April 2020 until September 2021. The first year was used as an establishment period and measurements took place in the second year.

The experiment contained 100 pots with a diameter of 63 cm filled with a mixture of 70 litres of sand and 20 litres of natural riparian soil collected from a nearby unpolluted riparian area (Dürrentalbach, 49.255048, 7.939021). Each pot was fertilized with 10 g of nitrogen fertiliser, N-P-K: 6-6-9 (ORGASAN, Cuxin DCM, Telgte, Germany) and 9 g of trace element fertilizer (Micromax Premium, Everris International, de Heerlen, Netherlands) once in each year. Plants were planted as rhizomes with one aboveground shoot. Rhizomes of both species originated from a riparian zone at the river Queich in Landau (49.198357, 8.096627). Rhizomes were washed and cut to approximately 5 cm, making sure that at least one node was included for regeneration. Four rhizomes of one of the two species were evenly distributed in each pot. The pots were arranged in a two-factorial randomized block design with plant species (*F. japonica* and *U. dioica*) and soil copper concentration being used as treatment factors. Five levels of $CuSO_4$ (Centrum Metal Odczynniki Chemiczne, Falenty, Poland) in 5 litres of tap water solution were mixed with the soil. The four nominal concentrations were 0, 90, 270, 810 and 2,430 mg kg^{-1}. Each treatment combination was replicated ten times. For more details about the experimental setup see Schmitz et al. (2023b).

2.2 Microbial biomass

For quantification of total fungal and bacterial biomass, soil samples collected in September 2021 were freeze-dried (Alpha 1–2 LD plus, Christ, Osterode am Harz, Germany) and up to 500 mg was prepared (ADJ 200-4, Kern & Sohn, Balingen, Germany). Total DNA was isolated using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). The manufacturer's protocol was modified to increase DNA yield as follows: after homogenisation, the samples were incubated at -80 °C for 10 min, then at 65 °C for 5 min, before being centrifuged, with final elution in 50 µL DES. qPCR reactions were performed in triplicates including standards and negative controls in each run on a Mastercycler® ep realplex S (Eppendorf, Hamburg, Germany). The internal transcribed spacer region 2 (ITS2) including 5.8S of the nuc-rDNA was selected as well-established target for Fungi and the 16S rRNA gene for Bacteria (Manerkar et al. 2008). In each run, a standard curve was generated from 4 known concentrations of the target sequence using the common soil organisms *Trichoderma viride* for Fungi (374 bp; CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTC AACCCTCGAACCCCTCCGGGGGTCGGCGTTGGGGATCGGCCCTTTACGGGGCCGGCCCCGAAATACAG TGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAGCGCGGCGCGTCCA CAGCCGTTAAACACCCCAAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAA GCATATCAATAAGCGGAGGAAAAGAAACCA; Rubin 2010) and *Escherichia coli* for Bacteria (470 bp; AAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACG GTAACAGGAAGAAGCTTGCTTCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCC TGATGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAG GCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT ACGGGACGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGA AGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCAT TGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAACTCCGTG; Manerkar et al. 2008) (Eurofins Genomics, Ebersberg) including 7 bp end degradation buffer. DNA extracts were diluted 1:50 and 1:200 for Fungi and Bacteria, respectively. Each reaction was performed using clear 0.2-mL 8-tube strips covered with clear flat optical 8-cap strips (Sarstedt, Nümbrecht, Germany) in a total volume of 10 µL, containing 5 µL PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 2 µL diluted template, 1.5 µL ddH2O and 0.75 µL of each primer (10 pmol µL-1). The following primers were used: ITS3F/ITS4 (White et al. 1990) for fungi, E8F/E533R (Baker et al. 2003) for bacteria, respectively. The PCR protocol employed an initial activation of 2 min at 50 °C and 2 min at 95 °C, followed by 40 cycles of 95 °C 15s, 55 °C 15s, 72 °C 1 min (Feckler et al. 2017). Raw data was analysed using Mastercycler ep Realplex version 2.2 (Eppendorf, Hamburg, Germany). The amount SYBR [copies] of the triplicates was averaged for every sample. Final sample DNA concentration was calculated by comparing the sample Cycle threshold CT value to the standard curve using the formula $1/eC$t1/eCt.

2.3 Soil invertebrates

Berlese funnels were used for sampling the soil invertebrate community. 200 ml of fresh soil per pot from collected bulk soil was filled into a Berlese funnel for one week in September 2021. A constant light source was attached to the top of the funnel to induce downward movement for the soil organisms. Organisms were collected at the

bottom of the funnel in vials containing 20 ml 70% ethanol and the collected invertebrates were identified to Class level. By far the most individual-rich groups relevant to the belowground ecosystem were Nematoda (56.8 % of all individuals) and Collembola (12.2 %; see Supplemental 1), and we focussed on these two groups in our analyses.

2.4 Soil biological activity

The soil biological activity was measured using the bait-lamina method. The method measures the soil biological activity using the degradation of cellulose in the soil (Kratz 1998). To measure the cellulose degradation, PVC sticks containing small holes along the length of the stick (16 cm x 0,5 cm x 0,1 cm; terra protecta, Berlin, Germany; from now on referred to as bait-lamina sticks) were placed in 60 pots. Within each pot, five sticks were arranged in a circle, resulting in a total of 300 bait-lamina sticks. The bait-lamina sticks contained a mixture of 13 g Cellulose (Carl Roth, Karlsruhe, Germany), 3 g Agar (Rapunzel, Legau, Germany), 2 g Bentonite (Carl Roth, Karlsruhe, Germany) and 2 g wheat bran (DM, Karlsruhe, Germany). The mixture was dissolved in 20 ml tap water and applied to the holes in the bait-lamina sticks until they were filled. This application was repeated twice afterwards to ensure a consistent filling of the bait-lamina sticks. Afterwards, the sticks were dried at room temperature for 72 h. The sticks were placed in the pots in July 2021 and remained in the pots for 13 days. Five pots for each plant species and copper concentration combination were selected, except for the highest copper concentration since there were no viable plants in this concentration. After removing each stick, the cellulose mixture was checked, and the state was recorded. The percentage of degraded holes in the bait sticks was used as an indicator for the soil biological activity (see Figure S1 for an example of degraded holes in a bait lamina stick).

2.5 Soil and root fungal diversity and ecological groups

Soil and root samples from the pots were collected in September 2021. In total, 72 soil and 38 root samples equally distributed across all copper treatment levels were collected and dried at 50 °C for 48 h. The dried samples were sent to the external company Sinsoma GmbH (Kirchgasse 6, Voels, Austria) for DNA extraction and analysis. DNA extraction was performed using the Magattract Power Soil Kit (Qiagen, Hilden, Germany) based on manufacturer protocols. The primers for the PCR were ITS3tagmix1-5 and ITS4ngs. The PCR was performed using the protocol described in Tedersoo et al. (2015). Information for the bioinformatic analysis of the samples can be found in supplementary material S5. The provided table containing OTU information for 1485 OTUs was used for subsequent analysis.

Based on the genera provided in the OTU table, functional traits were assigned using the FungalTraits database (Põlme et al. 2020) as it provides a more reliable fungal functional assignment than other databases regarding both quality and quantity (Tanunchai et al. 2023). The obtained information in the column primary lifestyle was then further simplified into four main categories: endophytes (END), mycorrhiza (MYC), parasites (PAR) and saprobes (SAP). We disregarded 381 OTUs with not provided primary lifestyle in FungalTraits as well as six lichen species, resulting in a total of 1098 OTUs with assigned functional traits. Further details about this approach can be found in Schmitz et al. 2023a.

2.6 Data analysis

The data was analysed using R version 4.0.5 for the statistical analysis. Linear mixed effects models (lmer) were created using the package “lme4” (Bates et al. 2015). First, models investigating the effect of the plant species (*F.*

japonica and *U. dioica*) and the interaction between the copper treatment and the plant species were created for all dependent variables. In a second step, models investigating the effect of the copper treatment on the dependent variables were created separately for each plant species. Outliers for each model were removed with the romr.fnc function of the "LMERConvenienceFunctions" package. We excluded outliers with residual at a distance greater than 2.5 standard deviations from the residuals mean.

The dependent variables were the abundance of soil invertebrates (Nematoda and Collembola), the soil biological activity (measured as percentage of degradation of bait lamina sticks) and the microbial biomass of bulk and rhizosphere soil measured by the qPCR approach. Here, we investigated the fungal and bacterial biomass measured as the geometric mean of the PCR forming units (PFU) obtained by the analysis as described in 2.2 as well as the fungi:bacteria ratio in both soil types. The data was log transformed to account for non-normal distribution and the random effect "block" was included to account for the variability because of the position on the experimental site. Significance levels were calculated using the ANOVA command from the "car" package (Fox & Weisberg 2019).

The analysis of the fungal functional groups based on the metabarcoding analysis used the glmmTMB package Version 1.1.9 (Brooks et al. 2017). We created generalized linear mixed models using a negative binomial distribution to account for count data (Lindén & Mäntyniemi 2011). Models for soil and root fungi under the presence of *F. japonica* compared to *U. dioica* were created seperately. We used the total OTU richness and the OTU richness in each functional group (endophytes, mycorrhiza, parasites and saprobes) as response variables in both soil and root samples. The explanatory variables in the models were the plant species and the copper treatment of the soil. The block the samples were obtained from was included as a random effect in all models to account for the randomized block design.

3 Results

3.1 Microbial biomass, abundance of soil invertebrates and soil biological activity

In the bulk soil, the presence of the invasive plant *Fallopia japonica* significantly altered the fungal and microbial biomass compared to *Urtica dioica*. Both decreased by more than 15% under *F. japonica* (Table 1, Figure 1, 2). In the rhizosphere soil, the biomass of both fungi and bacteria as well as the fungi:bacteria ratio was significantly affected by *F. japonica*. The biomass was lowered by more than 20% (fungi) and 40% (bacteria) respectively. The fungi:bacteria ratio was skewed much more strongly towards fungi compared to *U. dioica* (Table 1, Figure 3, 4). While soil biological activity was decreased by about 6 % under *F. japonica*, the abundance of soil invertebrates as well as the fungi:bacteria ratio was not significantly affected (Table 1). The interaction between plant and copper treatment was only significant for three of the response variables. The soil biological activity ($Chi^2 = 99.58$, $p < 0.001$) and the fungal biomass for both the bulk soil ($Chi^2 = 17.29$, $p = 0.008$) as well as the rhizosphere soil ($Chi^2 = 19.73$, $p = 0.003$). The biological activity decreased more strongly with increasing copper concentration under *F. japonica* compared to *U. dioica* (Figure 5). For both *F. japonica* bulk and rhizosphere soil, copper concentration led to a stronger decrease of fungal biomass in the 90 mg kg^{-1} treatment than in *U. dioica* soil.

For *F. japonica*, the copper treatment had a significant effect on the abundance of Collembola (Table 2, Figure 6). The abundance of Collembola increased under the lower copper treatments (90 and 270 mg kg^{-1}) but caused a decrease in the highest copper treatment of 810 mg kg^{-1}. Soil biological activity decreased with increasing copper

concentration in both the *F. japonica* and *U. dioica* pots (Table 2, Figure 5). Under *F. japonica*, the fungal biomass in the bulk soil only decreased for the first copper treatment but increased at higher copper treatments starting at 270 mg kg^{-1} (Table 2, Figure 1). Under *U. dioica*, the only other variable affected by the copper treatment was the fungi:bacteria ratio in the bulk soil. Here, the ratio of fungi to bacteria decreased at the first copper treatment but increased with later treatments (Table 2, Figure 7).

3.2 Soil and root associated fungi

The average number of OTUs in the soil samples was about 30% higher under *U. dioica* compared to *F. japonica* pots. Few OTUs for endophytes and mycorrhiza have been detected in both root and soil samples, the majority of OTUs was found in the saprobic group (Table 3). In the soil samples, presence of *F. japonica* significantly impacted the total OTU richness and the OTU richness of parasites compared to *U. dioica*. Under *F. japonica*, the copper treatment significantly affected the overall OTU richness as well as that of parasites and saprobes (Table 4a). The OTU richness was highest at the intermediate copper treatment levels of 270 mg kg^{-1} (Figure 8). The copper treatment under *U. dioica* only affected the total OTU richness and the richness of saprobes (Table 4b). Here, OTU richness was lower at the intermediate copper treatment levels compared to both the next lower and next higher treatment (Figure 9).

In the root samples, OTU richness was significantly affected by the presence of *F. japonica* for the overall OTU richness, mycorrhiza and saprobes (Table 3). The OTU richness was higher under *U. dioica* compared to *F. japonica* in most groups, except for parasites (Table 3). In the root samples, the copper treatment only affected the parasitic fungi under *U. dioica* (Table 4b), where higher OTU richness was found at the highest copper treatment level (810 mg kg^{-1}) (Figure 9).

4 Discussion

4.1 Impact of *Fallopia japonica* on soil communities and biological activity

As hypothesised, we found lower microbial biomass in invasive *F. japonica* pots than in native *U. dioica*. In the bulk soil, *F. japonica* drastically reduced both the fungal and the bacterial biomass. Our results support findings of Stefanowicz et al. (2021), who also found a decrease in both bacterial and fungal biomass under *F. japonica*, *Fallopia japonica* is known to produce secondary metabolites such as phenolic compounds, that affect the microbial community via root exudation (Bardon et al. 2016, 2017; Stefanowicz et al. 2021, 2022). This is in line with the Novel weapon hypothesis, stating that many invasive plants are able to use allelopathic compounds to displace native species (Kalisz et al. 2021). Another example for this is *Centaurea maculosa,* which uses phytotoxins to supress growth and germination in native species (Bais et al. 2003). In general, fungal and bacterial communities are known to be influenced by plant invasion (e.g. higher biomass under woody plant invasion; Liao and Boutton 2008). In the rhizosphere soil, only the bacterial biomass was reduced which caused the fungi:bacteria ratio to be shifted towards higher proportion of fungi. Surprisingly, the biomass of fungi was not affected in the rhizosphere. As described in Schmitz et al. (accepted), only certain fungi seem to be able to associate with *F. japonica* directly. Their biomass might increase without competition from other soil microbes and therefore the total biomass of fungi might remain similar compared to *Urtica dioica*.

As expected, the soil biological activity was lower under *F. japonica* compared to *U. dioica*. Soil biological activity, and especially decomposition, largely depends on the occurrence and biomass of fungi (Žifčáková et al. 2016; Xu

et al. 2019). The reduced soil biological activity is thus most likely a consequence of the lower microbial biomass in the bulk soil of the invasive species. Stefanowicz et al. (2016) also showed a negative effect of *F. japonica* on soil microbial activity.

In contrast to our hypothesis, *Fallopia japonica* presence did not affect the abundance of soil invertebrates compared to *U. dioica*. This was unexpected, because of frequently reported impacts of plant invasions on the soil fauna (Belnap et al. 2005; Biederman and Boutton 2009; Abgrall et al. 2019). For example, it has been found that Collembola respond to changes in the vegetation, including plant invasion (Salamon et al. 2004; Wissuwa et al. 2012). The absence of an impact in our study might be due to the fact that the effect of invasive plants on the soil fauna depends strongly on the habitat type (Abgrall et al. 2019) because soil invertebrates are also influenced by abiotic soil properties (Cassagne et al. 2003; Bedano 2004). Additionally, the soil fauna community in this study was likely not representative of a natural community in the field. While the abundance of soil arthropods did not differ, a potential study of the diversity might reveal further differences between the communities found under each plant.

In the soil, the presence of *F. japonica* impacted the OTU richness of fungi in this study for the total number of OTUs and for parasites. This corresponds with previous studies that have shown that invasive plants such as *F. japonica* can influence fungal diversity (Wolfe and Klironomos 2005; Si et al. 2013). Additionally, in the roots, *F. japonica* had significantly reduced OTU richness in the total number of OTUs as well as mycorrhiza and saprobes. This confirms observations from a previous field study that showed reduced numbers of root assoicated fungi in *F. japonica* (Schmitz et al. 2023a). Especially regarding mycorrhiza, this might be due to incompatabilities between the invasive plant and local fungi (Callaway et al. 2011). Additionally, the secondary metabolites produced by *F. japonica* can negatively impact soil fungi (Garnica et al. 2022). In this study, few endophytic or mycorrhizal species were detected in the samples, which might further amplify this effect. The most abundant group of fungi in this study, saprobic species, was also reduced under *F. japonica*. Since these fungi are responsible for processes such as litter degradation, this indicates that only a subset of species is able to use the invasive plant as a substrate. This has previously been found by Mincheva et al. (2014). *F. japonica* litter was found to be a low-quality substrate and had a different fungal composition compared to litter from native sites. In the wild, this could significantly affect ecosystem processes in areas invaded by *F. japonica* by reducing the diversity of fungal species.

4.2 Impact of copper contamination on soil communities associated with different plant species

Copper contamination affected Collembola abundance positively at lower concentrations and negatively at higher concentrations under *F. japonica*. This is in line with our hypothesis that higher copper concentrations negatively impact the abundance of soil arthropods. However, unexpectedly intermediate concentrations seem to increase the abundances. In other studies, for instance Filser et al. (2000) also found that many collembolan species tended to be more abundant in the Cu-enriched soil of up to 240 mg kg^{-1}. At higher concentrations of more than 1000 mg kg^{-1} the abundance of Collembola was reduced in forests impacted by a copper smelter (Haimi & Siira-Pietikäinen 1996; Kuznetsova 2009). This pattern has also been observed for other heavy metals such as Zink (Strojan 1978).

Contrary to our expectations that increasing copper contamination causes a loss of biomass in fungi and bacteria, the bulk soil fungal biomass increased under higher copper treatment levels and only decreased at lower concentrations under *F. japonica.* This is unexpected since copper is commonly used as a fungicide and is highly toxic to fungi and should therefore also be more susceptible to copper toxicity than bacteria, as we hypothesized.

A possible reason for this finding might be that fungi that are more resistant to copper pollution are able to produce higher biomass due to competitive advantages against fungi species that are less resistant to heavy metals and therefore are no longer able to survive. Such heavy metal-resistant fungi species have previously been found (Iram et al. 2015; Tam 1995). While only affecting the fungal biomass significantly in the bulk soil, copper contamination affected the fungal biomass in both bulk and rhizosphere soil. The effect of copper also differed between *F. japonica* and *U. dioica*. The decrease in biomass in the lowest treatment (90 mg kg^{-1}) was higher in *F. japonica* compared to *U. dioica* for both soil types. This indicates that the fungal community associated with *U. dioica* is more resistant to disturbance compared to *F. japonica*. This might be because the presence of *F. japonica* itself is already detrimental to microbial communities (see above), while *U. dioica* is not negatively affecting the microbial communities. As for the bacterial biomass, the lack of effect of copper might be due to the higher tolerance against copper than fungi (Flemming & Trevors 1989).

Under *U. dioica*, the bulk soil fungi:bacteria ratio decreased under lower concentrations but increased at higher copper concentrations. This indicates that fungi are more abundant at lower copper concentrations while bacteria are dominant at higher ones, however the abundance of bacteria and fungi itself did not differ significantly. While this is in line with our hypothesis that fungi are more affected by higher copper concentrations, unlike the findings for *F. japonica*, the lack of a direct negative impact of copper contamination on soil microbes is surprising. The possible reasons as to why these direct impacts for fungi are not present under *U. dioica* are likely similar to those under *F. japonica*. Biomass of soil fungi is likely made up of different species under high and low copper contamination, leading to no visible pattern. For bacteria the reason might again be their generally higher tolerance to copper. The changes in the ratio of fungi to bacteria might be because fungi that directly interact with the plant are found more commonly at low copper concentrations and are able to suppress other groups of microorganisms and dominate the microbial community (Murali et al. 2021). However, higher concentrations that are toxic to fungi reduce the abundance of such fungal species and benefit more tolerant bacterial groups instead, shifting the fungi:bacteria ratio. The presence of copper resistant bacteria has previously been found in the soil and even in the rhizosphere (Chen et al. 2005; He et al. 2010; Kools et al., 2008).

As we expected, the biological activity decreased more with copper concentrations under both *F. japonica* and *U. dioica*. The decrease in soil activity was more severe under *F. japonica* compared to *U. dioica*. The reason for this is likely that F. japonica suppresses soil activity as described above which is compounded by the negative impact copper has on soil microorganisms. Since these soil microbes are the main driver of soil activity, a loss in abundance of especially fungi in the bulk soil is expected to cause a reduction in the soil activity. This is in line with other studies that also found a reduction of soil activity in response to heavy metal pollution (Hattori 1992; Nwuche & Ugoji 2008). The increase of fungal biomass in the bulk soil under *F. japonica* at high copper contamination did not translate to a increase in soil activity, indicating that the fungal species present at high copper concentrations are not able to fulfil the same function as those at low concentrations.

The OTU richness of fungi was affected by the copper treatment in the soil. Significant effects were observed for the samples from both *F. japonica* and *U. dioica* pots. In *F. japonica*, the total OTU richness as well as richness of parasites and saprobes were lower at the low treatment levels (0 and 90 mg kg^{-1}) compared to intermediate levels. In *F. japonica* especially, the 270 mg kg^{-1} treatment showed a very high OTU richness compared to the other copper treatments (Figure 8). This indicates that the initial application of copper will strongly impact the

fungal community. Since copper is widely used as a fungicide, the decrease of fungal diversity at higher copper treatments is expected (Johnson 1935). However, contrary to expectations, at higher concentrations OTU richness and abundance increased in this study. This could be due to more metal resistant fungi being able to establish when the initially dominant species are reduced by copper stress. This has previously been found to be a major driver of adaptation to copper stress in metal rich environments (Babich & Stotzky 1985, Gadd 1993). While the diversity and abundance in the soil seems to peak at intermediate treatments, the microbial activity is highest when no copper is added to the pots. This indicates that while the fungi might be able to subsist, essential soil functions such as degradation are lost at high copper concentrations. Additionally, Chu et al. 2010 found that addition of organic material increases the abundance of soil fungi regardless of soil copper contamination. This could be another reason that mainly saprobic and parasitic species are present at higher copper treatments. Other studies have also shown that heavy metal pollution of up to 400 mg kg^{-1} can have positive impact on fungal growth (Morkunas et al. 2018). In the root samples, only the parasitic fungi were significantly affected by copper pollution. For *F. japonica*, OTU richness was highest at the high and low treatment level, while the OTU richness in *U. dioica* increased with increasing copper treatment. A metal tolerating plant such as *F. japonica* (Schmitz et al. 2023b) may be able to use intermediate levels of copper stress to increase defences against biotic stressors such as parasitic fungi. Morkunas et al. 2018 found in a review that plant defences against biotic and abiotic stressors may interact positively or negatively and abiotic stressor may be used against parasites or herbivores. However, at very high heavy metal contaminations, this might no longer be sustainable. The high diversity of root associated parasites in *F. japonica* contradicts the enemy release hypothesis, as it seems that the native *U. dioica* is better able to defend against parasitic fungi without the added copper stress. This further indicates that copper pollution can have cascading effects in the field by negatively impacting the native plant population.

5 Conclusion

We found that as expected, fungal and bacterial biomass as well as microbial activity are lower under the invasive *Fallopia japonica* compared to *Urtica dioica*. However, no impact on the soil arthropod abundance has been observed, which might be due to the nature of the habitat in our study.

Increasing copper contamination had variable effects depending on the plant species in our study. In *F. japonica* it reduced soil arthropod (Collembola) abundance but increased fungal biomass. In *U. dioica* higher copper contamination shifted the fungi:bacteria ratio towards a higher proportion of bacteria. Only the microbial activity was negatively affected by copper contamination in both plant species.

Overall, it seems that plant invasion lowers the soil microbial activity which is further intensified by copper contamination even when microbial biomass and OTU richness itself seems to not always be affected negatively by copper in this study. This indicates that while the abundance of fungi may not decrease, ecosystem functions are lost under higher copper concentrations, which indicates a shift in the community composition. This result should be further investigated. The loss of soil functions is detrimental to the ecosystem and could cause loss of major soil functions such as litter degradation over time. In combination with the better performance of *F. japonica* under copper stress (Schmitz et al. 2023b), this could have compounding impacts when dealing with plant invasion. As the microbial community is already impacted in sites with heavy metal contamination, these sites are also more easily invaded by *F. japonica* which further impacts the microbial community in the soil.

Data availability statement

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Table 1 Comparison of the effect of presence of the plants *Fallopia japonica* and *Urtica dioica* on soil biota. The mean and standard deviation for each variable is presented alongside the results of the corresponding linear mixed model (Chi^2 and p value). Values significant at the 0.05 threshold are given in bold.

Dependent variable	***F. japonica***	***U. dioica***	**Chi^2**	**P**
Nematoda	4.60 ± 6.08	7.50 ± 10.21	0.03	0.871
Collembola	1.60 ± 3.75	1.00 ± 2.29	0.28	0.600
Soil biological activity (%)	68.44 ± 16.78	72.50 % ± 19.10	5.83	**0.016**
Fungal biomass (bulk)	7544.47 ± 12385.52	8963.48 ± 9493.47	7.65	**0.006**
Bacterial biomass (bulk)	772448.9 ± 729408.5	956866.7 ± 618937.6	5.38	**0.02**
Fungi:bacteria ratio (bulk)	0.55 ± 1.29	0.01 ± 0.01	2.37	0.123
Fungal biomass (rhizosphere)	18554.59 ± 24183.05	24506.32 ± 22499.02	5.85	**0.016**
Bacterial biomass (rhizosphere)	897317.0 ± 1003124.0	1542961.0 ± 821367.5	8.88	**0.003**
Fungi:bacteria ratio (rhizosphere)	89.15 ± 374.55	0.02 ± 0.02	5.78	**0.016**

Table 2 Comparison of the effect of the copper treatment on soil biota separately for *Fallopia japonica* and *Urtica dioica*. Model results of the linear mixed model (Chi^2 and p value) are given for each variable. Values significant at the 0.05 threshold are given in bold.

	Fallopia japonica		***Urtica dioica***	
Dependent variable	**Chi^2**	**P**	**Chi^2**	**P**
Nematoda	0.76	0.859	2.63	0.452
Collembola	7.91	**0.048**	2.27	0.519
Soil biological activity	61.05	< **0.001**	73.91	< **0.001**
Fungal biomass (bulk)	15.38	< **0.001**	0.73	0.867
Bacterial biomass (bulk)	0.60	0.899	1.59	0.661
Fungi:bacteria (bulk)	2.20	0.532	22.44	< **0.001**
Fungal biomass (rhizosphere)	3.29	0.350	6.79	0.079
Bacterial biomass (rhizosphere)	2.01	0.569	4.26	0.235
Fungi:bacteria (rhizosphere)	3.55	0.314	6.10	0.107

Table 3 Comparison of the effect of presence of the plants *Fallopia japonica* and *Urtica dioica* on soil and root fungi total OTU richness and OTU richness in the four functional groups. The mean and standard deviation for each variable is presented alongside the results of the corresponding model (Chi^2 and p value). Values significant at the 0.05 threshold are given in bold.

Dependent variable	***F. japonica***	***U. dioica***	**Chi^2**	**P**
Soil fungi				
Total	17.06 ± 26.72	25.50 ± 26.54	4.42	**0.04**
Endophytes	0.06 ± 0.25	0.25 ± 0.62	0.86	0.35
Mycorrhiza	1.00 ± 1.90	1.92 ± 2.71	2.92	0.09
Parasites	4.31 ± 5.71	7.58 ± 7.23	4.99	**0.03**
Saprobes	11.69 ± 19.61	15.75 ± 16.91	2.60	0.11
Root fungi				
Total	29.43 ± 40.50	30.75 ± 16.62	3.88	**0.05**
Endophytes	0.29 ± 0.49	0.50 ± 0.58	0.90	0.34
Mycorrhiza	1.57 ± 3.74	2.50 ± 1.73	3.91	**0.05**
Parasites	10.57 ± 13.97	8.00 ± 5.23	0.63	0.43
Saprobes	17.00 ± 23.22	19.75 ± 10.31	5.76	**0.02**

Table 4a Comparison of the effect of the copper treatment (in mg kg^{-1}) on soil and root fungi total OTU richness and OTU richness in the four functional groups under *Fallopia japonica*. Model results of the corresponding mixed model (Chi^2 and p value) are given for each variable. Values significant at the 0.05 threshold are given in bold.

	Soil fungi		**Root fungi**	
Dependent variable	**Chi^2**	**P**	**Chi^2**	**P**
Total	36.27	< **0.001**	2.27	0.52
Endophytes	0	1	0	1
Mycorrhiza	0	1	0	1
Parasites	26.65	< **0.001**	5.07	0.17
Saprobes	20.73	< **0.001**	0.57	0.90

Table 4b Comparison of the effect of the copper treatment (in mg kg^{-1}) on soil and root fungi total OTU richness and OTU richness in the four functional groups under *Urtica dioica*. Model results of the corresponding mixed model (Chi^2 and p value) are given for each variable. Values significant at the 0.05 threshold are given in bold.

	Soil fungi		**Root fungi**	
Dependent variable	**Chi^2**	**P**	**Chi^2**	**P**
Total	30.63	< **0.001**	9.91	**0.007**
Endophytes	0	1	0	1
Mycorrhiza	0.76	0.94	1.69	0.43
Parasites	7.79	0.10	4.58	0.10
Saprobes	18.06	**0.001**	0.44	0.80

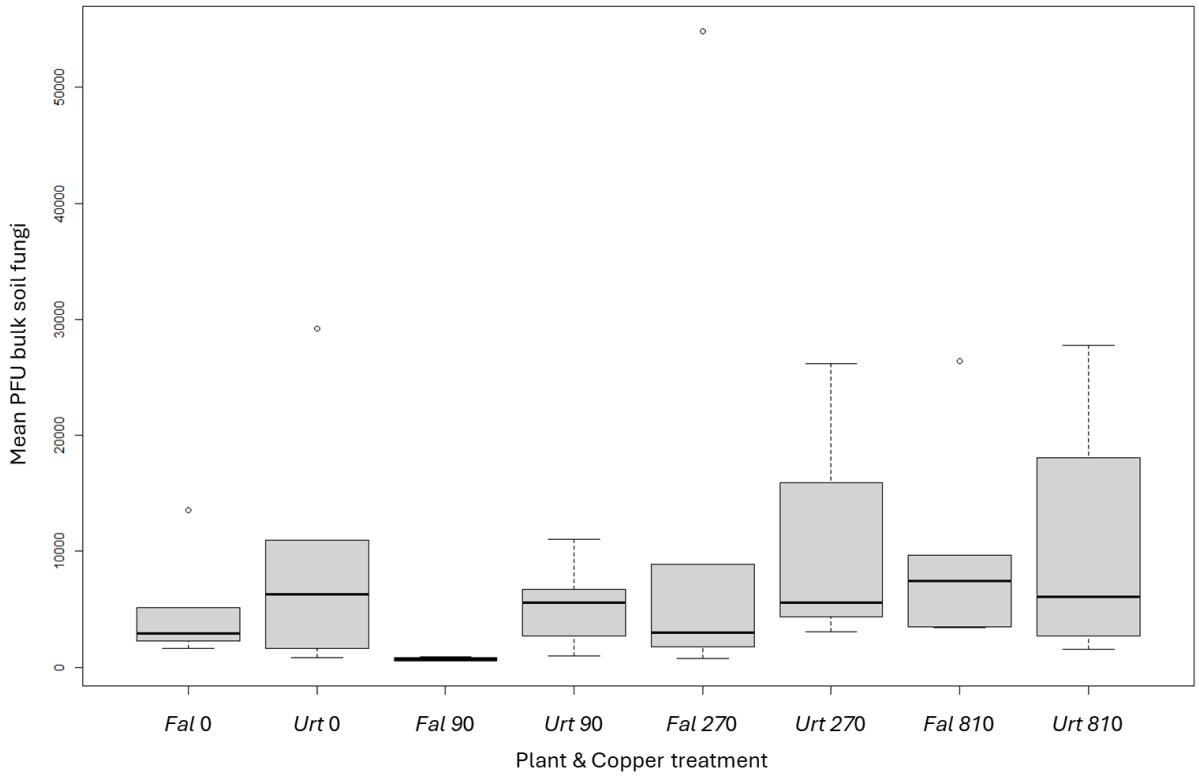

Fig. 1 PCR forming units (PFU) as a measurement of microbial biomass for fungi in the bulk soil. On the x axis, *Fal* refers to *F. japonica* samples and *Urt* to *U. dioica* samples. The number refers to the copper treatment in mg kg^{-1} soil. Each box represents the interquartile range (IQR) and the whiskers show the absolute maximum and minimum of the data except for outliers. The median values are represented by the black line.

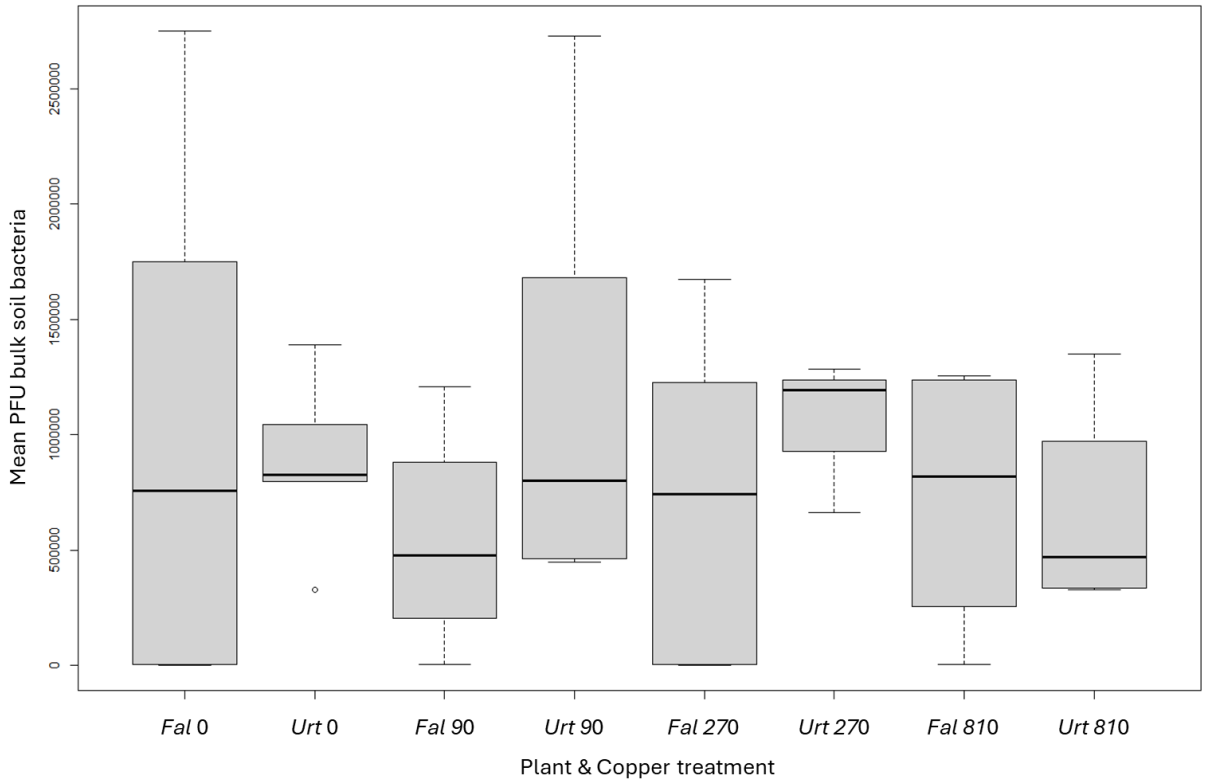

Fig. 2 PCR forming units (PFU) as a measurement of microbial biomass for bacteria in the bulk soil. On the x axis, *Fal* refers to *F. japonica* samples and *Urt* to *U. dioica* samples. The number refers to the copper treatment in mg kg^{-1} soil. Each box represents the interquartile range (IQR) and the whiskers show the absolute maximum and minimum of the data except for outliers. The median values are represented by the black line.

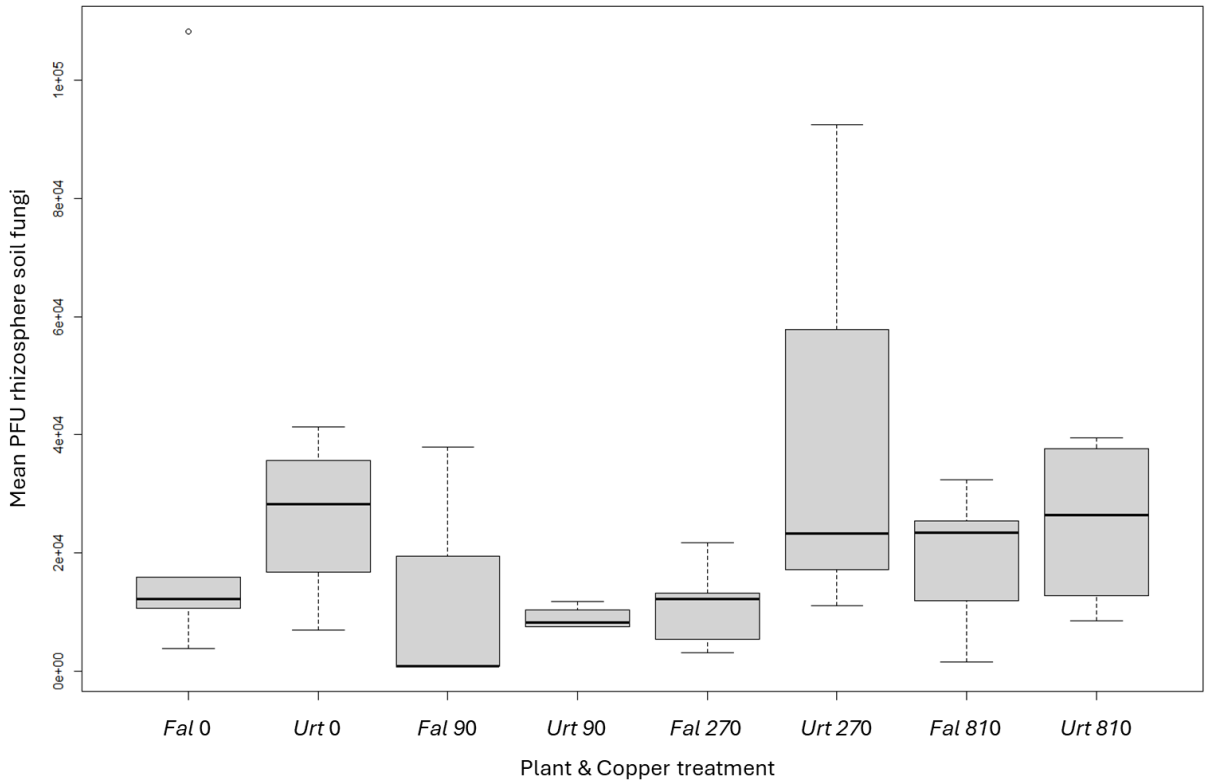

Fig. 3 PCR forming units (PFU) as a measurement of microbial biomass for fungi in the rhizosphere soil. On the x axis, *Fal* refers to *F. japonica* samples and *Urt* to *U. dioica* samples. The number refers to the copper treatment in mg kg^{-1} soil. Each box represents the interquartile range (IQR) and the whiskers show the absolute maximum and minimum of the data except for outliers. The median values are represented by the black line.

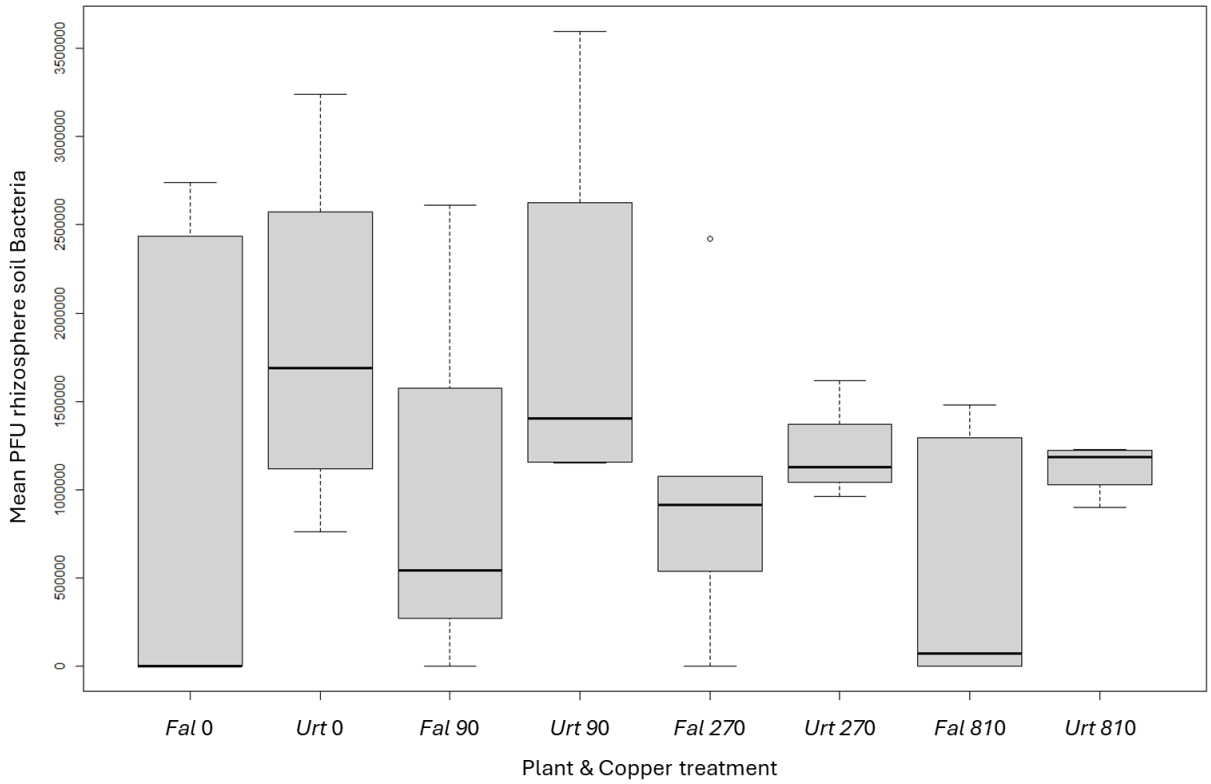

Fig. 4 PCR forming units (PFU) as a measurement of microbial biomass for bacteria in the rhizosphere soil. On the x axis, *Fal* refers to *F. japonica* samples and *Urt* to *U. dioica* samples. The number refers to the copper treatment in mg kg^{-1} soil. Each box represents the interquartile range (IQR) and the whiskers show the absolute maximum and minimum of the data except for outliers. The median values are represented by the black line.

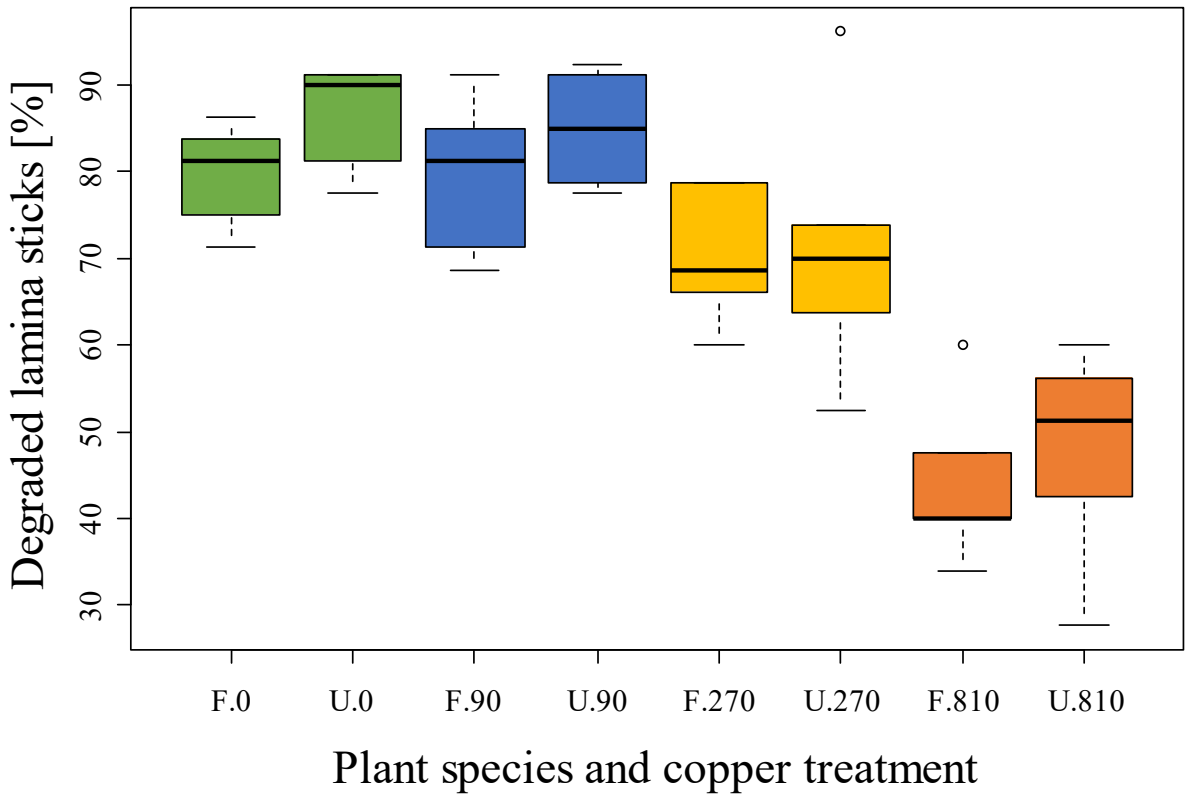

Fig. 5 Soil biological activity measured by bait lamina stick degradation for each copper treatment and plant species combination (*Fallopia japonica* as "F" and *Urtica dioica* as "U"). Each copper treatment in mg kg^{-1} is represented by color (0 = green, 90 = blue, 270 = yellow, 810 = orange). Each box represents the interquartile range (IQR) and the whiskers show the absolute maximum and minimum of the data except for outliers. The median values are represented by the black line.

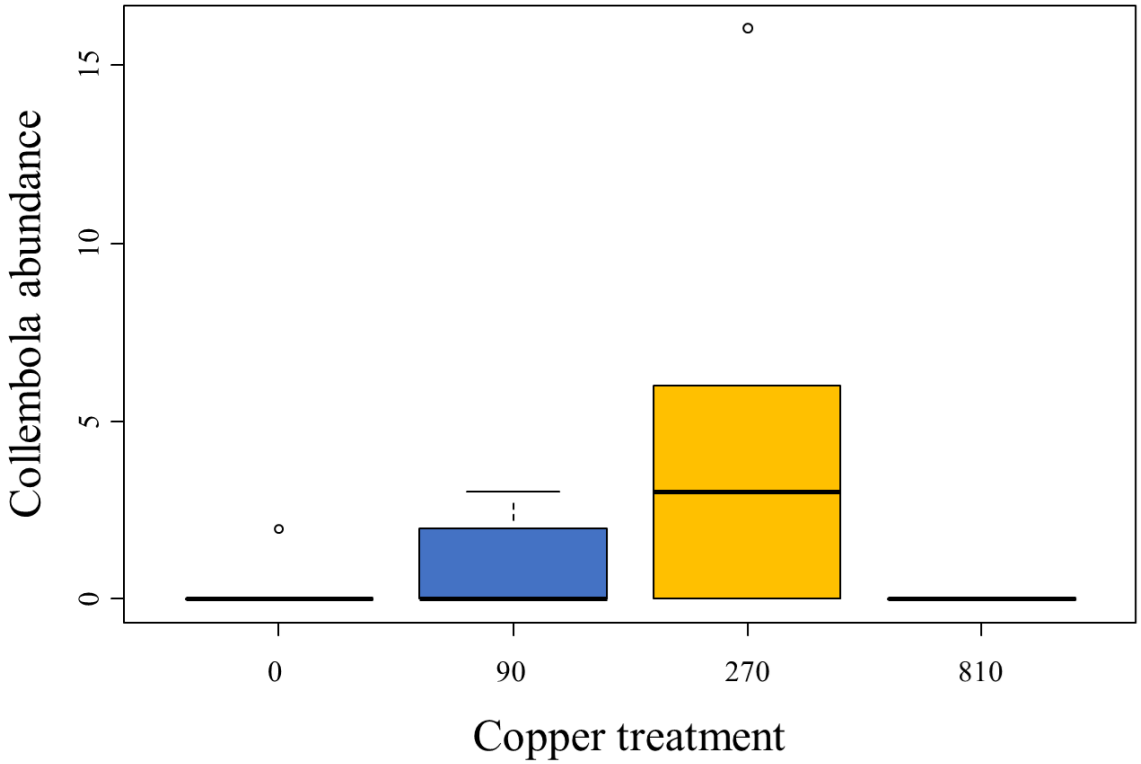

Fig. 6 Collembola abundance in the bulk soil under *F. japonica*. Each copper treatment in mg kg^{-1} is represented by color (0 = green, 90 = blue, 270 = yellow, 810 = orange). Each box represents the interquartile range (IQR) and the whiskers show the absolute maximum and minimum of the data except for outliers. The median values are represented by the black line.

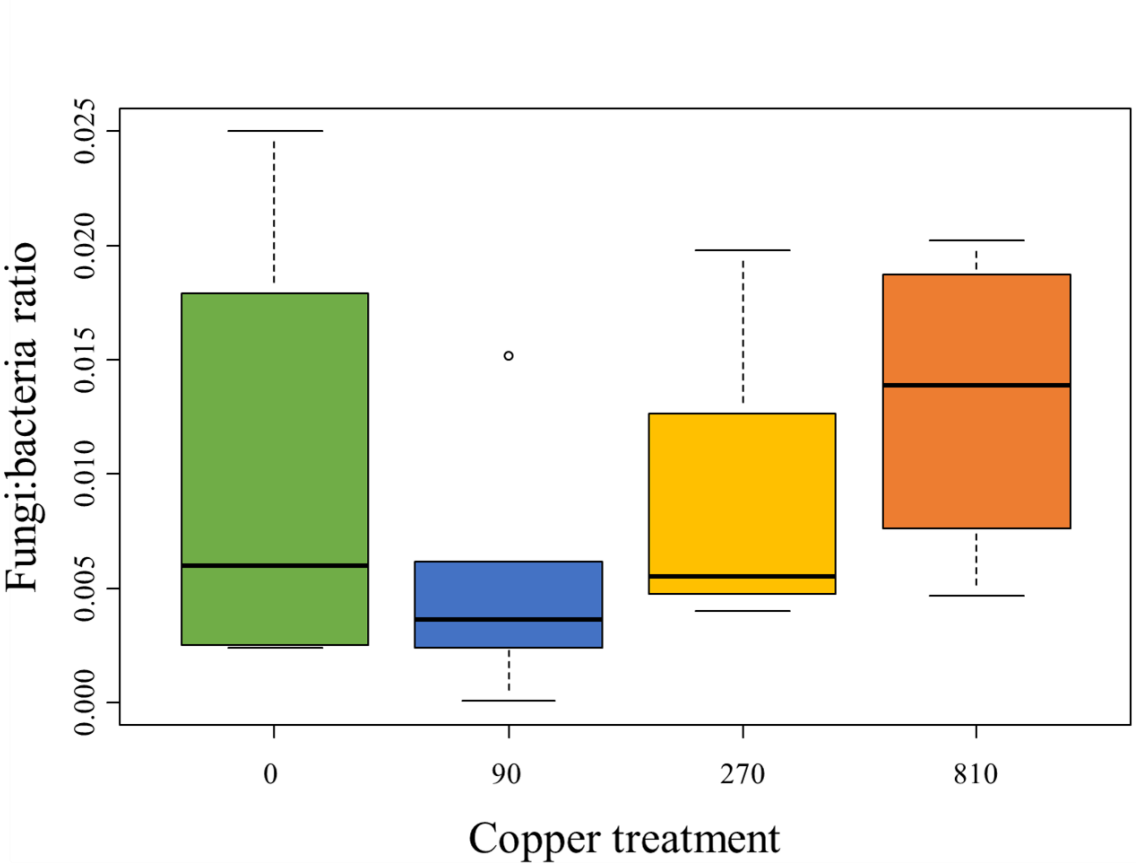

Fig. 7 Fungi:bacteria ratio in the bulk soil under *U. dioica.* Each copper treatment in mg kg^{-1} is represented by color (0 = green, 90 = blue, 270 = yellow, 810 = orange). Each box represents the interquartile range (IQR) and the whiskers show the absolute maximum and minimum of the data except for outliers. The median values are represented by the black line.

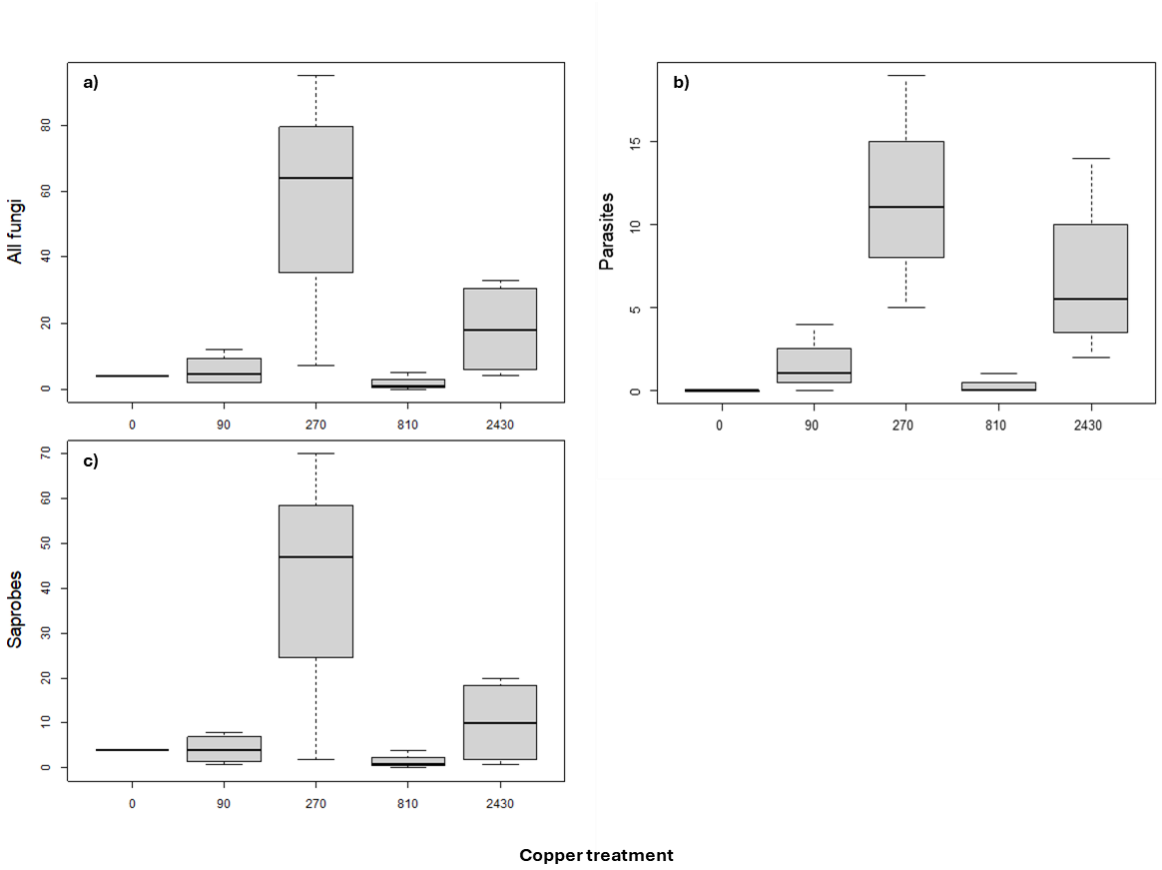

Fig. 8 OTU richness in the soil samples under *Fallopia japonica* for all groups (a), parasites (b) and saprobes (c) under each copper treatment level. The copper treatment in mg kg^{-1} is shown on the x axis. Each box represents the interquartile range (IQR) and the whiskers show the absolute maximum and minimum of the data except for outliers. The median values are represented by the black line.

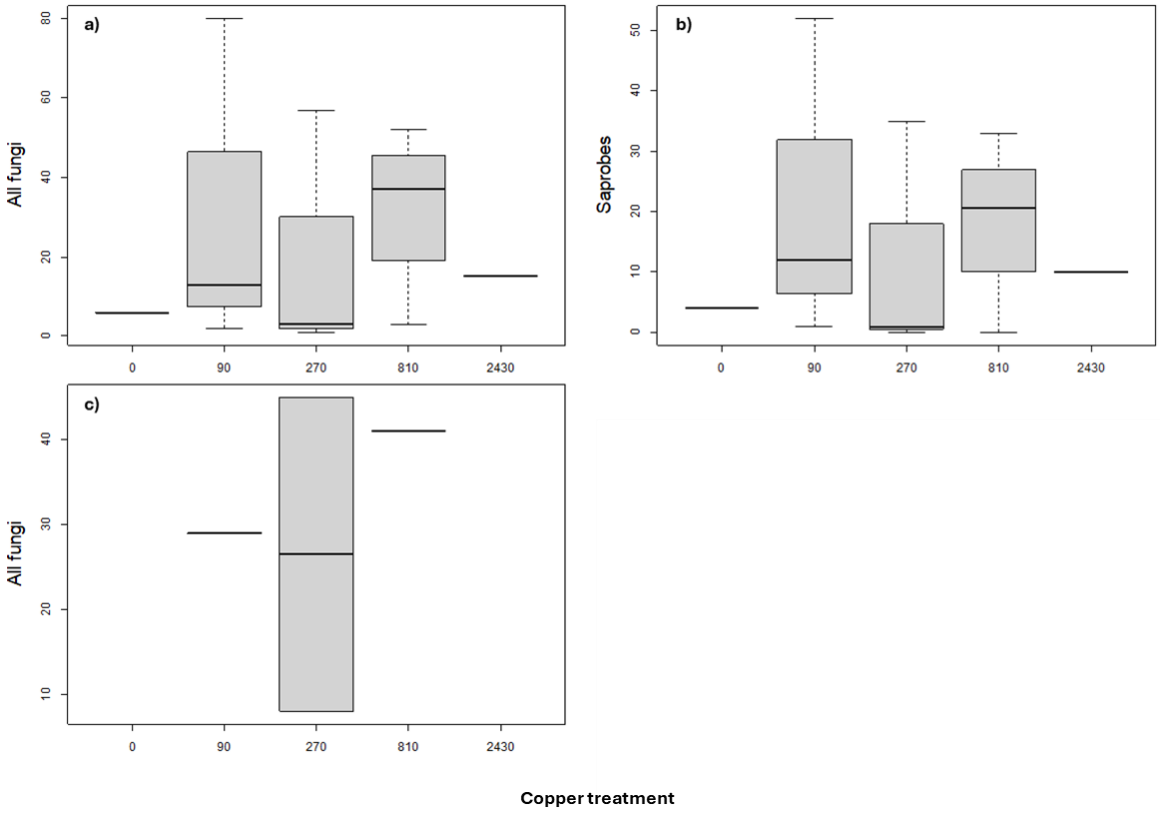

Fig. 9 OTU richness in the soil and root samples under *Urtica dioica* for all groups in the soil (a), parasites in the soil (b) and all groups in the root samples (c) under each copper treatment level. The copper treatment in mg kg^{-1} is shown on the x axis. Each box represents the interquartile range (IQR) and the whiskers show the absolute maximum and minimum of the data except for outliers. The median values are represented by the black line.

Supplement

The digital resources S1 to S5 are provided in the attached CD disk.

Fig. S1 Example picture of a degraded bait lamina stick.

Digital resource S1 Soil arthropods abundances for each soil sample. Sample id is an identifier for each sample. Block states the position of the sample within the randomized block design. Plant is the focal plant present in the corresponding pot ("Fallopia" for *Fallopia japonica* and "Urtica" for *Urtica dioica*). Copper gives the corresponding copper treatment in mg kg^{-1}. The following columns give the abundance of the stated invertebrate taxa in the sample. "Others" refers to invertebrate that do not fall into any of the given taxa and "Total" is the sum of all taxa.

Digital resource S2 Biomass of soil microorganisms for each soil sample. Sample id is an identifier for each sample. Block and Pot state the position of the sample within the randomized block design. Soil type identifies if the sample is from bulk ("Bulk") or rhizosphere ("Rhiz") soil. Plant is the focal plant present in the corresponding pot ("Fallopia" for *Fallopia japonica* and "Urtica" for *Urtica dioica*). Copper gives the corresponding copper treatment in mg kg^{-1}. Bacteria PFU are the PCR forming units (PFU) for bacteria in the sample, corresponding to the biomass of bacteria. The same for the Fungi PFU, corresponding to the fungal biomass. The fungi:bacteria ratio is the fraction of fungal to bacterial PFU.

Digital resource S3 Soil activity measured using bait lamina sticks. Sample id is the identifier of the sample. Block and Pot state the position of the sample within the randomized block design. Plant is the focal plant present in the corresponding pot ("F" for *Fallopia japonica* and "U" for *Urtica dioica*). Copper gives the corresponding copper treatment in mg kg^{-1}. The percentage of degradation (corresponding to the soil activity) in each pot is given in the column Degradation ratio.

Digital resource S4 Mean and standard deviation of OTU richness of the defined functional groups under both the plant and copper treatment. Total refers to the sum of all functional groups. Abbreviations are as following: endophytes (END), mycorrhiza (MYC), parasites (PAR) and saprobes (SAP). Overall is the OUT richness of all plant treatments combined. When NA is given, there was insufficient data to calculate mean and standard deviation.

Digital resource S5 Information about the bioinformatical analysis of the metabarcoding samples provided by Sinsoma GmbH (Voels, Austria)