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**Title:**

Canopy facilitation outweighs elemental allelopathy in a metalliferous system during an exceptionally dry year.

**Authors**

Hugo Randé<sup>1</sup>, Richard Michalet<sup>1</sup>, David Nemer<sup>1</sup>, Florian Delerue\*<sup>1</sup>

<sup>1</sup>Univ. Bordeaux, CNRS, Bordeaux INP, EPOC, UMR 5805, F-33600 Pessac, France

**\*Corresponding author:**

fdelerue@bordeaux-inp.fr;

ORCID: 0000-0002-9809-5321

Hugo Randé: 0000-0003-1707-1107

Richard Michalet: 0000-0002-6617-4789

David Nemer: 0000-0002-2140-0180

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## **Author contributions**

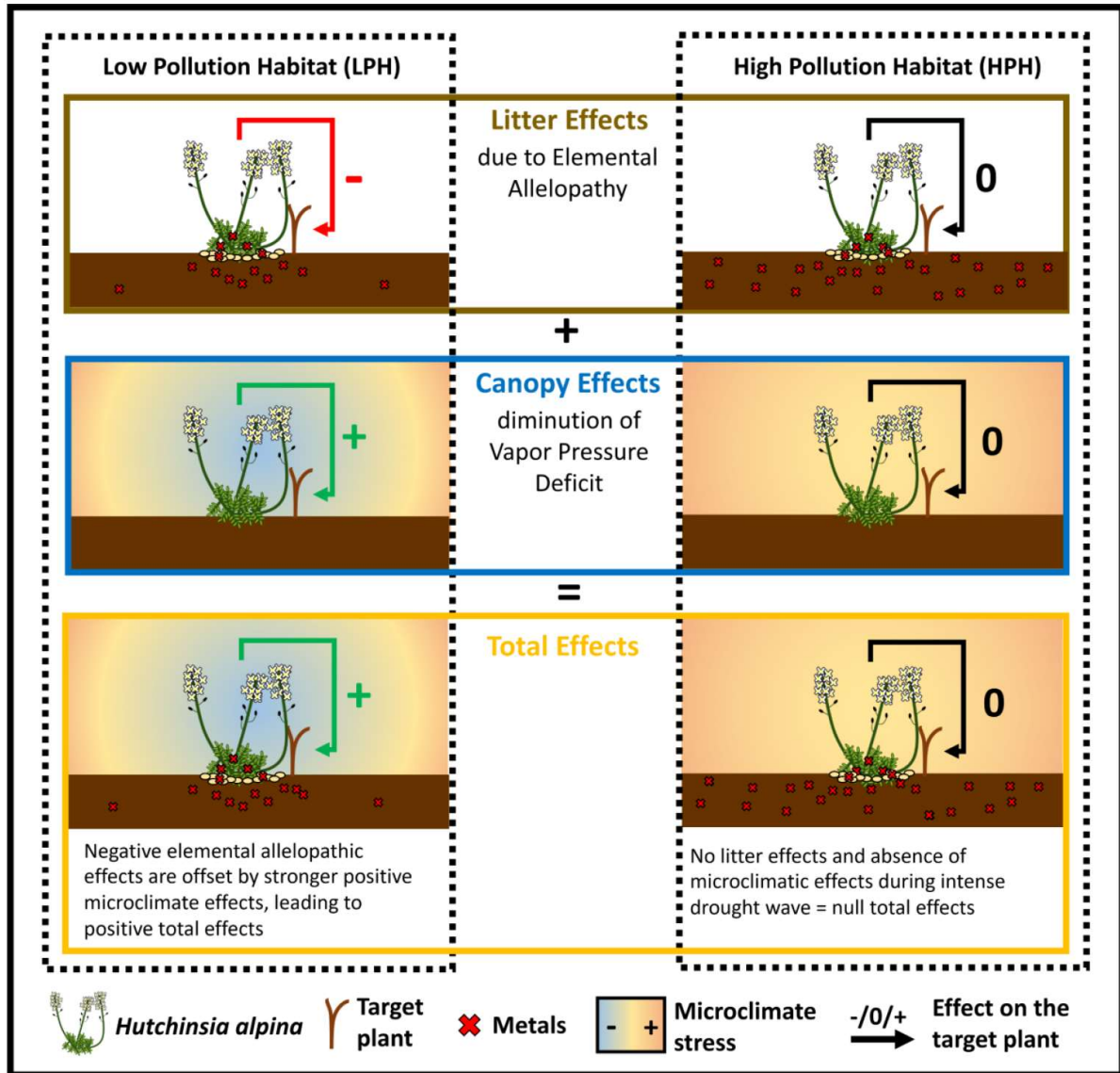
Hugo Randé, Florian Delerue and Richard Michalet conceived the study. Hugo Randé, Florian Delerue, David Nemer and Richard Michalet implemented the field experiment and collected the data. Hugo Randé and Florian Delerue analysed the data. All authors contributed significantly to the writing of the manuscript and gave their approval.

## **Data availability**

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.kwh70rzd4> (Randé *et al.*, 2024).

## **Conflict of interest**

None of the authors have a conflict of interest.



Elemental allelopathic effects occur when a metal-hyperaccumulating species interacts with less metal-tolerant neighbours in habitats with low metal availability in soils. The same metallophyte species simultaneously alleviates microclimatic stress for neighbours during dry episodes. These positive canopy effects outweighed negative litter effects during a particularly harsh drought and heatwave. In highly polluted habitats, both kinds of effects were absent.

## Summary

- The Elemental Allelopathy hypothesis states that the metal-rich litter of hyper-accumulating species may have a detrimental impact on neighbouring plants giving a competitive advantage to hyper-accumulators. Here, we aim to specify the conditions of application of this hypothesis in realistic field conditions, and its relative importance compared to other positive effects found in metalliferous systems.
- We disentangled the litter-induced elemental allelopathy and canopy effects of two metallophyte species (*Arenaria multicaulis* and *Hutchinsia alpina*) with different levels of leaf Zn and Cd accumulation on two ecotypes of *Agrostis capillaris* with different levels of metal tolerance. The experiment was conducted in two habitats with contrasting pollution levels in a former mining valley in the Pyrenees (France).
- The metallophyte species that accumulates more metals (*Hutchinsia alpina*) showed a strong elemental allelopathy effect on the target with lower metal tolerance in the habitat with lower pollution level, while the metallophyte species that accumulates less metals (*Arenaria multicaulis*) had no litter effect. Both metallophyte species had positive canopy effects likely due to improvement of micro-climatic conditions. The drought that occurred during the experiment may have influenced these canopy effects, increasing their importance during the course of the study. For *Hutchinsia alpina* the positive canopy effects were stronger than the negative litter effects, resulting in overall positive effects on both target ecotypes.
- *Synthesis.* Our results brought a better understanding of the occurrence of elemental allelopathy in metallophyte communities and its relative importance as compared to micro-climatic facilitation in a global warming context.

## Keywords:

plant-plant interactions, facilitation, microclimate amelioration, stress-gradient hypothesis elemental allelopathy hypothesis, metal hyper-accumulation, stress tolerance.

## Introduction

Metalliferous ecosystems develop spontaneously on metal-rich outcrops and derived soils (Ernst, 1990; Harrison & Rajakaruna, 2011; Reeves *et al.*, 2018) or on soils impacted by anthropogenic mining or smelting activity (Hou *et al.*, 2020) in relation to the exploitation of ore bodies. Because metals beyond a certain available concentration are toxic for most living organisms, metallophyte communities are composed of plant species or ecotypes that have developed specific adaptations to cope with metal-related stress (Baker, 1981). Some of them, the hyper-accumulators, have the counter-intuitive ability to hyper-concentrate metals in their leaves close to the photosynthetic apparatus (Baker, 1981; van der Ent *et al.*, 2013). Therefore, it has attracted the attention of plant ecologists who proposed several hypotheses to explain its selection through evolution.

Plant-plant interactions are crucial for the assemblage of plant communities, driving their extant composition and diversity (Grime, 1979; Brooker *et al.*, 2007). Indeed, plants by their presence and growth modify their immediate environments, which can positively influence their neighbours (i.e. facilitation) or worsen their development (i.e. interference and competition for resources). Consistently, Boyd & Martens (1998) proposed the Elemental Allelopathy hypothesis, in relation with plant-plant interactions in metalliferous systems, to explain hyper-accumulation by some metallophyte species. This hypothesis states that hyper-accumulating species has the ability to increase soil toxicity in their immediate environment and close soil surface through production and decomposition of a metal hyper-rich litter, which ultimately negatively affects neighbouring plants (Boyd & Jaffré, 2001; Morris *et al.*, 2009). These potential allelopathic effects called “Elemental Allelopathy” rely on the use of the metallic element cycles (absorption, translocation in leaves, elimination in litter and decomposition) at the own advantage of the metallophyte species. Corresponding negative effects have been supported by several greenhouse (Jaffé *et al.*, 2018; Mohiley *et al.*, 2020, Yang *et al.*, 2023) and field studies (Mehdawi *et al.*, 2011a). However, results of dedicated case studies are not all consistent. Morris *et al.* (2006) failed to observe a negative elemental allelopathy effect close to *Acroptilon repens*. On the contrary, Zhang *et al.* (2005) showed on the same plant that Elemental Allelopathy can operate on a short time scale, soon after litter fall and the beginning of its decomposition. Additionally, if this cycle (absorption of metallic elements from the whole soil volume accessible by roots, translocation and elimination in litter) is repeated over time, it can create patches of high contamination close to hyper-accumulators. However, as Elemental allelopathy has mainly been observed in greenhouse studies, the

conditions under which it can be observed in spontaneous metalliferous communities are less known and remain to be studied.

More in details, two important directions of research are needed to better understand the role and importance of Elemental Allelopathy in metalliferous systems. Firstly, because these negative litter effects are not always detected, its conditions of application (in which habitats these effects can be found and between which interacting plants) have to be specified as stated above. Different arguments can be depicted from the literature. In metalliferous areas, a metal pollution gradient is often created by the heterogeneity of mineral deposits. Along these gradients, an important species turnover takes place and diverse interactions are at stake involving plants with different levels of metal-tolerance and different adaptations to cope with metal stress (Wang *et al.*, 2013, Nemer *et al.*, 2022, Randé *et al.*, 2023a). Rationally, Elemental Allelopathic effects are more likely produced by the species accumulating the highest level of metals in their leaves, and species or ecotypes with low metal-tolerance are the most likely to be sensitive to these effects (Mehdawi *et al.*, 2011a, Mohiley *et al.*, 2020). Additionally, Mohiley *et al.* (2021) demonstrated in a greenhouse study that competition for light can stimulate leaf metal-accumulation. In realistic field condition, such competition for light is expected in communities with high aboveground vegetation biomass (Grime, 1973; Tilman, 1985; Michalet *et al.*, 2023) more likely to be found in habitats with intermediate to low level of metal-stress. Finally, in habitats with high metal-stress, the potential increase of metal availability close to hyper-accumulating plants is likely to be negligible compared to the very high level of metal-availability already present in soils (see the greenhouse studies of Morris *et al.*, 2006; Jaffe *et al.*, 2018). Therefore, our first objective was to precise the conditions of application of the Elemental Allelopathy in realistic field conditions.

Secondly, the Elemental Allelopathy Hypothesis appears contradictory with the accumulating evidences of positive plant-plant interactions in metalliferous ecosystems (e.g. Dominguez *et al.*, 2015; Nemer *et al.*, 2022; Randé *et al.*, 2023a). Metalliferous systems, as other stressed systems, are known to be particularly prone to positive interactions (Nemer *et al.*, 2022, 2023; Randé *et al.*, 2023a). Indeed, the Stress Gradient Hypothesis (SGH; Bertness & Callaway, 1994), a dominant theory in plant ecology, proposes that competition should shift to facilitation as environmental stress increases. Interestingly, one of the main process described in many case studies that brought evidence of facilitation in metal-stressed environments is mitigation of microclimatic stress by nurse (effect) plants (Zvereva & Kozlov, 2007; Dominguez *et al.*, 2015), enabling target (beneficiary) plants to better cope with metal-

pollution. Such microclimate improvements are particularly efficient under the canopies of some metallophyte species (Ginocchio *et al.*, 2003; Randé *et al.*, 2023b) tolerant to metal-exposure stress. In accordance with studies made in various stressed environments (Liancourt *et al.*, 2005; Gross *et al.*, 2010), these facilitative effects mainly benefit less metal-tolerant species or ecotypes (Nemer *et al.*, 2022, 2023; Randé *et al.*, 2023a).

Therefore, it is likely that in metalliferous systems multiple and potentially opposite effects can operate simultaneously, as shown in a variety of environments (Callaway & Walker, 1997; Pugnaire & Luque, 2001; Michalet *et al.*, 2015). For example, Randé *et al.* (2023b) showed that both positive microclimatic improvement by nurse canopy and elemental allelopathy effects by the very same species can happen simultaneously, pointing the importance to disentangle the contribution of these two types of effects in the net effect of plant-plant interactions. Therefore, our second objective was to disentangle Elemental Allelopathy from positive canopy effects generally detected in metalliferous ecosystems, and to evaluate the importance of both kinds of effects at different positions along a metal-pollution gradient. Elemental allelopathy being detectable on relatively short time scale (one vegetative season) due to the effect of litter decomposition (Zhang *et al.*, 2005), we isolated litter effects comparing the response of target plants in places where benefactor canopy has been removed but controlling for the presence of the benefactor plant litter (Fig. 1). Controlling the litter presence directly in a field study provides a realistic approach to estimate potential allelopathic effect compared to greenhouse studies using leaf leachates as a surrogate of litter decomposition effects (Mohiley *et al.*, 2020). Additionally, we studied benefactor plants canopy effects using the standard removal method comparing the response of target plants with and without the benefactor plant canopy (and with presence of litter in both situations, Fig. 1).

Gathering all the arguments scattered in the literature, we predicted (first hypothesis) that Elemental Allelopathy effects should be detected in areas with intermediate to low levels of metal-availability and for pairs of interacting species involving a species with a very high ability to accumulate metals in its leaves (the effect species) and a species or ecotype sensitive to metal-stress (the target species). Regarding more specifically canopy effects, consistent with recent studies, we expect that positive microclimatic improvements will increase with increasing metal stress, and particularly for the target plants less tolerant to metals (second hypothesis). As Elemental Allelopathy should decrease and facilitation via micro-climatic improvement should increase along gradients of metal stress, we predicted overall total negative effects at low level of metal stress, and overall total positive effects at high level of metal stress

(third hypothesis). This third hypothesis is consistent with the Stress Gradient Hypothesis, which predicts a shift from negative to positive plant-plant interaction as stress increases. In order to test our hypotheses, we used the above-mentioned methodology to explore the variations of canopy and litter effects of two metallophyte species with different leaf metal accumulation abilities (*Hutchinsia alpina* with higher leaf metal accumulation and *Arenaria multicaulis* with lower leaf metal accumulation) on two ecotypes of the grass *Agrostis capillaris* (one tolerant to metal pollution and the other less tolerant) in two habitats with different levels of pollution in a former mining valley in the French Pyrenees with Zn, Pb and Cd contamination.

## Material and methods

### *Study site*

The study site is located in the former mining valley of Sentein in the French Pyrenees (N42° 49.388' E0° 55.625', 1100 m a.s.l.). The climate in the region is warm oceanic as confirmed by a weather station (WS-GP1 - Delta-T Devices) situated less than 2 km from the study site with total precipitation of 1038 and 929 mm and mean temperature of 9.11 and 11.37 °C in 2021 and 2022 respectively. Important mining activity for the extraction of zinc and lead occurred in this valley between the 1850s and 1960s and stopped more than 60 years ago, leaving several hotspots with severe metal contamination (Stephant-Champigny *et al.*, 2015). This experiment took place in one of these highly contaminated areas and covered nine months of monitoring (from the 27<sup>th</sup> October 2021 to 2<sup>nd</sup> August 2022). Within this region, former studies (Nemer *et al.*, 2023; Randé *et al.*, 2023a) confirmed the presence of strong heterogeneity in soil contamination, with zones highly contaminated showing few hyper-tolerant metallophyte species, and zones less contaminated dominated by grass species (Fig. S1). We initially planned to run the experiment throughout a full year until the end of October 2022, but we decided to end up the experiment earlier because of the exceptional drought and heatwave that occurred in the northern hemisphere in summer 2022 (Lu *et al.*, 2023), and then to avoid massive and complete mortality of our target plants. This climatic event strongly affected metalliferous grasslands in the valley and led to a collapse of all effects related to plant-plant interactions evaluated during this summer period (Delerue *et al.*, in prep a). In our case, continuing the experimentation would likely have jeopardized the ability to measure the different effects we were looking at if all of our target plants were dead.

### *Experimental design*

#### Spatial replication and habitats with contrasting levels of soil pollution

This experiment was performed within an area with several hot spots of contamination (around 230 m<sup>2</sup> each, 20 m apart from each other). All these hotspots have the same exposition and slight slope (10%), and are positioned below a former mining trail (Fig. S1a). We benefited from this spontaneous spatial replication and used three different hot spots as blocks for this experiment, each hotspot presenting different types of habitats with contrasting levels of soil pollution (Fig. S1b): “High Pollution Habitats” in their centre (Fig. S1c); and “Low Pollution

Habitats” in their periphery. These two contrasting habitats were easily distinguishable based on their plant community composition, and their contrasting levels of pollution (Nemer *et al.*, 2022; Randé *et al.*, 2023a). HPH were dominated by the two metallophyte species *Hutchinsia alpina* (hereafter ‘*H. alpina*’) and *Arenaria multicaulis* (hereafter ‘*A. multicaulis*’), with few individuals of the grass *Agrostis capillaris*; LPH were reversely dominated by different grass species including *Agrostis capillaris* (hereafter ‘*A. capillaris*’), and lower presence of the above-mentioned two metallophyte species.

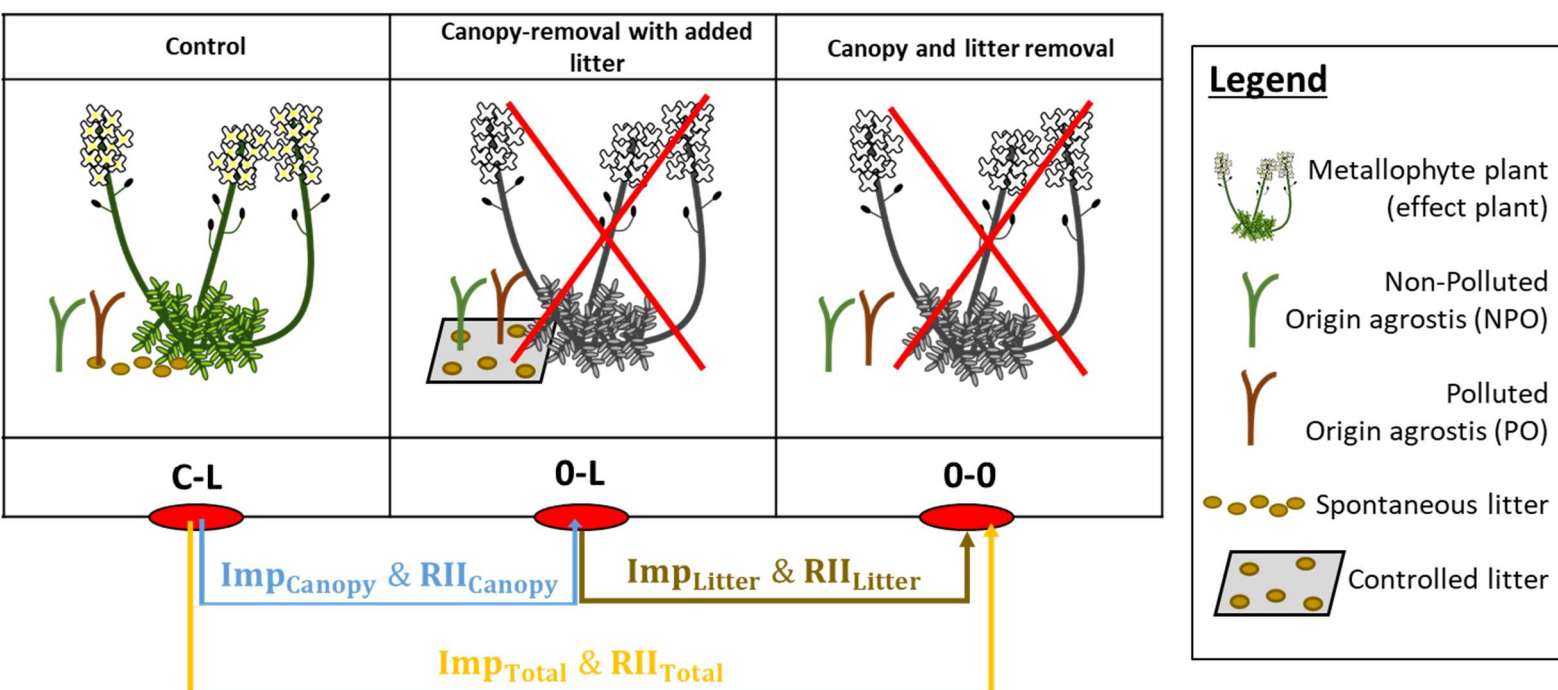
#### Metallophyte species, target species ecotypes and neighbouring modalities

We used the two metallophytes *H. alpina* and *A. multicaulis* (Fig. S2) as plants producing the effects in this study. In each habitat of each block, we distinguished eight plots (approximately one square meter each, four plots for each species) corresponding to areas with presence of several individuals of the two metallophytes. *A. multicaulis* and *H. alpina* can both present high concentration of metals (Zn, Pb and Cd) in their leaves in the valley, *H. alpina* being expected to reach higher concentrations (Delerue *et al.*, in prep b). We used the easy-to-transplant grass species *A. capillaris* as the target species. We used two different ecotypes of *A. capillaris* with assumed different metal tolerances for this experimentation based on their distribution: one ecotype from a similar metal polluted site situated nearby (less than 1 km) on a similar substrate (hereafter Polluted Origin ecotype, or PO) and the other one from a non-polluted grassland next to the experimental site (hereafter Non-Polluted Origin ecotype, or NPO). The ecotypic differentiation of this species with exposure to excess of metal/metalloids is well established in the literature and can be detected on very small spatial distances (Nicholls & McNeilly, 1985; Symeonidis *et al.*, 1985), which in turn can modulate their response to plant-plant interactions (Nemer *et al.*, 2022).

We collected and transplanted in each plot mid-October 2021 an *A. capillaris* individual of each ecotype in three neighbouring modalities (Fig. 1 and Fig. S3): 1) Control modality: the transplantations were made under the canopy of the metallophyte individuals (average canopy diameter of both metallophyte species was approximately 15 cm, and the target was exposed to both canopy and litter effects (C-L)); 2) Canopy-removal with added litter modality: the target individuals were transplanted in a zone with the presence of the metallophyte where the aerial parts of the metallophyte plant individual were cut at the ground level and spontaneous litter was removed; then an addition of a standardized amount of litter was performed (0-L); 3)

Canopy and litter removal: the target individuals were transplanted in a zone with the presence of the metallophyte where the aerial parts of the metallophyte plant individual were cut at the ground level and the spontaneous litter was removed (0-0). In each plot, the different neighbouring modalities were set up at a minimum distance of 20 cm from the others. A total of 288 target individuals (3 blocks x 2 habitats x 2 metallophyte species x 3 neighbouring modalities x 2 targets ecotypes x 4 replicates) was transplanted.

**Fig. 1** Presentation of the different neighbouring modalities and the calculation of the different Importance Indices. To calculate  $Imp_{Canopy}$  and  $RII_{Canopy}$ , and, thus, quantify the effects of the



plant canopy, we compared the survival performance between (C-L) and (0-L) modalities. To calculate  $Imp_{Litter}$  and  $RII_{Litter}$ , and to quantify the plant litter effects, and, thus, elemental allelopathy, we compared the survival performance between (0-L) and (0-0) modalities. To integrate both of these effects, and, thus, to have the total plant effects, we compared the survival performance between (C-L) and (0-0) modalities.

To control for litter effects, we choose to standardise the amount and quality of litter in the neighbouring modality (0-L). We harvested leaves of the two metallophytes in late September 2021, directly in the three blocks of the experiment in the two contrasting habitats. Leaves from the same species and habitat from the three blocks were pooled and dried (50°C, three days). Then, a weight of 0.80 grams of dried litter was placed in litter bags of 10 x 6 cm, and these litter bags were placed in the field in the (0-L), modality (Fig. S3b). At the end of the experiment (2 August 2022), the litter bags were collected, the remaining litter was washed,

dried and weighed back at the lab to calculate the litter decomposition rate for each bag. A complementary litter quantification survey in the autumn showed that on a similar 10 x 6 cm surface, the amount of dried litter varied little between species and habitat, and was an average of 0.51g. Since the whole quantity of litter was added all at once at the beginning of the experiment, this higher amount of 0.80g appeared realistic to encompass the litter fall that should occur throughout the whole duration of the experiment. Note that this litter manipulation could not be done in (C-L) modality not to disturb the metallophyte species individuals. Finally, we also controlled for litter bags effect by comparison of additional *A. capillaris* transplants without and with empty bags (2 ecotypes X 2 habitats X 3 blocks X 2 replicates), confirming the absence of effect of empty litter bags ( $P=0.65$ ).

Functional traits, leaf metal accumulation of metallophyte species and corresponding metal concentration in soils

Five individuals of each of the metallophyte species were randomly chosen in one hotspot of contamination to measure morphological traits related to the leaf resource economics functional axis (Díaz *et al.*, 2016). This functional axis being strongly related to leaf life span and soil resource acquisition (Wright *et al.*, 2004) it could help understanding the different species effects on metal cycles and metal concentrations in soils. Briefly, fresh leaves of both metallophyte species were harvested, leaf fresh weight was measured with a portable scale and leaf area (LA, cm<sup>2</sup>) was determined with a portable scanner and the Winfolia software (Regents Instruments, Quebec, Canada). The leaves were then dried at 65°C during 48h before weighting. Then, we calculated Leaf Dry Matter Content (LDMC, %) ([Fresh weight/Dry weight] × 100) and Specific Leaf Area (SLA, cm<sup>2</sup> g<sup>-1</sup>; leaf area/leaf dry weight).

Additionally, we characterized leaf metal-accumulation of metallophyte species as follow. For each metallophyte species and each habitat, leaves from several individuals were collected in the three blocks and pooled together. From these four different kinds of leaves (2 Metallophyte species X 2 Habitats), 10 replicates were separated and analysed per metallophyte species and per habitat type. For each replicate, 0.25g of leaves were washed with de-ionized water, dried (48h at 65°C) and ground in order to determine leaf metal concentration (Zn, Cd, Pb) of the two metallophyte species according to the habitat type (HPH or LPH). This was made by microwave assisted digestion by HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> (4:1 v: v, respectively) followed by

quantification of the metal concentrations by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

To quantify the available metals (Zn, Cd, Pb) in the topsoil layer under both metallophyte species, a total of 96 soil samples (3 blocks x 2 habitats x 2 metallophyte species x 8 replicates) was harvested with a little hand auger (2 cm in diameter, 5 cm deep). An extraction with 0.11 M acetic acid was performed, following Rauret *et al.* (1999) on those samples. We chose extraction by acetic acid because polluted soils in the valley are largely carbonated. Then, corresponding solutions were analysed by ICP-AES. Finally, we calculated a bioconcentration factor (shoot:soil ratio) for each metallophyte species in each habitat to highlight the ability of each species to accumulate metals according to the level of metals availability in soils.

#### *Plant–plant interactions measurement and analysis*

We used the survival of *A. capillaris* individuals by the end of the experiment (see Fig. S4 and Table S1) to estimate the effects of the different treatments. The survival rate of the four replicates in each block was calculated for each combination of treatments (the metallophyte nurse (*H. alpina* or *A. multicaulis*), the habitat (HPH or LPH), the ecotype (NPO or PO) and the neighbouring modality ((C-L), (0-L) or (0-0)). Then, we calculated the Importance interaction index (Imp, Seifan *et al.*, 2010) and Relative Interaction Index (RII, Armas *et al.*, 2004) to estimate litter and canopy effects of both metallophyte species (Fig. 1). The advantage of Imp index compared to the commonly used RII is that it estimates how much the difference in plant response between neighbouring modalities is due to the effect considered (e.g. litter effect) relative to all other factors potentially at stake. Therefore, this index is particularly appropriate to fulfill our first objective to precise the conditions of application of the Elemental Allelopathy in realistic field conditions. It will show the importance of litter effects in explaining target performance in different treatments and at different pollution levels. From the initial Imp calculation proposed by Seifan *et al.* (2010), we derived several Imp to delineate the different effects at stake comparing neighbouring modalities as follows (see also Fig. 1):

$$(1) Imp_{Litter} = \frac{X_{(0-L)} - X_{(0-0)}}{|X_{(0-L)} - X_{(0-0)}| + |X_{(0-0)} - X_{max}|}$$

where  $X_{(0-L)}$  is the Survival in the (0-L) treatment,  $X_{(0-0)}$  is the Survival in the (0-0) treatment, and  $X_{max}$  is the highest Survival found in the experiment, considering all the neighbouring modalities, both metallophyte species and habitats.

$$(2) Imp_{canopy} = \frac{X_{(C-L)} - X_{(0-L)}}{|X_{(C-L)} - X_{(0-L)}| + |X_{(0-L)} - X_{max}|}$$

where  $X_{(C-L)}$  Is the Survival in the (C-L) neighbouring modality.

$$(3) Imp_{Total} = \frac{X_{(C-L)} - X_{(0-0)}}{|X_{(C-L)} - X_{(0-0)}| + |X_{(0-0)} - X_{max}|}$$

Finally, we calculated similarly different kind of Relative Interaction Intensity indices for litter ( $RII_{Litter}$ ), canopy ( $RII_{canopy}$ ) and total effects ( $RII_{Total}$ ) (See legend of Fig. S6 for corresponding equations). Note that positive values of these indices show a beneficial effect and negative values show a negative impact.

### *Statistical analyses*

All analyses were done using R version 4.2.1 (R Core Team, 2022). In all cases, we checked the homogeneity and normality of model residuals to respect the conditions of application of linear modelling. During these analyses, the block effect was first included in the statistical models. The block effect was eliminated for models that were not affected by it, including Imp and RII models. The different models were simplified using a stepwise model selection based on the AIC criterion (stepAIC function, MASS package in R). The rates (decomposition and survival) were arcsine transformed before analysis.

We performed two-way ANOVAs (with posthoc Tukey pairwise multiple comparisons) to test the effect of metallophyte species (*A. multicaulis* or *H. alpina*), the Habitat (HPH or LPH) and their interaction on metal concentrations in the topsoil layer. Similar two-way ANOVAs with the same factors were performed to test their effects on metal concentrations in leaves and on litter decomposition rate. Nurse plant trait values (SLA, LDMC) were compared with Wilcoxon–Mann–Whitney U tests (nonparametric tests, N=5) to test for differences between both metallophyte species.

We performed four-way ANOVAs to evaluate the effect of the metallophyte species considered, the habitat, the *Agrostis* ecotype (NPO and NO), the neighbouring modality ((C-L), (0-L), (0-0)) and their interactions on the final target survival rates.

We performed three-way ANOVAs to evaluate the effect of the metallophyte species considered, the Habitat, the *Agrostis* ecotype (NPO and NO) and their interactions on the different Imps and RIIs. The effects on each kind of indices (Litter, Canopy, Total) were analysed separately. A second complementary analysis was performed using one sample t-tests to test for difference of Imps and RIIs from zero for the combination of treatments of interest identified by the three-ways ANOVAs.

## Results

### *Functional characterisation and leaf metal accumulation of metallophyte species and metal concentration in soils*

There were significant effects of the Habitat and Metallophyte species treatments on Zn and Cd concentrations in leaves ( $p < 0.001$ , Table S2), with higher concentrations of both metals in HPH and in *H. alpina* (Fig. 2a, b). Values of Zn concentration were even found to overcome the hyper-accumulation threshold ( $3000 \text{ mg.kg}^{-1}$ ; Krämer, 2010) in both habitats for both species, with much higher concentration for *H. alpina*. Similar results were found for Cd, confirming lower concentrations in LPH than HPH for both metallophyte species, and lower Cd concentrations in *A. multicaulis* than in *H. alpina* ( $p < 0.001$ , Table S2). There was also a significant interaction between the Habitat and Metallophyte species treatments for Cd concentrations, with a higher difference between habitats for *H. alpina* than for *A. multicaulis*. The results for Pb concentration in leaves were also similar (data not shown). The bioaccumulation ratio (concentration in leaves/concentration in soil, Fig. 2a, b) showed that *H. alpina* have a higher ability to accumulate metals than *A. multicaulis* for both Zn and Cd. *H. alpina* bioaccumulation ratio was also higher in the LPH than in the HPH (0.95 vs. 0.48 respectively). The two species showed marginally significant differences in LDMC and SLA ( $W=22$  and  $W=4$ ;  $p=0.055$  and  $p=0.095$ , respectively), with higher LDMC and lower SLA for *A. multicaulis* than *H. alpina* (Fig. 2c). Litter of *H. alpina* showed a higher decomposition rate during the experiment compared to *A. multicaulis* (88 and 82% respectively,  $p=0.003$ , Fig. 2d, Table S3).

There were significant differences between Habitats in Zn and Cd availability in soils under metallophyte species ( $p < 0.001$  for both metals, Table S4, similar result for Pb not shown), with an obvious higher metal concentration in HPH than LPH (Fig. 2e, f). Considering values for LPH only, there were also significant differences in soil metal availability between the two metallophyte species ( $p = 0.035$  and  $p = 0.016$  for Zn and Cd availability respectively, Fig. 2e, f; same trend ( $p = 0.096$ ) for Pb not shown). Bioavailability was 1.6 times higher for Zn ( $7760 \pm 1126$  vs.  $4890 \pm 777$  mg kg<sup>-1</sup>) and 1.4 times higher for Cd ( $17.27 \pm 1.68$  vs.  $11.92 \pm 1.12$  mg kg<sup>-1</sup>) under *H. alpina* than under *A. multicaulis*.

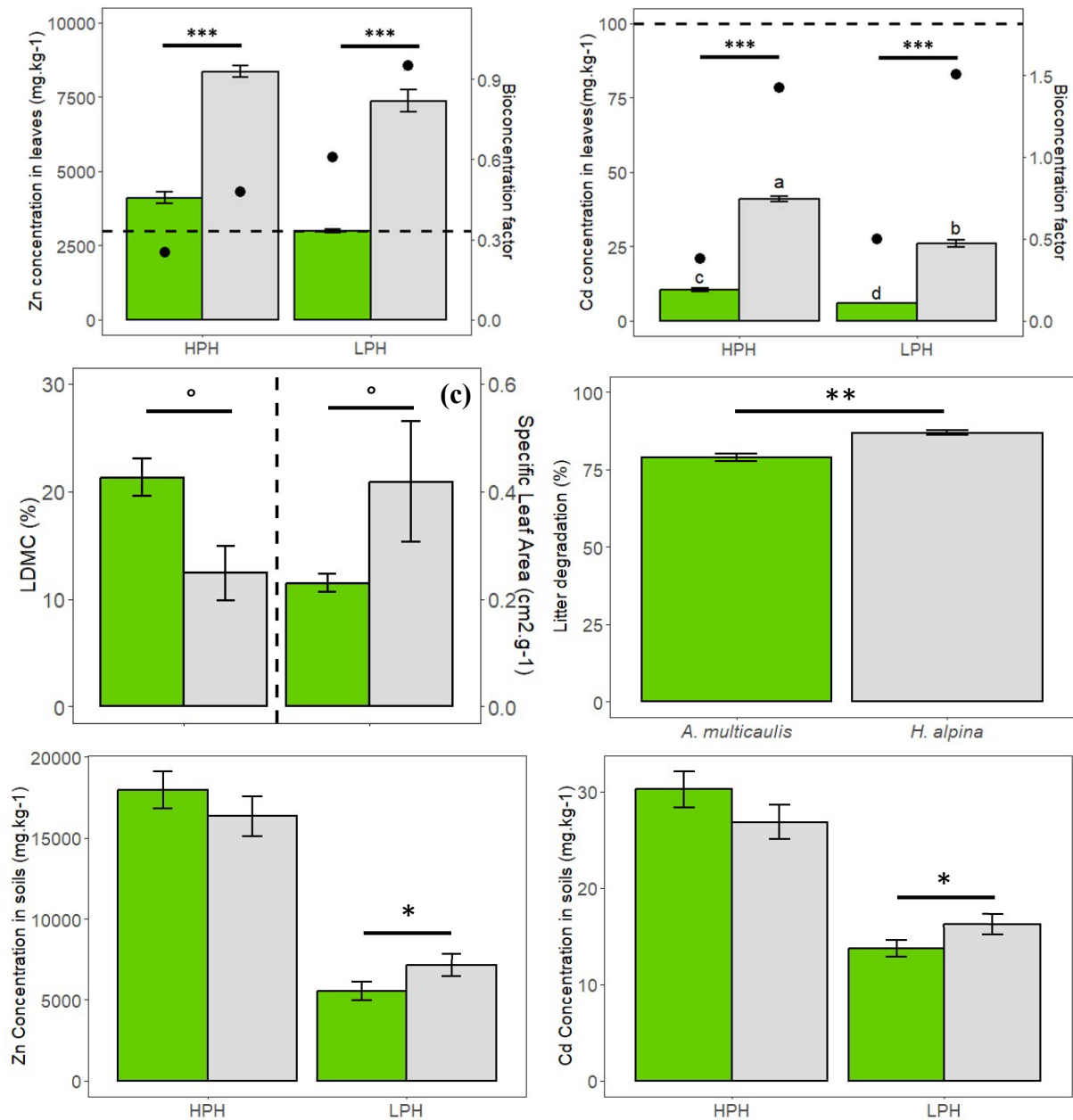


**Fig. 2** Trait differences between the two metallophyte species and soil metal availability variation. Means  $\pm$  SE are shown (*A. multicaulis*: green bars; *H. alpina*: grey bars). a, b) variation in Zn (a) and Cd (b) concentration (mg. kg<sup>-1</sup>) in the leaves of both metallophyte plants in the two habitats (HPH and LPH). In b) panel, different letters show the results of the posthoc Tukey HSD tests for the Habitat X Metallophyte species significant interaction. The dashed lines represent the suggested hyper-accumulation threshold for each element (Krämer, 2010). The Bioconcentration factors (the ratio between the metal concentration in the plant leaves and in the soil) are indicated the black points (see the second y-axis on the right) for both metallophyte species and habitats (one value for each combination). c) Variation of Leaf Dry Matter Content (% , left y-axis) and Specific Leaf Area (cm<sup>2</sup> g<sup>-1</sup>, right y-axis) of both

metallophyte species. Results of Wilcoxon–Mann–Whitney U tests are shown ( $^{\circ}$ ,  $P < 0.1$ ). d) Variation of litter decomposition (%) between both metallophyte plants. The Metallophyte species X Habitat interaction was not represented because not significant. e, f) variation in Zn (e) and Cd (f) bioavailability in soils ( $\text{mg. kg}^{-1}$ ) under both metallophyte plants (Control modality) in the two habitats (High Pollution and Low Pollution Habitats). In panels a), b), e), f), the results of one-way ANOVA for the effect of Metallophyte species on different traits within each habitat are shown above the bars (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

### *Survival of transplants in relation with Litter, Canopy effects and Total effects*

Considering the variation of  $\text{Imp}_{\text{Litter}}$  with the different treatments, there was a significant Habitat effect ( $p=0.002$ , Table 1a), due to overall negative values in LPH ( $p=0.024$  for the t-test on  $\text{Imp}_{\text{Litter}}$  different from 0 in LPH), versus neutral values in HPH. There was a marginally significant interaction between Habitat and Metallophyte species ( $p=0.077$ , Table 1a), with negative  $\text{Imp}_{\text{Litter}}$  values for *H. alpina* in LPH ( $p=0.036$  for the t-test) and neutral values for the same Metallophyte species in HPH and for *A. multicaulis* in both habitats (Fig. 3a). There was also a marginally significant *Agrostis* ecotype effect ( $p=0.087$ , Table 1a), with negative  $\text{Imp}_{\text{Litter}}$  values for NPO *A. capillaris* ( $p=0.043$  for the t-test) and neutral values for PO *A. capillaris*. Although the three-way interaction between Habitat, Metallophyte species and *Agrostis* ecotype was not significant,  $\text{Imp}_{\text{Litter}}$  for NPO *A. capillaris* in *H. alpina* and in LPH was significantly different from 0 ( $p=0.019$  for the t-test, Fig. S5).

Considering the variation of  $\text{Imp}_{\text{Canopy}}$  with the different treatments, there was a significant Habitat effect ( $p=0.028$ , Table 1b), due to the overall positive canopy effect in LPH ( $p<0.001$  for the t-test on  $\text{Imp}_{\text{Canopy}}$  in LPH) and more neutral values in HPH (Fig. 3b). There was also a marginally significant interaction between Habitat and Metallophyte species ( $p=0.097$ , Table 1b), with positive  $\text{Imp}_{\text{Canopy}}$  for *A. multicaulis* in both LPH ( $p=0.006$  for the t-test) and HPH ( $p=0.084$  for the t-test), and  $\text{Imp}_{\text{Canopy}}$  for *H. alpina* switching from positive ( $p<0.001$  for the t-test) in LPH to neutral in HPH (Fig. 3b). This interaction was also significant for final survival rates ( $p=0.001$ , Table S1), with a decrease in target survival in *H. alpina* when metal stress is increased (Fig. S4a).

Considering the variation of  $\text{Imp}_{\text{Total}}$  with the different treatments, there was a marginally significant interaction between Habitat and *Agrostis* ecotype ( $p=0.052$ , Table 1c), with  $\text{Imp}_{\text{Total}}$  values switching from positive to neutral for NPO *A. capillaris* from LPH to HPH,

whereas values remained positive in both habitats for PO *A. capillaris* (Fig. 3c). The same interaction was also significant for final survival rates ( $p=0.025$ , Table S1), with lower survival for NPO *Agrostis* than for PO *Agrostis* in HPH (Fig. S4b). Survival rates in the different neighbouring treatments ( $p<0.001$  for the Neighbouring modality effect, Table S1) confirmed the majority of positive total and canopy effects, with higher survival rates in (C-L) treatment than in (0-L) and (0-0) treatments (Fig. S4c). The interaction between Metallophyte species and *Agrostis* ecotype had a significant effect on survival rates ( $p=0.047$ , Table S1) with higher survival for NPO *Agrostis* in *H. alpina* than in *A. multicaulis* (Fig. S4d), but this interaction was not found on RII and Imp. The same interactions for RII as for Imp of Fig. 3 (see Fig. S6), show that the results were less significant but consistent with the Imp results (Table S5).

**Table 1** Results of the three-way ANOVAs for the effects of the Habitat, Metallophyte species and *Agrostis* ecotype treatments and their interactions on Imp<sub>Litter</sub> (a), Imp<sub>Canopy</sub> (b) and Imp<sub>Total</sub> (c). Models have been simplified using the stepAIC function (MASS package).

| <b>(a)</b>                                               |      | Imp <sub>Litter</sub> |              |    |
|----------------------------------------------------------|------|-----------------------|--------------|----|
| Factors                                                  | dF   | F                     | P            |    |
| Habitat                                                  | 1,19 | 11.77                 | <b>0.002</b> | ** |
| Metallophyte species                                     | 1,19 | 3.24                  | <b>0.087</b> | .  |
| <i>Agrostis</i> ecotype                                  | 1,19 | 3.24                  | <b>0.087</b> | .  |
| Habitat X Metallophyte species                           | 1,19 | 3.47                  | <b>0.077</b> | .  |
| <b>(b)</b>                                               |      | Imp <sub>Canopy</sub> |              |    |
| Factors                                                  | dF   | F                     | P            |    |
| Habitat                                                  | 1,16 | 5.79                  | <b>0.028</b> | *  |
| Metallophyte species                                     | 1,16 | 0.67                  | 0.421        |    |
| <i>Agrostis</i> ecotype                                  | 1,16 | 0.21                  | 0.650        |    |
| Habitat X Metallophyte species                           | 1,16 | 3.10                  | <b>0.097</b> | .  |
| Habitat X <i>Agrostis</i> ecotype                        | 1,16 | 0.70                  | 0.412        |    |
| Metallophyte species X <i>Agrostis</i> ecotype           | 1,16 | 0.82                  | 0.377        |    |
| Habitat X Metallophyte species X <i>Agrostis</i> ecotype | 1,16 | 2.10                  | 0.166        |    |
| <b>(c)</b>                                               |      | Imp <sub>Total</sub>  |              |    |
| Factors                                                  | dF   | F                     | P            |    |
| Habitat                                                  | 1,16 | 1.11                  | 0.307        |    |
| Metallophyte species                                     | 1,16 | 2.61                  | 0.125        |    |
| <i>Agrostis</i> ecotype                                  | 1,16 | 0.16                  | 0.690        |    |
| Habitat X Metallophyte species                           | 1,16 | 1.38                  | 0.256        |    |
| Habitat X <i>Agrostis</i> ecotype                        | 1,16 | 4.39                  | <b>0.052</b> | .  |
| Metallophyte species X <i>Agrostis</i> ecotype           | 1,16 | 1.20                  | 0.289        |    |
| Habitat X Metallophyte species X <i>Agrostis</i> ecotype | 1,16 | 1.97                  | 0.178        |    |

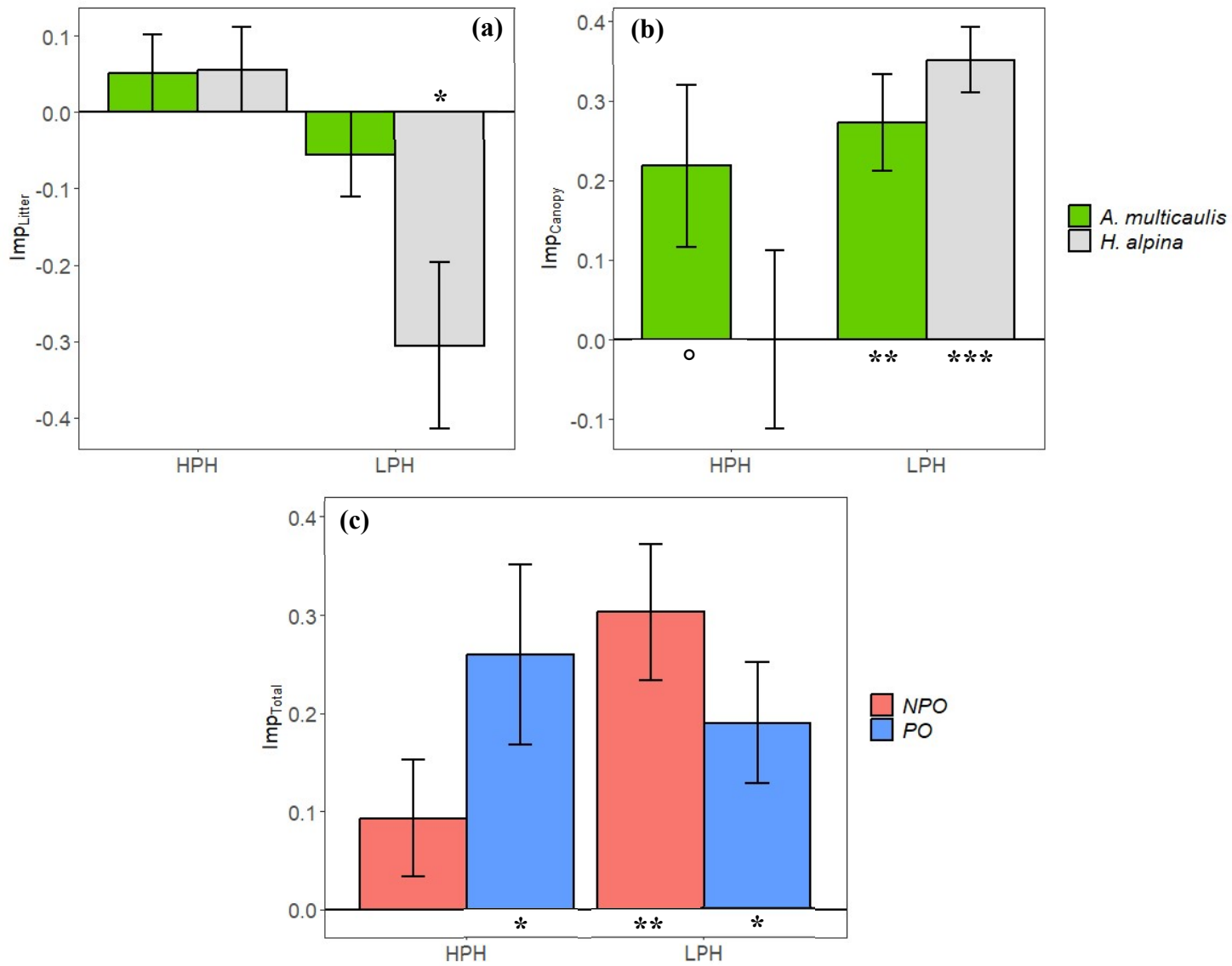


**Fig. 3** Variation of Importance Indices in both habitats according to Metallophyte species or *Agrostis* ecotype. Means  $\pm$  SE of the different Importance Indices (ImpCanopy, ImpTotal, ImpLitter) are shown. Differences from 0 are shown below each bar (one-sample t-test; °,  $P < 0.1$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). a) ImpLitter. b) ImpCanopy. c) ImpTotal.

## Discussion

Consistent with our first hypothesis, we observed a negative litter effect for the target ecotype with lower metal-tolerance (*Agrostis* NPO), exposed to the litter of the metallophyte species with higher leaf metal accumulation (*H. alpina*) and in situation of low pollution (in LPH). Regarding variation of canopy effects, we observed a habitat effect different to that assumed in the second hypothesis, with no increase of facilitation with increasing pollution. Contrary to our expectation for the third hypothesis, we did not observe any positive total effects with increasing pollution, since both canopy and litter effects were higher and lower in low and highly polluted habitats, respectively, leading to an absence of variation in total effects between habitats. Finally, only positive total effects were detected in this study, showing that when elemental allelopathy occurred, it was outweighed by more important canopy effects.

### *Differences in functional traits and effects of Metallophyte species*

The two metallophyte species present in our experimental sites differed regarding their functional traits and leaf metal-accumulation. *H. alpina* had a lower LDMC and a higher SLA and litter decomposition rate than *A. multicaulis*. LDMC and SLA are functional traits strongly related to the leaf Resource Economics axis and soil resource acquisition, with acquisitive species having lower LDMC (and nutrient rich short-lasting leaves) and higher SLA compared to conservative species with nutrient poor long-lasting leaves (Poorter & De Jong, 1999, Diaz *et al.*, 2016; Michalet *et al.*, 2023). Note that a larger sample size would have likely led to more significant differences in the functional traits of these two metallophyte plants. Consistently, *H. alpina* produced a litter easier to decompose (Cornwell *et al.*, 2008). Additionally, consistent with another study in the same valley (Delerue *et al.*, in prep b), we found that the more acquisitive species (*H. alpina*) accumulates more metals in its leaves compared to the less acquisitive species (*A. multicaulis*). Both species showed important leaf Zn concentration, above the accepted threshold to detect hyper-accumulating species (Krämer, 2010). In LPH, very high leaf Zn concentrations were maintained only for *H. alpina*, in relation with a higher bio-concentration ratio confirming the hyper-accumulating ability of this species. In summary, *H. alpina* has shorter-lasting leaves, easier to decompose, and with higher metal concentration, even on substrate with lower metal-availability: this species is more likely to produce negative litter effects by elemental allelopathy, as observed in this study. Recurrent production of this

metal-rich litter can even lead to a detectable increase of metal availability in the topsoil layer, as also shown here in LPH by comparison with *A. multicaulis*.

### *Elemental allelopathy: where, by whom and for whom?*

Empirical evidences of the elemental allelopathy are rather scarce in the literature, and most of the demonstrations concern hyper-accumulation of Selenium (Medhawi *et al.*, 2011a; Schiavon & Pilon-Smits, 2016), with some noticeable examples involving Zn hyper-accumulation (Bert *et al.*, 2000; Mohiley *et al.*, 2021) highlighting the need to precise the conditions of application of this hypothesis. Here, in agreement with the first hypothesis, we observed elemental allelopathic effects under very specific conditions. First, these effects were observed only for *H. alpina*, the species with highest acquisitive and accumulating capacities of the experiment, and having the faster litter decomposition. Secondly, elemental allelopathy was more pronounced for the *Agrostis* ecotype harvested in a non-metallicolous population and, thus, with no specific adaptations to cope with metal stress (see consistent results in Mohiley *et al.*, 2020 and Mehdaoui *et al.*, 2011a). Note that survival of this ecotype was particularly low in highly polluted habitats compared to the other ecotype (Fig. S4b, d), which is consistent with its assumed lower metal-tolerance. Finally, we observed these negative effects in habitats with low pollution, at the end of the ecological niche of the considered metallophyte species, and where metal accumulating species and less-tolerant plants interact. In such low-metalliferous environments, in the context of the study at rather low altitude and in mild environmental conditions, dominant species are expected to be more competitive than stress-tolerant (Grime, 1974; Nemer *et al.*, 2021). Therefore, elemental allelopathic effects could be particularly important to face the higher competitive interactions in corresponding habitats. Consistently, Mohiley *et al.* (2021) found an increase of Zn-accumulation by *Arabidopsis halleri* in case of competition for light. In summary, elemental allelopathy likely operates in habitats where negative interactions are frequent. However, in this study, the total effects of plant-plant interactions in LPH were not negative, but positive, which can appear contradictory. Note that former studies in the same valley and in similar LPH constantly found competitive interactions (Nemer *et al.*, 2022, 2023; Randé *et al.*, 2023), confirming that competition should be effective in LPH. In our study, the particularly extreme dry and hot event in spring and early summer 2022 likely lead to this exceptional switch from competitive to facilitative interactions as observed in the case of seasonal increase of drought stress (Delerue *et al.*, 2015).

### *Impact of the drought and heat wave on Canopy effects*

The exceptional heatwave that strokes the Northern hemisphere in 2022 likely contributed to other results contradicting our second hypothesis regarding the evolution of canopy effects between LPH and HPH. In opposition to former studies (Yang *et al.*, 2015; Nemer *et al.*, 2022; 2023), we did not find an increase of positive canopy effects with increasing pollution (Fig. 3b). During the heatwave, temperatures were 4 to 5 °C above the last 40 years mean in western Europe (Ibebuchi & Abu, 2023), with persistent anticyclonic conditions that exacerbated the drought in this region from April to June (Faranda *et al.*, 2023). Consequences of this extreme event caused serious damage to plant communities in the region, which strongly modified plant-plant interactions (Delerue *et al.*, in prep a). Michalet *et al.* (2014) proposed that in case of very intense stress, facilitative interactions should collapse, because of the decrease ability of nurse plants to mitigate stressful conditions for beneficiaries (Le Bagousse-Pinguet *et al.*, 2014). Such collapse of facilitation in case of intense drought and heatwave has already been reported in different contexts (Koyama & Tsuyuzaki, 2013; Guignabert *et al.*, 2020). In summary, a rational explanation for our results may be: i) a switch from competitive to facilitative canopy effects in low polluted habitats, as observed in the case of important seasonal drought stress (Delerue *et al.*, 2015), ii) a collapse of facilitative effects in HPH under *H. alpina* because of the very intense stress due to the combined effect of drought and important pollution (Fig. 3b). Noteworthy, canopy effects were better maintained in HPH for the other metallophyte species *A. multicaulis*. This species being more conservative according to the value of its functional traits, it is likely more drought-stress tolerant. It may have suffered less from the intense drought at stake, and have kept on provided microclimatic shelter.

### *Total effects as a synthesis of litter and canopy effects*

These unexpected results regarding canopy effects likely due to the extreme climatic conditions also likely explains why we were unable to validate the third hypothesis. Indeed, canopy effects being strongest in intermediate habitats, where the negative elemental allelopathy effects were also at play, we did not find a difference in total effects (Imp<sub>Total</sub>) between the two habitats. Results more consistent with the Stress Gradient Hypothesis and the third hypothesis, with important negative interactions in LPH (due to competition and elemental allelopathy) and positive interactions in HPH (due to positive canopy effects) may be more probable in years

with regular climatic conditions. The decrease of facilitation observed in this study in HPH seems to rather have affected the less tolerant targets (NPO *A. capillaris*), although it was for this ecotype that the strongest facilitation was expected in corresponding habitats (Fig. 3c; consistent with Nemer *et al.*, 2022, 2023). This less tolerant ecotype growing in a more benign environment is also likely more sensitive to different kind of stresses, including drought, and could have not benefited from potential facilitation in HPH because of the too harsh conditions due to multiple stresses.

We designed a field study to evaluate the effects due to Elemental Allelopathy in realistic field conditions, and to estimate their importance compared to canopy effects. We, thus, controlled litter quantity and its decomposition in (0-L) neighbouring modalities to estimate litter effects by comparison with (0-0) neighbouring modalities. Therefore, the litter effects were estimated in situations where the plant canopies were always absent, while canopy effects were estimated by comparisons of situations where litter was always present (Fig. 1). This is because we did not manipulate litter presence under nurse canopies not to disturb nurse plants. Further studies should investigate if both litter and canopy effects are simply additive or antagonistic or synergistic. In the latter case, it will be important to assess if the results reported here regarding the type of effect could change somehow according to the presence of the other compartment at stake (canopy or litter). Another limitation of our study is due to the litter addition all at once at the beginning of the experiment. It might slightly have increased the observed litter effects compared to the natural litter accumulation that takes place more gradually in spontaneous situations. But whatever the complexity of litter effects and of their interactions with plants canopies, they were taken into account in the calculation of total effects comparing the condition without canopy and litter to the condition with canopy and litter. Overall, when litter effects were detected, they were outweighed by more important canopy effects leading to overall total positive effects.

#### *On the ecological importance of Elemental Allelopathy*

Finally, because total effects were found to be positive, we may think that elemental allelopathy is not an important interaction in ecosystems with either important or low presence of metals in soils. However, this argument has to be tempered in regards of the exceptional climatic conditions during this study. As previously said, positive canopy effects were likely exacerbated

by this context, jeopardizing the possibility to reach negative total effects. In a climate change context, an increase of extreme climatic episodes like observed in 2022 is expected (Zscheischler *et al.*, 2020). On the one hand, negative allelopathic effects produced by hyper-accumulating species in habitats with low metal-stress may be offset by positive canopy effects as observed here. On the other hand, plant interactions may more frequently switch from competing to facilitation in such habitat (Brooker, 2006), and the need of allelopathic effects to withstand competition may be less pronounced. Additionally, it is worth mentioning that elemental allelopathic effects may be just one of the weapons available from a more diverse arsenal to withstand competition. For instance, the defense provided by metal-hyper-accumulation can lead to a decrease in some herbivores and pest pressure compared to their neighbours in low polluted habitats (Mohiley *et al.*, 2020) which may provide an additional competitive advantage. In summary, the evolution and importance of plant-plant interactions involving hyper-accumulating species in a global warming context is still an open question.

Still, we did find allelopathic effect produced by the metallophyte species with higher leaf metal accumulation and where we expected it: in low polluted habitats. It has been shown by phylogenetic studies that many hyper-accumulating species or populations had some pre-adaptations to metal tolerance before they colonized and adapted locally to metal-rich environments (Manara *et al.*, 2020). Meyer *et al.* (2016) showed that a gene directly involved in hyper-accumulation (Heavy Metal ATPase 4) was present in ancient non-metallicolous populations of *Arabidopsis halleri*, suggesting that its ability to hyper-accumulate Zn or Cd has evolved before colonization of metal-rich environments. Elemental Allelopathy, necessary to withstand competition in such habitat, may have contributed to selection of hyper-accumulating ability in these ancient populations, provided a simultaneous strong tolerance to metal exposure that helped colonization of metal-rich habitats that came afterwards (Meyer *et al.*, 2016). But, in any case, metal hyper-accumulation has evolved several times in different lineages and for different reasons and drivers (Manara *et al.*, 2020). Elemental Allelopathy may be only one of those drivers that operated only in some specific contexts.

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## Supplementary Information

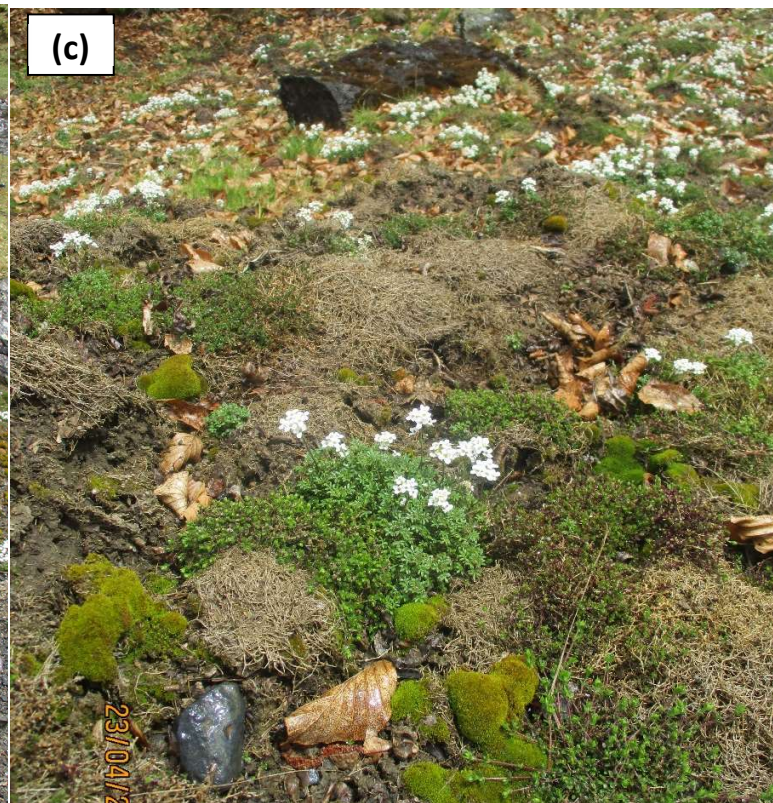
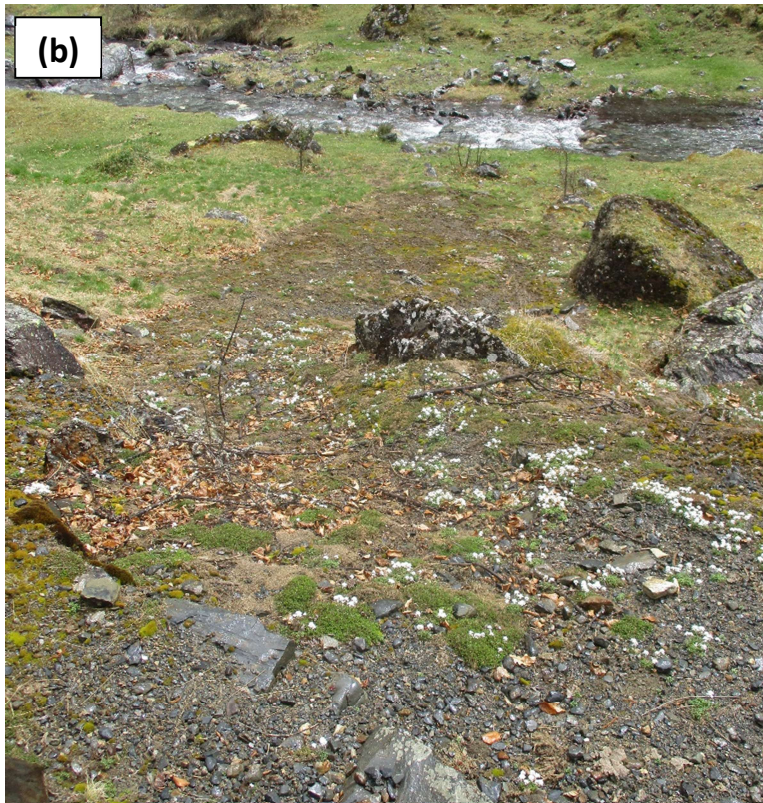
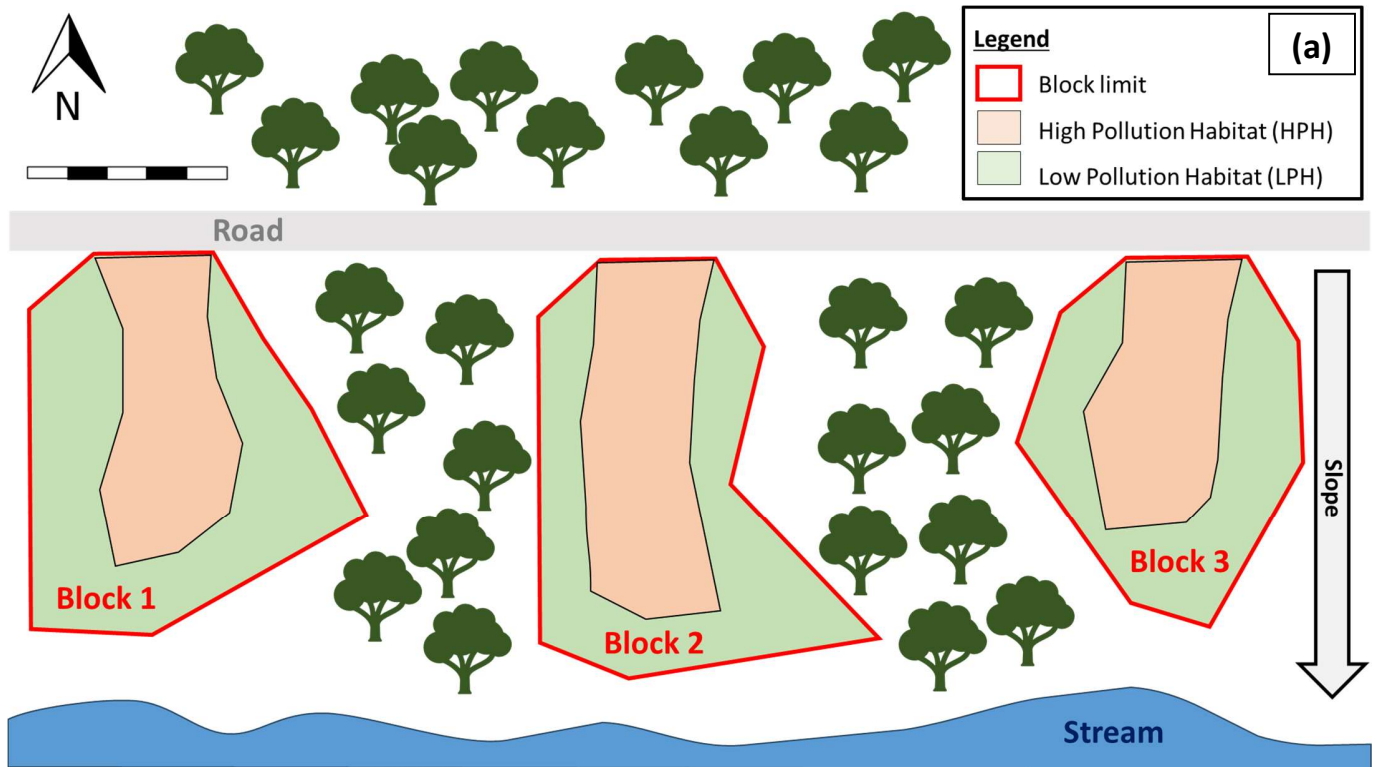


**Fig. S1** Illustration of the studied system.

a) Schematic representation of the study site; b) Picture of one block used for this study with areas heavily impacted by metal pollution (HPH, in the foreground) with smaller and sparse vegetation, mainly composed of metallophyte species (*A. multicaulis* and *H. alpina*) and less impacted areas surrounding them with denser vegetation (LPH, in the background) dominated by grass species but where metallophyte species are also present; c) Picture of the HPH, where *H. alpina* (species in flower) and *A. multicaulis* are growing side by side. Photo credit: Florian Delerue.



**Fig. S2** The two dominant metallophyte plant species. a) *Hutchinsia alpina*; b) *Arenaria multicaulis*. (Photo credit: Valentin Mauro)

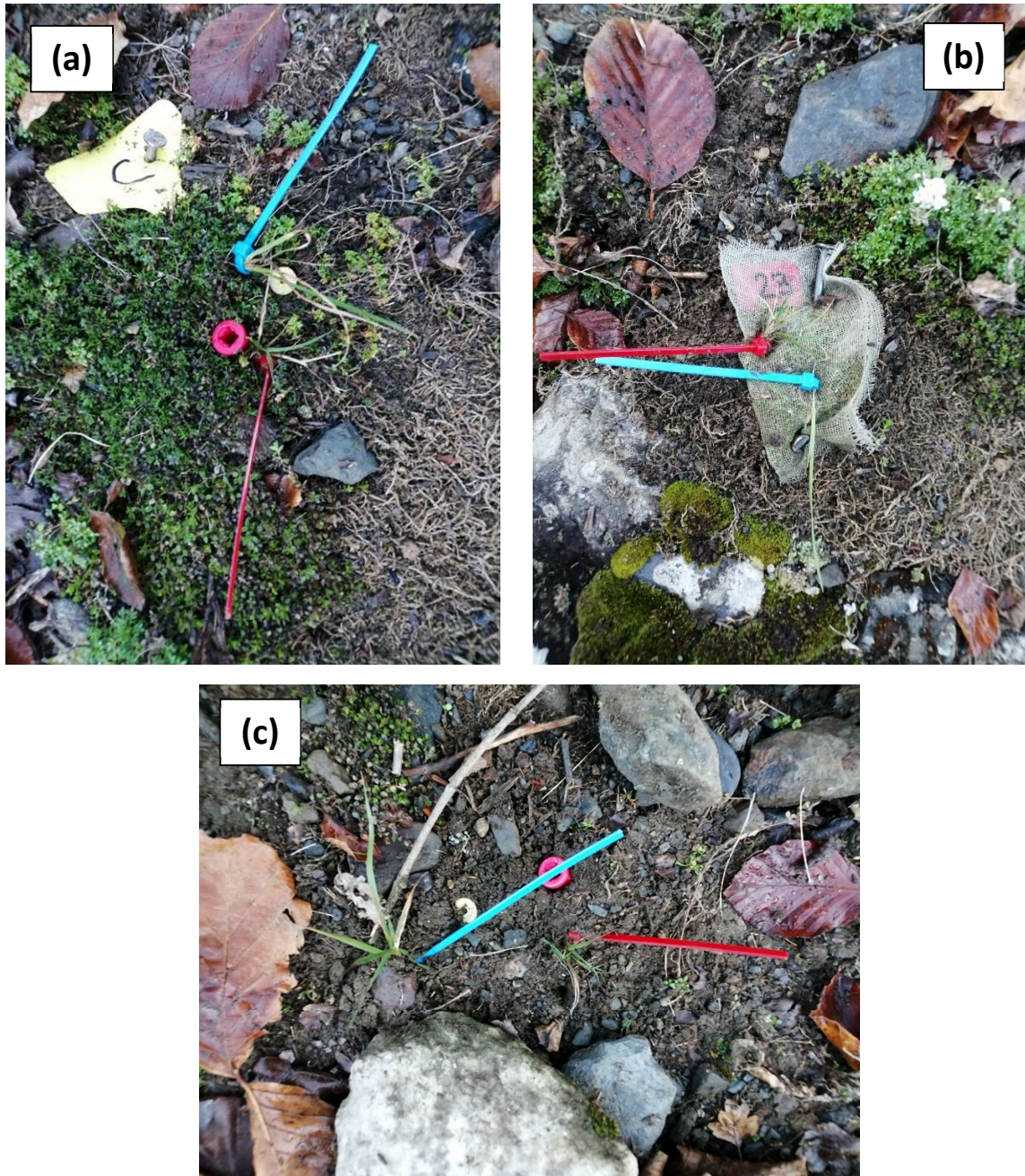


**Fig. S3** Pictures of the different neighbouring modalities used in the experiment.

*A. capillaris* individuals transplanted are marked with coloured cables (red for Polluted Origin ecotype and blue for Non-Polluted Origin ecotype). a) Control modality (C-L); b) Canopy-removal with added litter modality (0-L); c) Canopy and litter removal modality (0-0).

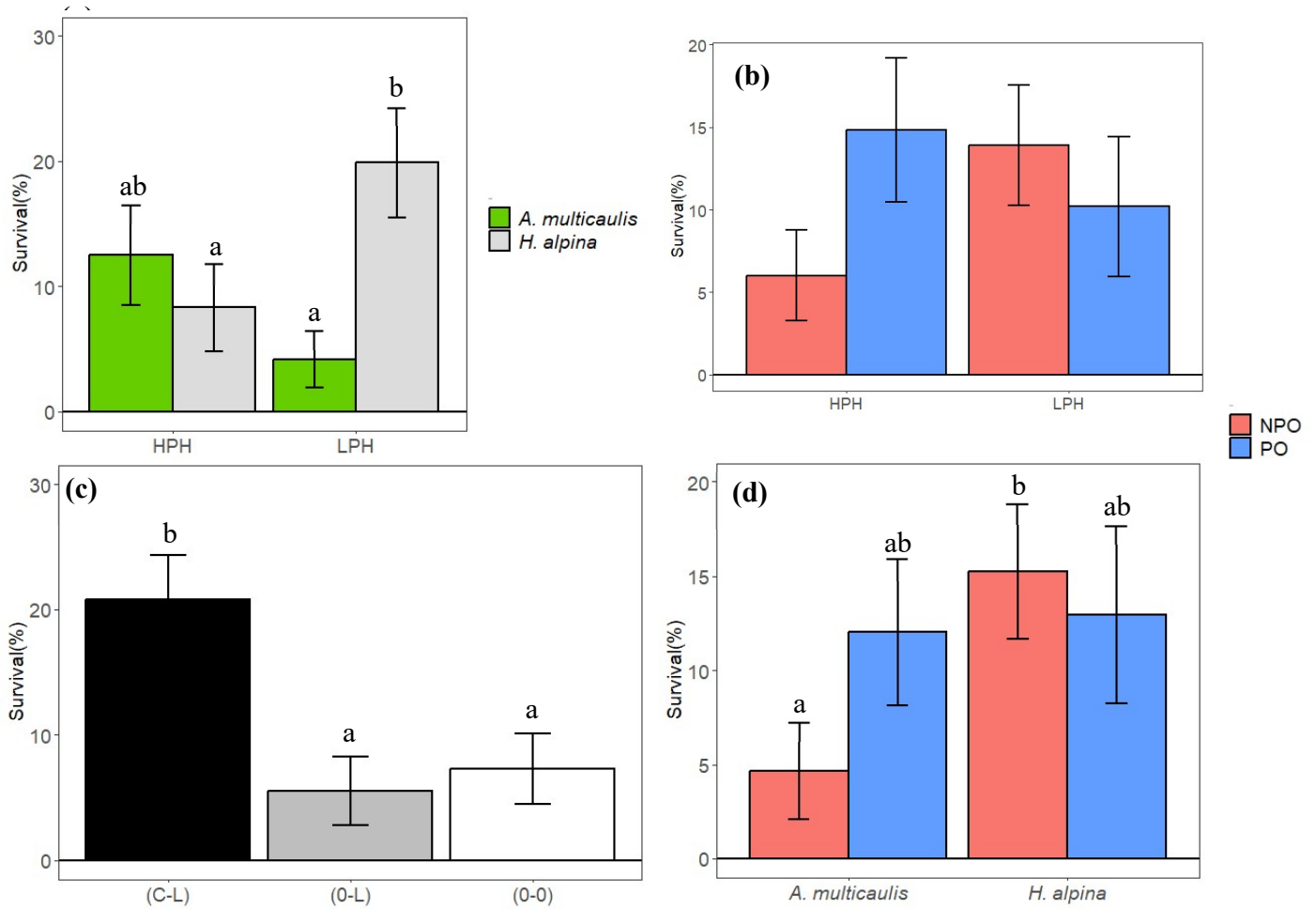
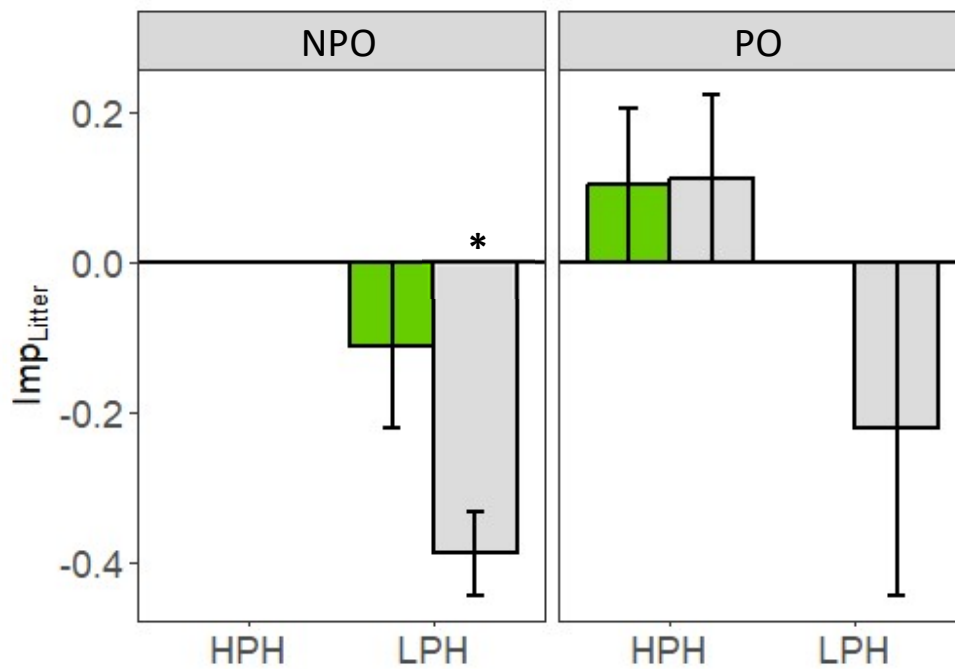


**Fig. S4** Target Survival (%) at the end of the experiment in relation to neighbouring modalities, habitats, metallophyte species and ecotypes.

The different panels illustrate the significant results found for target survival (Table S1). Means  $\pm$  SE are shown. a) effect of the interaction between habitat and metallophyte species. b) effect of the interaction between the kind of habitat and *Agrostis* ecotype. c) effect of neighbouring modality. d) effect of the interaction between metallophyte species and *Agrostis* ecotype. In all panels, different letters indicate significant difference between groups (posthoc Tukey HSD tests).

**Fig. S5.** Variation of Litter Importance Index depending on habitat, *Agrostis* ecotype and metallophyte species.

Means  $\pm$  SE of Litter Importance Index ( $\text{Imp}_{\text{Litter}}$ ) in both habitats (HPH and LPH) for the two ecotypes of *A. capillaris* (NPO and PO) according to metallophyte species (*A. multicaulis*: green bars; *H. alpina*: grey bars). Differences of  $\text{Imp}_{\text{Litter}}$  from 0 are shown next to each bar when significant (one-sample t-test; \* $P < 0.05$ ).



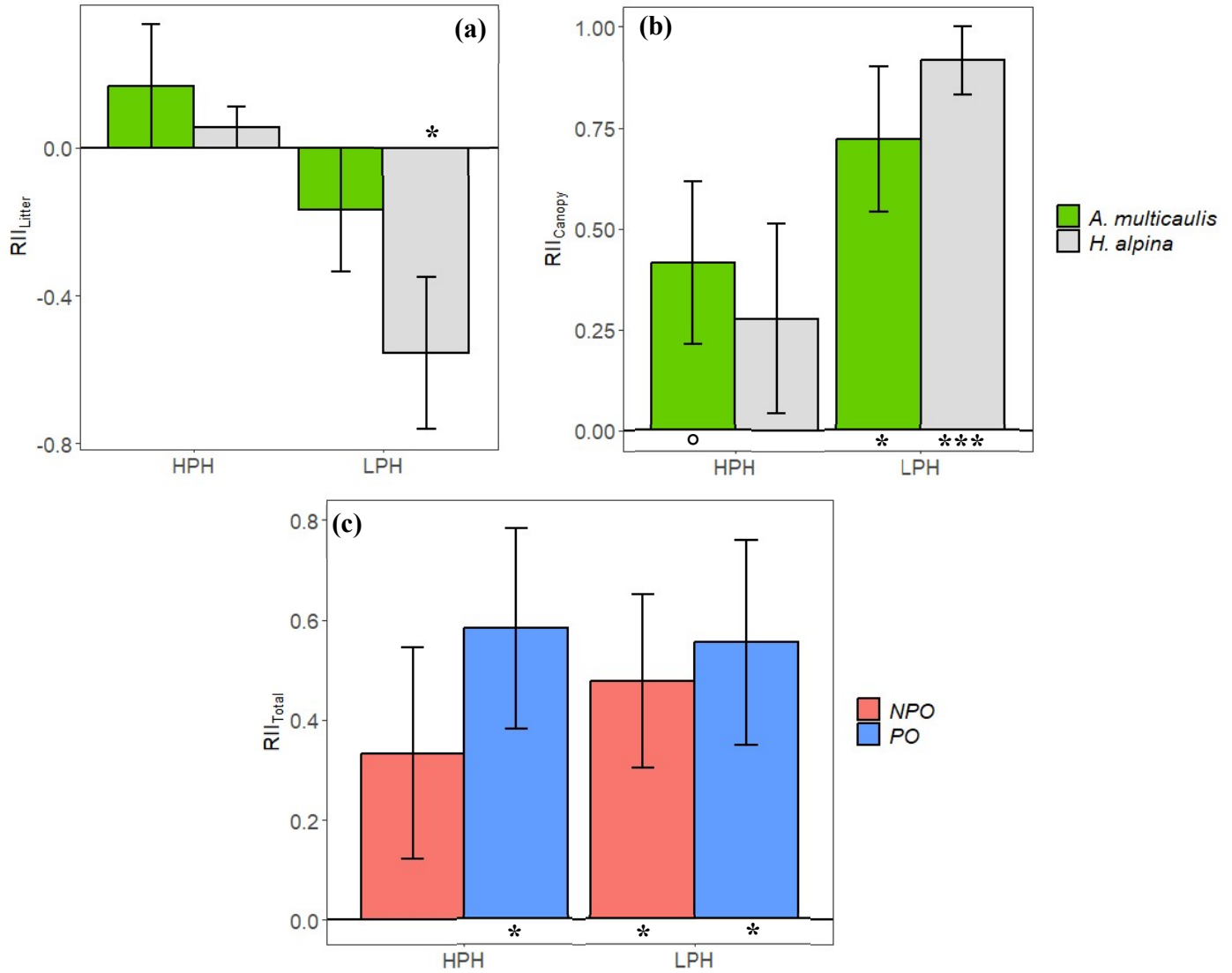


**Fig. S6** Variation of RII Indices in both habitats according to Metallophyte species or *Agrostis* ecotype.

Means  $\pm$  SE of the different RII Indices (RII<sub>Canopy</sub>, RII<sub>Total</sub>, RII<sub>Litter</sub>) are shown. Differences from 0 are shown below each bar (one-sample t-test; °,  $P < 0.1$ ; \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ). a) RII<sub>Litter</sub>. b) RII<sub>Canopy</sub>. c) RII<sub>Total</sub>. Below are the equations for calculating the different types of RII:

$$RII_{Litter} = \frac{(X_{0-L} - X_{0-0})}{(X_{0-L} + X_{0-0})} \quad RII_{Canopy} = \frac{(X_{C-L} - X_{0-L})}{(X_{C-L} + X_{0-L})} \quad RII_{Total} = \frac{(X_{C-L} - X_{0-0})}{(X_{C-L} + X_{0-0})}$$

where  $X_{0-L}$  is the value of the Survival when growing in the Canopy-removal with added litter modality,  $X_{0-0}$  is the value of the Survival when growing in Canopy and litter removal modality and  $X_{C-L}$  is the value of the Survival when growing in the Control modality.

| Factors                                                                          | Survival rate |       |                     |
|----------------------------------------------------------------------------------|---------------|-------|---------------------|
|                                                                                  | dF            | F     | P                   |
| Habitat                                                                          | 1,46          | 0.34  | 0.562               |
| Metallophyte species                                                             | 1,46          | 3.76  | <b>0.058</b> .      |
| <i>Agrostis</i> ecotype                                                          | 1,46          | 0.25  | 0.617               |
| Neighbouring modality                                                            | 2,46          | 11.57 | <b>&lt;0.001***</b> |
| Habitat X Metallophyte species                                                   | 1,46          | 10.96 | <b>0.001 **</b>     |
| Habitat X <i>Agrostis</i> ecotype                                                | 1,46          | 5.31  | <b>0.025 *</b>      |
| Metallophyte species X <i>Agrostis</i> ecotype                                   | 1,46          | 4.14  | <b>0.047 *</b>      |
| Habitat X Neighbouring modality                                                  | 2,46          | 1.90  | 0.160               |
| Metallophyte species X Neighbouring modality                                     | 2,46          | 0.96  | 0.389               |
| <i>Agrostis</i> ecotype X Neighbouring modality                                  | 2,46          | 0.96  | 0.389               |
| Habitat X Metallophyte species X <i>Agrostis</i> ecotype                         | 1,46          | 1.73  | 0.194               |
| Habitat X Metallophyte species X Neighbouring modality                           | 2,46          | 1.07  | 0.349               |
| Habitat X <i>Agrostis</i> ecotype X Neighbouring modality                        | 2,46          | 0.59  | 0.553               |
| Metallophyte species X <i>Agrostis</i> ecotype X Neighbouring modality           | 2,46          | 0.18  | 0.828               |
| Habitat X Metallophyte species X <i>Agrostis</i> ecotype X Neighbouring modality | 2,46          | 1.58  | 0.215               |

**Table S1** Results of the four-ways ANOVAs for the effects of the Habitat, Metallophyte species, *Agrostis* ecotype, Neighbouring modality and their interactions on Survival rate. The block effect was kept in this statistical model since it was marginally significant (P=0.058), but not shown in the table. The rates were arcsine transformed before analysis.

| <b>a) Zn</b>                   | dF   | F     | P                   |
|--------------------------------|------|-------|---------------------|
| Habitat                        | 1,36 | 19.9  | <b>&lt;0.001***</b> |
| Metallophyte species           | 1,36 | 337.3 | <b>&lt;0.001***</b> |
| Habitat X Metallophyte species | 1,36 | 0.1   | 0.772               |

| <b>b) Cd</b>                   | dF   | F     | P                   |
|--------------------------------|------|-------|---------------------|
| Habitat                        | 1,36 | 126.8 | <b>&lt;0.001***</b> |
| Metallophyte species           | 1,36 | 854.3 | <b>&lt;0.001***</b> |
| Habitat X Metallophyte species | 1,36 | 36.1  | <b>&lt;0.001***</b> |

**Table S2** Results of the two-way ANOVAs on the effects of the Habitat, Metallophyte species and their interactions on metal concentration in leaves.

| Factors                        | Litter degradation |       |                 |
|--------------------------------|--------------------|-------|-----------------|
|                                | dF                 | F     | P               |
| Habitat                        | 1,7                | 0.39  | 0.549           |
| Metallophyte species           | 1,7                | 19.42 | <b>0.003 **</b> |
| Habitat X Metallophyte species | 1,7                | 1.29  | 0.292           |

**Table S3** Results of the two-ways ANOVAs for the effects of the Habitat, Metallophyte species, and their interactions on litter decomposition rate. The block effect was kept in this statistical model since it was significant ( $P=0.005$ ), but not shown in the table. The rates were arcsine transformed before analysis.

| <b>a) Zn</b>                   | dF   | F     | P                   |
|--------------------------------|------|-------|---------------------|
| Habitat                        | 1,44 | 61.38 | <b>&lt;0.001***</b> |
| Metallophyte species           | 1,44 | 2.35  | 0.132               |
| Habitat X Metallophyte species | 1,44 | 0.36  | 0.550               |

| <b>b) Cd</b>                   | dF   | F     | P                   |
|--------------------------------|------|-------|---------------------|
| Habitat                        | 1,44 | 42.82 | <b>&lt;0.001***</b> |
| Metallophyte species           | 1,44 | 2.47  | 0.122               |
| Habitat X Metallophyte species | 1,44 | 1.02  | 0.317               |

**Table S4** Results of the two-way ANOVAs for the effects of the Habitat, Metallophyte species, and their interactions on metal availability in soils estimated by acid acetic extraction.

| <b>(a)</b>                                               |      | RII <sub>Litter</sub> |                 |
|----------------------------------------------------------|------|-----------------------|-----------------|
| Factors                                                  | dF   | F                     | P               |
| Habitat                                                  | 1,16 | 10.39                 | <b>0.004 **</b> |
| Metallophyte species                                     | 1,16 | 2.91                  | 0.103           |
| <i>Agrostis</i> ecotype                                  | 1,16 | 4.35                  | <b>0.049 *</b>  |
| <b>(b)</b>                                               |      | RII <sub>Canopy</sub> |                 |
| Factors                                                  | dF   | F                     | P               |
| Habitat                                                  | 1,16 | 6.14                  | <b>0.024 *</b>  |
| Metallophyte species                                     | 1,16 | 0.02                  | 0.885           |
| <i>Agrostis</i> ecotype                                  | 1,16 | 0.19                  | 0.667           |
| Habitat X Metallophyte species                           | 1,16 | 0.76                  | 0.394           |
| Habitat X <i>Agrostis</i> ecotype                        | 1,16 | 0.34                  | 0.567           |
| Metallophyte species X <i>Agrostis</i> ecotype           | 1,16 | 0.34                  | 0.567           |
| Habitat X Metallophyte species X <i>Agrostis</i> ecotype | 1,16 | 1.72                  | 0.207           |
| <b>(c)</b>                                               |      | RII <sub>Total</sub>  |                 |
| Factors                                                  | dF   | F                     | P               |
| Habitat                                                  | 1,16 | 0.08                  | 0.773           |
| Metallophyte species                                     | 1,16 | 1.24                  | 0.280           |
| <i>Agrostis</i> ecotype                                  | 1,16 | 0.69                  | 0.416           |
| Habitat X Metallophyte species                           | 1,16 | 0.02                  | 0.878           |
| Habitat X <i>Agrostis</i> ecotype                        | 1,16 | 0.19                  | 0.667           |
| Metallophyte species X <i>Agrostis</i> ecotype           | 1,16 | 0.16                  | 0.687           |
| Habitat X Metallophyte species X <i>Agrostis</i> ecotype | 1,16 | 2.83                  | 0.111           |

**Table S5** Results of the three-way ANOVAs for the effects of the Habitat, Metallophyte species and *Agrostis* ecotype treatments and their interactions on RII<sub>Litter</sub> (a), RII<sub>Canopy</sub> (b) and RII<sub>Total</sub> (c). Models have been simplified using the stepAIC function (MASS package).