Relative contribution of canopy and soil effects between plants with different metal tolerance along a metal pollution gradient

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Abstract

Multiple effects, operating either on the long-term (soil-engineering effects) or on the short-term during plant life (microclimate modification or resources pre-emption), can act simultaneously and determine the outcome of plant-plant interactions. These diverse effects have not been disentangled along a gradient of metal/metalloid pollution, although this is crucial for understanding the dominant species turnover along the gradient, and thus the driving processes of facilitation recurrently found in metalliferous ecosystems, which could help improving ecological restoration of these degraded ecosystems.

Here, we experimentally assessed different short-term effects of two dominant forbs of highly polluted habitats (*Hutchinsia alpina* and *Arenaria multicaulis*, tolerant to metal stress) and two grasses of less polluted habitats (*Agrostis capillaris* and *Festuca rubra*, less tolerant to metal stress) on target plant species (the same as the dominant species mentioned above) transplanted along a large metal pollution gradient. Additionally, in highly polluted environments, we differentiated short- from long-term effects of the two metallicolous forbs, which had different abilities to concentrate metals in their leaves.

In line with other studies along metal gradients, variation of short-term interactions appeared to follow the Stress Gradient Hypothesis for plants less adapted to metal pollution ($p = 0.030$), with positive interactions dominating in most severe areas. Regarding long-term effects, the species with highest leaf metal-accumulation showed no negative effect contrary to the Elemental allelopathy Hypothesis. Long-term effects of the species with lower leaf-metal accumulation could not be determined because of the occurrence of an unexpected difference in

micro-habitat conditions (soil depth and humidity) for this species along the metal pollution gradient.

Increasing short-term facilitation along metal pollution gradients, which confirmed previous studies, is promising for improving conditions and restoring the most polluted environments. However, long-term results stressed the difficulty to quantify these effects given that these areas are highly fragmented and heterogeneous.

Keywords:

stress gradient hypothesis, elemental allelopathy hypothesis, metals and metalloids, stress tolerance, habitat heterogeneities, ecological restoration.

1. Introduction

Plant interactions play a crucial role in plant community assembly (Lortie et al. 2004; Brooker et al. 2007) but the strength and type of interactions depend on different drivers (Michalet et al. 2014). The Stress Gradient Hypothesis (hereafter SGH, Bertness and Callaway, 1994) predicts an increase in facilitative interactions and a decrease in competitive interactions as environmental stress (or disturbance) increases. For example, on soils rich in metals and metalloids (hereafter referred to as 'metals') several studies have shown that facilitation is the dominant plant interaction (Zvereva and Kozlov, 2004; Wang et al. 2013; Domínguez et al. 2015; Yang et al. 2015). Indeed, metal in excess in soils correspond to harsh habitats having strong negative impacts on plants, inducing communities with low diversity and degraded vegetation.

Species ability to cope with environmental stresses primarily drives their position along stress gradients and ultimately plant interactions. The trade-off between plant competitive and stress-tolerance abilities (Grime, 1979; Liancourt et al. 2005; Nemer et al. 2021) explains why most competitive species (also called effect species) dominate benign habitats whereas stress-tolerant species dominate stressful habitats. This is in line with the SGH and consistent with the recognized importance of competition between plants in low-stressed habitats, and the dominance of stress-tolerant species and the importance of facilitative interactions in more constrained environments. Moreover, plant interactions also depend on the sensitivity of companion species (also called response species) response to the environmental stress at stake. Facilitation in harsh ecosystems is particularly marked for those species that are sensitive to environmental stress and that rely on stress alleviation by neighbouring plants for their development. Actually, facilitation first benefits species with relatively low stress tolerance (and higher competitive ability), whose ecological optimum occurs in benign environments (Liancourt et al. 2005; Gross et al. 2010). Thus, the

61 outcome of plant interactions depends on the stress tolerance of both the dominant effect species
62 and the beneficiary response species (Nemer et al. 2021, 2023).

63 Additionally, the outcome of plant interactions is the balance of multiple combined effects
64 produced by a plant in its immediate vicinity, which could be either positive or negative (Callaway
65 and Walker, 1997). For example, Callaway et al. (1991) have shown in Central California that a
66 phenotype of *Quercus douglasii* had negative root-interference effects that overwhelmed its
67 positive shading effect for understorey herbs. Pescador et al. (2014) revealed that facilitation
68 through increasing nutrients could be offset by negative effects of light competition. Wang et al.
69 (2017) also found on the Tibet Plateau contrasting soil and canopy effects of the shrub *Dasiphora*
70 *fruticosa* on understorey herbs, with a trade-off between positive root effects and negative canopy
71 effects. All these studies highlight the diversity of co-occurring plant effects that can be separated
72 in two categories according to the time scale of the interactions (Noumi et al. 2016): i) "Short-term
73 Effects" (STE) acting during plant life and; ii) "Long-Term Effects" (LTE) acting at the time scale
74 of soil evolution processes and remaining after plants death. STE are for example microclimate
75 modifications (Guignabert et al. 2020) and effects related to competition for resources (light
76 interception, water and nutrient uptake, Callaway et al. 1991), whereas LTE correspond to
77 ecosystem-engineering effects (soil organic matter enrichment, Navarro-Cano et al. 2019b;
78 increase in nutrient availability, Pescador et al. 2014; improvement of water retention, Noumi et
79 al. 2010; but also accumulation of toxic compounds and allelopathic interference, Michalet et al.
80 2017).

81 The two aforementioned types of effect can be experimentally separated by combining the
82 two most common methods for quantifying interactions between plants: the observational method
83 (comparison of a target performance with neighbours and in naturally open areas) and the removal

84 method (comparison of a target performance with neighbours and in removed-neighbours
85 conditions). The removal method quantifies STE since soil evolution processes are identical with
86 neighbours and in recently removed-neighbours conditions. The observational method quantifies
87 the "Net Effects" (NE, i.e., the sum of STE and LTE). Finally, LTE are quantified by comparing
88 target responses in removed-neighbours versus open conditions, conditions that differ in soil
89 evolution processes due to effect plants. However, the observational method can only be applied
90 in habitats with naturally occurring open conditions (i.e., without soil fully covered by plants), and
91 therefore only in highly stressed or disturbed environments. In benign environments, only the
92 removal method can be applied to quantify plant interactions because vegetation cover is high and
93 open areas are absent. Competition being the most important interaction in such benign context,
94 the removal method is more adapted to evaluate corresponding competitive effects since they are
95 related to resource pre-emption and they occur during the life of the plant producing the effects
96 (and therefore corresponding to STE). However, in more constrained environments where both
97 methods are available, consideration of the method used to quantify neighbour effects is crucial
98 because different methods can provide contrasting results (Maestre et al. 2003, 2005). Michalet
99 (2006) argued that the removal method is more likely to reveal negative interactions compared to
100 the observational method in arid environments. Indeed, in degraded arid ecosystems with
101 important soil erosion, vegetated patches are generally located on deeper soils than open ones,
102 leading to a higher performance of target plants in Removal modality than in the open, explaining
103 these results. If these soil differences are due to ecosystem-engineering effects, they can be
104 appropriately quantified by LTE measurements. However, if they correspond to pre-existing
105 environmental differences (within-habitat environmental heterogeneity existing before the arrival

of the plants), then LTE measurements can provide misleading interpretation regarding plant interactions (Steinbauer et al. 2016, but see Michalet et al. 2023b).

In metal-rich environments (including mine tailings), the outcome of plant interactions rationally depends on the same drivers presented above. Indeed, as along other stress gradients, there is a strong turnover of dominant species with increasing metallic pollution, in relation with a trade-off between competitive abilities and metal-stress tolerance (Nemer et al. 2022). In low-polluted habitats, non-metallicolous species with strong competitive abilities are dominant, whereas in highly polluted habitats metallicolous species with metal-tolerance abilities dominate. Positive effects of dominant plants are therefore expected in metal-rich environments, especially for the less metal-stress tolerant target species or ecotypes. Actually, Nemer et al. (2022) found that ecotypes of the grass *Agrostis capillaris* from low polluted areas and with lower stress tolerance were more facilitated than ecotypes from highly polluted areas and with higher stress tolerance. Similarly, facilitative interactions have been shown to increase for targets having higher functional distance from metal-tolerant nurse plants, i.e., being presumably less stress-tolerant (Navarro-Cano et al. 2019a). However, metallicolous plant communities are often the result of very specific plant assemblages (Baumbach, 2012), suggesting the disappearance: i) of metallicolous species in more benign environments likely due to competitive exclusion, and ii) of less tolerant plant species in environments with highest level of metal stress.

Consistently with the ecological literature, we suggest that to depict the driving mechanisms of changes in plant-plant interactions along metal pollution gradients, the type of effect quantified has to be considered, and should help explaining the species turnover in plant communities observed along such gradients, and thus the different interactions at stake depending on the level of pollution. Most studies that have assessed facilitation in metal-rich environments have only used

129 the observational method and did not disentangle STE from LTE in the overall effects of plants on
130 their neighbours (Cuevas et al. 2013; Domínguez et al. 2015; Navarro-Cano et al. 2019b).
131 Furthermore, to cope with high metal content, some metallicolous species accumulates metals in
132 their leaves (Baker et al. 1981; Van der Ent et al. 2013), which can lead to specific soil effects such
133 as allelopathic effects that deserved to be considered. The Elemental allelopathy Hypothesis
134 proposed that important accumulation of metals in leaves and then release of important amount of
135 bio-available toxic compounds through litter decomposition afterwards can jeopardize the
136 development of neighbouring plants (Boyd and Jaffré, 2001; Morris et al. 2009). This is a LTE
137 specific to metal-rich systems. Thus, as in other harsh ecosystems, both STE and LTE can act
138 simultaneously in metal-rich environments. For instance, Randé et al. (2023) showed in a metal-
139 polluted slag heap that a nurse species with high amount of metals in its leaves, had negative LTE,
140 probably due to elemental allelopathy but had also positive STE due to microclimate amelioration.
141 This study was innovative because it explicitly disentangled LTE from STE in a metal-rich
142 ecosystem. However, this pioneer approach was conducted in a homogeneous contaminated
143 environment. Thus, the evolution of STE and LTE along metal-stress gradients remains to be
144 explored.

145 Regarding STE, several studies have shown that nurse plants in polluted habitats can
146 produce a fist kind of positive effects by alleviation of climatic stress and improvement of micro-
147 climatic conditions (Zvereva and Kozlov, 2007; Dominguez et al. 2015). This likely enables target
148 plants to better cope with metal-stress. In line with these results, alleviation of such climatic stress
149 during STE should be even more important as metal-stress increases. Consistently, we expect STE
150 to increase with metal pollution in soils, as observed in Nemer et al. (2022). Regarding LTE, the
151 expected effects likely depend on the nurse plant ability to accumulate metals in its leaves (Randé

et al. 2023). In case of a leaf-accumulating nurse plant, elemental allelopathy may be involved. This negative effect has been shown to operate more importantly on neighbouring plants with low level of metal-stress tolerance (El Mehdawi et al. 2011; Mohiley et al. 2020) and in environments with moderate level of metal presence in soils (Jaffe et al. 2018). For these reasons, along a metal pollution gradient, negative LTE (due to elemental allelopathy) are more likely to be observed at the intermediate part of the gradient because: i) neighbouring plants are not all strongly metal-stress tolerant and some can be sensitive to elemental allelopathy and ii) in the most severe polluted habitats, the additional release of toxic compounds through litter decomposition should be negligible compared to the overall excess of metal in soils (Morris et al. 2006). When nurse plants do not accumulate metals in their leaves, positive LTE can occur, mainly through improvement of soil conditions in particular of nutrient availability (Frérot et al. 2006) or microbial and enzymatic activity (Yang et al. 2015). As plants become increasingly stressed along metal gradient, in particular for non-metallicolous ones, it is expected that these positive LTE will become increasingly important along the stress gradient.

In this study, we investigated the changes in direction of both STE and LTE on target plants with different metal-tolerance along a metal stress gradient, in a former mining sector in the French Pyrenees. Specifically:

1. We aim to study the variation of STE along the gradient and according to the metal tolerance of the target species. We assumed that STE should be mainly negative in the more benign part of the gradient, in particular for metallicolous targets, and mainly positive in the most severe part of the gradient, in particular for non-adapted targets, consistent to the Stress Gradient Hypothesis.

Two additional assumptions were dedicated to LTE, in the most polluted plots:

2. We aim to study the variation of LTE for the most accumulating nurse species and according to the metal tolerance of the target species. We expect a decrease of potential negative elemental allelopathy effects, in particular for target species with low metal-stress tolerance, consistent to Elemental allelopathy Hypothesis.
3. We aim to study the variation of LTE for the least accumulating nurse species and according to the metal tolerance of the target species. We expect an increase of potential positive effects, in particular for target species with low metal-stress tolerance, consistent to the ecological literature related to positive soil effects in metal polluted environments."

2. Material and methods

2.1. Study site and metal pollution gradient

The study site is located in the "Cirque de La Plagne", Sentein, Ariège, France. This area has been marked by significant mining activity between the 1850s and 1950s for the extraction of zinc and lead ore. The site for this experiment (42°49′N, 0°56′E) was a former ore-washing area located at 1100 m a.s.l. in the Pyrenean mountain range. It is composed by mineral deposits contaminated with heavy metals (Zn, Pb and Cd and other metals in excess like Cu, As and Al, see metal pollution range in Supplementary Table S1). The meteorological conditions quantified during the time of the experimentation and the growing season (mid-April to mid-October 2021) by a WS-GP1 weather station (Delta-T Devices) located less than 1 km from the study site were 13.9°C for mean temperature and 485 mm for total precipitation.

Within the site, the mineral deposits are heterogeneous, creating a Metal Pollution Gradient (hereafter MPG, Supplementary Table S1). The total concentration of Pb and Zn increased from

198 0.03% and 0.12% in least polluted habitats to 0.75% and 3.85% in most polluted habitats,
199 respectively (mass of metal per mass of soil). Vegetation volume (the product of vegetation cover
200 and mean vegetation height) varied from 8 $dm^3.m^{-2}$ in the most polluted areas to 257 $dm^3.m^{-2}$ in
201 the least polluted areas. Along this gradient, there was a strong turnover in dominant species: in
202 the most impacted areas, there was a sparse vegetation consisting only of metallicolous species,
203 whereas in the less polluted areas, there was a more consistent vegetation consisting only of non-
204 metallicolous species. In the intermediate habitats, there was a mixture of metallicolous and non-
205 metallicolous species, depending on the level of pollution of the environment (Supplementary Fig.
206 S1).

207 Several variables related to soil metal content (total Zn, Pb and available Zn, Pb, Cd with
208 acetic acid extraction) and more complex environmental variables (soil texture, organic matter,
209 C/N, pH, soil moisture, vegetation volume) have been collected in our study site in 2020 and 2021
210 to produce kriging maps for each of these variables over the whole site. For each of the
211 experimental plots of this study (see next paragraph), their precise position were determined using
212 a differential GPS (© Leica GS10). Then, we used the prediction on the kriging maps of all
213 environmental variables to estimate their value for each plot. Finally a Principal Component
214 Analysis for all these variables enabled to build a synthetic metric (PCA_{MPG}) summarizing the
215 variation of environmental variables between plots and quantifying their position along the metal
216 pollution gradient (Supplementary Fig. S2).

217

218 *2.2. Experimental design*

To replicate our treatments, the experimental site was separated in four blocks each including the whole metal-pollution gradient (Fig. 1A & B). In each block, and in order to assess and compare the outcome of plant-plant interactions along this gradient, four plots of approximately 1 m^2 have been determined (2 plots in highly polluted areas, where metallicolous species dominate, and 2 plots in less polluted areas, where non-metallicolous species dominate). For each plot, we selected two nurse species that were the two most dominant species. In the polluted plots, the dominant metallicolous species were *Hutchinsia alpina* (hereafter *H. alpina*) and *Arenaria multicaulis* (hereafter *A. multicaulis*). Analyses of leaves samples, collected in the immediate vicinity of the study area (less than 1 km), and after careful washing with deionised water, showed significant differences in terms of leaf metal concentration between these two species. *H. alpina* accumulated 4 times more Cd (40.87 ± 0.98 vs 10.46 ± 0.51 $mg.kg^{-1}$), more Pb (1481 ± 35 vs 913 ± 43 $mg.g^{-1}$), and two times more Zn (8351 ± 200 vs 4098 ± 196 $mg.kg^{-1}$) than *A. multicaulis*. *H. alpina* is a species from the *Brassicaceae* family known to present many hyper-accumulating species (Reeves et al. 2018), and for which previous study have also confirm its very high ability to accumulate Zn in its leaves (Delerue et al., in prep). For the plots in lowest polluted areas, the two dominant nurse species used were the grass species *Agrostis capillaris* (hereafter *A. capillaris*) and *Festuca rubra* (hereafter *F. rubra*).

For plant interactions measurements we choose to apply both the removal and observational procedures (Michalet, 2006) in order to separate STE from LTE. Thus on April 19th, 2021, we selected in each plot, 12 individuals of each nurse species, and transplanted one individual of each target species into each of these nurse individuals ("Control" neighbouring modality). We removed the canopy of half of the nurse individuals by cutting the nurse plants at the ground level ("Removal" neighbouring modality). We also transplanted 12 individuals of each target species in

242 open areas (naturally without vegetation, “Open” neighbouring modality, i.e., observational
243 method) close to the nurse plant individuals, and only in highly polluted plots (because there were
244 open areas only in these microhabitats) of each block (Fig. 1C). To test the role of the target metal-
245 tolerance in the outcome of plant-plant interactions, we transplanted as target species a
246 metallicolous (*H. alpina*) and a non-metallicolous (*A. capillaris*) species along the whole gradient.
247 In highly polluted plots, we transplanted similarly another non-metallicolous target (*F. rubra*) to
248 strengthen the study of non-metallicolous targets response to metallicolous nurse plant effects
249 (hypothesis 1). In lowest polluted plots another metallicolous target (*A. multicaulis*) was also
250 transplanted to strengthen the study of metallicolous targets response to non-metallicolous nurse
251 plant effects (hypothesis 1). All transplanted individuals were harvested in their preferred habitat
252 (polluted areas for *H. alpina* and *A. multicaulis* and low-polluted areas for *A. capillaris* and *F.*
253 *rubra*) (Fig. 1C).

254

255 *2.3. Plant–plant interactions measurement and analysis*

256 Three monitoring of targets survival were done during the experiment (11 May, 29 July and 14
257 October 2021) allowing us to give for each individual a Survival Performance index (hereafter SP)
258 at the end of the experiment as follow: 0 for individuals dead at the first monitoring, 1 for
259 individuals dead between the first and second monitoring, 2 for individuals dead between the
260 second and final monitoring and 3 for individuals still alive at the end of the experimentation. This
261 SP index allows to integrate the survival over time. In each plot, SP was calculated for each
262 modality (each target species in each neighbouring modality in each nurse species in each plot). SP
263 for target species with similar metal tolerance (metallicolous M or non-metallicolous NM) were

also averaged. Then, in all plots, we use the Relative Interaction Intensity (RII, Armas et al. 2004) to quantify the STE of the nurse species as follows:

$$RII_{Short-term} = \frac{(X_{Control} - X_{Removal})}{(X_{Control} + X_{Removal})}$$

where $X_{Control}$ is the value of SP in the Control modality and $X_{Removal}$ is the value of SP in the Removal modality. In highly polluted plots, to disentangle the role of LTE and STE on net effects, we calculated two other RIIs, following Michalet et al. (2015):

$$RII_{Long-term} = \frac{(X_{Removal} - X_{Open})}{(X_{Removal} + X_{Open})}$$

$$RII_{Net} = \frac{(X_{Control} - X_{Open})}{(X_{Control} + X_{Open})}$$

where X_{Open} is the value of SP when growing in the Open subplots.

The three RII indices can vary between -1 and 1, with positive values showing positive outcomes (facilitation) and negative values showing negative outcomes (competition).

2.4. Soil depth and humidity measurements

Micro-environmental heterogeneity existing before the arrival of the nurse plants can provide misleading interpretation of LTE measurements (Steinbauer et al. 2016). Thus, in polluted plots with open areas, we checked this micro-environmental variation by measuring several variables related to soil depth below the two nurse species (*H. alpina* and *A. multicaulis*) and in nearby open areas. First, in each polluted plot, a volumetric soil moisture measurement (in %) was recorded on July 29, 2021 with the "Soil Moisture Kit" probe (from Delta-T Devices) in the three Control and Open neighbouring modalities of both nurse species. Additionally, on 18 November

2022, we randomly selected five individuals of each nurse species. Then, we measured soil depth using a 18cm-nail sunk to bedrock into the soil with a hammer for each nurse plant individual (Control modality) and in the closest Open microhabitat.

2.5. Statistical analysis

All analyses were done using R version 4.0.3 (RCore Team, 2017). First, we conducted a principal component analyses on the soil variables of all plots to examine how soil conditions (metals and other environmental variables) varied in the experimental design. This enabled the quantification of a synthetic variable for each plot (the position of the plots on the 1st PCA axis, named PCA_{MPG} hereafter, see Supplementary Fig. S2) integrating all the environmental variation along the Metal Pollution Gradient (MPG) in the studied site. During analyses of all response variable (see below), we used linear mixed-effects models with plot ID nested in block ID as random factors.

We analysed the variations of $RII_{Short-term}$ for Metallicolous (M) and Non-Metallicolous (NM) targets (hypothesis 1) with PCA_{MPG}, Target tolerance (M or NM) and their interaction as fixed factors. The same model was fitted for Survival Performance (SP) response with Control and Removal neighbouring modalities as additional fixed factor. To further distinguish LTE and STE for nurse species with different ability of leaf metal accumulation (hypotheses 2 and 3), we analysed the variations of RIIs (STE, LTE and NE) in highly polluted plots (with Open neighbouring modality). We analysed the variations of RIIs with PCA_{MPG}, Target tolerance (M or NM), Nurse species, Effect type (STE, LTE or Net effects) and their interactions as fixed factors. The same model was fitted for Survival Performance (SP) response with Control, Removal and Open as additional fixed factor. In highly polluted plots, we also analysed the variation of soil

moisture and depth in two neighbouring modalities (Control and Open) for *A. multicaulis* and *H. alpina* with PCA_{MPG}, Nurse species, Neighbouring modality and their interactions as fixed factors.

3. Results

3.1. Environmental gradient

The first axis of the PCA conducted on soil and environmental variables explained 67.6% of the whole variance of the different variables. All metal/metalloids elements were strongly negatively correlated to this first axis, whereas vegetation volume (Vol), organic matter content (OM) and the amount of fine mineral particles (Clay-Silt) were all positively correlated to it (Supplementary Fig. S2). Thus, the positions of the plots on this first PCA axis was a good integrator of all environmental variations along the Metal Pollution Gradient in this study. For further analysis, the PCA_{MPG} metric for each plot corresponds to the opposite of their position on the 1st PCA axis so that metal stress increased with PCA_{MPG}.

3.2. Short-term interactions along the whole metal pollution gradient (hypothesis 1)

There was a significant metal pollution gradient (PCA_{MPG}) per Target tolerance interaction ($P<0.01$, Table 1) on $RII_{Short\text{-}term}$ (Fig. 2A), because RII of Non-Metallicolous targets significantly increased with increasing pollution, whereas RII of Metallicolous targets did not change along the gradient. STE for NM targets switched from negative to positive, whereas STE for M targets were mainly negative along the whole gradient (mean $RII_{short\text{-}term} = -0.12 \pm 0.05$; t-test for difference from zero: $p= 0.054$). This is consistent with the significant interaction between PCA_{MPG}, Target tolerance (M or NM) and Neighbouring modality on SP ($p= 0.01$, Supplementary Table S2). The difference in SP between Removal and Control modalities increased with increasing stress for M

328 targets. Specifically, values increased significantly with pollution in the Removal modality, but
329 only marginally significantly in the Control for M targets (Fig. 2B, left panel). In contrast, SP for
330 NM targets was higher in Removal than Control modality in low polluted plots but higher in the
331 Control than the Removal modality in highly polluted conditions (Fig. 2B, right panel).

332 *3.3. Short- and long-term interactions along the most polluted part of the gradient (hypotheses*
333 *2 and 3)*

334 In the most polluted plots, there was a significant interaction between the Nurse species and Target
335 tolerance treatments ($p < 0.001$, Table 2) on RII_{Net} because overall RII_{Net} were not different from
336 0 for NM targets in both nurse plants, while RII_{Net} were negative for M targets in *A. multicaulis*
337 and stayed neutral for M targets in *H. alpina* (Fig. 3). There was also a significant interaction
338 between the Nurse species, Target tolerance and PCA_{MPG} ($p= 0.002$, Table 2), because RII_{Net}
339 increased significantly for NM targets in *H. alpina* (Fig. 3A) with increasing pollution, whereas
340 there was a marginal increase in RII_{Net} for M targets in *A. multicaulis* (Fig. 3B).

341 Additionally, we found a significant interaction between the Metal Pollution Gradient
342 (PCA_{MPG}), Nurse species and type of effect (type of RII) considered ($p= 0.04$, Table 2) on RII
343 values. It indicated that the different kind of RIIs in most polluted plots varied differently for both
344 nurse species along the pollution gradient. For *A. multicaulis* nurses, we found a significant
345 increase in STE but no variation (slight and not significant decrease) in LTE leading to not-
346 significant change in RII_{Net} along the MPG gradient (Fig. 4B). For *H. alpina* nurses both STE and
347 LTE tended to increase (not significantly though) leading to a significant increase of RII_{Net} (Fig.
348 4A). Between both nurse plants, STE were different according to the response species tolerance
349 ($p= 0.03$, Supplementary Table S3), with negative $RII_{Short\text{-}term}$ values for M targets in both nurses
350 species and significantly different from values of NM targets in *A. multicaulis* (Supplementary Fig.

S3). SP responses showed a significant decrease in the Open modality with increasing pollution near *H. alpina* (Fig. 4C) whereas no changes occurred in the Open modality near *A. multicaulis* (Fig. 4D). This difference of SP variation along the gradient for the two nurse plants contribute to the slight modification of LTE variation with increasing pollution between the two nurse plants (Fig. 4A & B, p= 0.027 for the interaction between MPG and Nurse species on $RII_{Long\text{-}term}$).

3.4. Micro-habitat heterogeneity (soil depth and moisture) in the highly polluted part of the gradient

There was a significant (p= 0.01, Supplementary Table S4) and marginally significant (p= 0.06, Supplementary Table S5) interactions between the pollution metric PCA_{MPG}, Nurse species and Neighbouring treatments for soil depth and soil moisture, respectively. Soil depth did not change with increasing pollution for *H. alpina*, neither in Control nor in Open modalities (Fig. 5A), whereas it marginally increased in the Open modality but marginally decreased in the Control modality (Fig. 5B) for *A. multicaulis*. A similar trend was observed for changes in soil moisture with increasing pollution, with a significant increase in the Open modality (Fig. 5D) close to *A. multicaulis*, but not close to *H. alpina* (Fig. 5C).

4. Discussion

Regarding variation of Short-Term Effects along the whole metal pollution gradient, we did not observe a clear increase of competition for Metallicolous targets in the least polluted habitats (hypothesis 1). However, STE tended to vary accordingly to the SGH for Non-Metallicolous targets (hypothesis 1). Regarding Long-Term Effects, we did not observe any negative LTE for *H. alpina*, the most metal-accumulating nurse plant, contrary to what was expected in hypothesis 2.

373 Additionally, we could not conclude regarding LTE for *A. multicaulis*, the least metal-
374 accumulating plant, because of the occurrence of a local environmental heterogeneity and, thus,
375 different soil conditions in Control and near open modalities (hypothesis 3).

376 *4.1. Short-term interactions along the whole gradient*

377 Concerning the first hypothesis in the most benign areas, at first glance, the absence of increasing
378 competitive interactions for metallicolous targets as pollution and stress decrease might appear
379 contradictory with the ecological literature. Indeed, higher competitive interactions are expected in
380 low-stressed habitats (Grime, 1973; Bertness and Callaway, 1994; Michalet et al. 2023a). However,
381 we did find competitive interactions for metallicolous targets in low-polluted habitats, and the
382 maintenance of such interactions over the whole gradient explains the absence of variation of STE
383 for these targets. More precisely, it has been suggested that in the most stressed habitats for plant
384 growth, all plants should be highly stress-tolerant, and more prone to interact neutrally or
385 competitively because they do not depend on stress alleviation for their development (Liancourt et
386 al. 2005, 2017). It could explain the maintenance of competitive interactions for metal-tolerant
387 targets in highly polluted plots.

388 Regarding Non-Metallicolous targets, results of STE suggest a validation of the Stress
389 Gradient Hypothesis in the context of this study, with $RII_{short\text{-}term}$ increasing along the pollution
390 gradient and switching from negative to positive values. This facilitation for NM targets on STE in
391 most polluted plots was largely driven by the effects of the nurse *A. multicaulis*. Indeed, this
392 increase of STE was slighter within the nurse *H. alpina*. Furthermore, $RII_{Short\text{-}term}$ for NM targets
393 in *A. multicaulis* in the most polluted plots were higher than those of the M targets in both nurse
394 plants (Supplementary Fig. S3). One reason for the higher STE in *A. multicaulis* may be that these
395 species have a denser and looser canopy (Supplementary Fig. S4), which may promote

microclimate improvements. This overall increase in STE was mostly due to an increase in survival in the Control modality (Fig. 2B, right panel), the SP responses in the Removal modality remaining stable. This increase of NM targets survival in the nurse plant canopy confirms the lower competitive effect of stress-tolerant metallicolous nurse plant as compared to more competitive grasses dominating in more benign areas (Liancourt et al. 2005; Nemer et al. 2021, 2022). Additionally, this raises the question of why the Survival Performance in Removal modality did not decrease. *F. rubra* and *A. capillaris* species have also been studied for their tolerance to metals/metalloids (see for instance Baker, 2002). Even if our transplants were harvested in low-polluted areas, they likely have a level of tolerance to metals, even if not as pronounced as for the dominant species present in the most polluted areas. Additionally, it is reasonable to assume that a longer duration of our experiment could have led to a higher mortality of the transplants in the Removal modality.

Overall, the results regarding STE are consistent with previous studies. The SGH has been validated in several polluted contexts (e.g. Domínguez, 2015; Yang et al. 2015); however positive interactions are more probable for the less-adapted targets that are sensitive to the stress present in the corresponding habitats (Liancourt et al. 2005; Liancourt and Tielbörger, 2011; Qi et al. 2018). Consistently, Nemer et al. (2022) working with different ecotypes of a same target species found that the less metal-tolerant ecotype was facilitated in high-polluted areas.

4.2. Environmental differences in highly polluted plots between Control and Open modalities

Looking at the Survival Performances of transplants, it decreased significantly in the Open modality close to *H. alpina* nurses with increasing pollution. However, it did not decrease

418 significantly in the Open modality close to *A. multicaulis*. The Open modality aimed at representing
419 the environment before soil engineering modification by any nurse plant. Thus, Open modality
420 should not lead to contrasting results for transplant survival, either they are close to one nurse
421 species or another one. These differences can rationally be related to the differences observed in
422 the soil characteristics in the Open modality close to the different nurse plants. Indeed, when
423 pollution increased, soil depth did not change in Open modality close to *H. alpina* (Fig. 5A), as
424 well as in Control modality of this nurse plant. In contrast, soil depth marginally increased in the
425 Open modality close to *A. multicaulis* when pollution increased, with an opposite marginal
426 decrease in the Control modality. This opposite variation in soil depth beneath the plant and in
427 nearby open areas shows that when the pollution increased, *A. multicaulis* settled in shallower areas
428 surrounded by deeper open areas. Whatever the reason of this subtle modification of microhabitat
429 preference as pollution increased, it leaded to an increase of the moisture content in the Open
430 modality close to *A. multicaulis* as pollution increased, likely in relation with a higher water holding
431 capacity (Fig. 5D). Finally, this increase of soil moisture is consistent with the better Survival
432 Performance of the targets in the Open of *A. multicaulis* nurse plots, while we expected a decrease
433 of survival with the increase of pollution (as observed in Open modality close to *H. alpina*, Fig.
434 4C). Since this specific Survival Performance was used to calculate the RII for the LTE (difference
435 between the 'Open' and 'Removal' modalities) and NE (difference between the 'Open' and
436 'Control' modalities), values of these indices may have been influenced not by the nurse plant
437 effect itself, but rather by the difference of habitat in the Open modality compared to the other
438 modalities, and having influenced the target plants performance. Therefore, the interpretation of
439 corresponding LTE and NE RIIs for *A. multicaulis* nurses have to be handled with care.

440

441 *4.3. Variation of Long-Term Effects in highly polluted plots*

442 Hypotheses 2 and 3 were dedicated to investigate the variation of soil engineering effects according
443 to nurse plant leaf metal accumulation and the level of soil pollution. The hypothesis 2 regarding
444 potential elemental allelopathy effects, concerns in priority LTE produced by *H. alpina*, the species
445 with highest leaf metal accumulation. The hypothesis 3 relative to potential positive LTE should
446 concern in priority *A. multicaulis* because of its lower level of leaf metal accumulation. However,
447 the amelioration of soil humidity in Open condition with increasing pollution close to *A. multicaulis*
448 may have over-estimated the targets performance in the Open in the most polluted conditions and,
449 thus, minimized potential positive LTE (i.e., the difference between Removal and Open modality).
450 If this bias occurred, it may have affected the NM targets in priority, since they were stressed in
451 the corresponding habitat and therefore more sensitive to improving conditions. In summary, based
452 on our results, further experimentations controlling for environmental micro-heterogeneity are
453 necessary to answer hypothesis 3.

454 Regarding, *H. alpina* and hypothesis 2, we found no soil heterogeneities for this nurse plant
455 between the Open and Control modality, providing appropriate conditions for interpretation of
456 LTE. We did not find negative LTE consistent with the Elemental allelopathy Hypothesis, neither
457 for Metallicolous nor Non-Metallicolous targets. In fact, most results confirming elemental
458 allelopathy comes from controlled studies (El Mehdawi et al. 2011; Mohiley et al. 2020), and
459 studies regarding this hypothesis are not equivocal (Morris et al. 2006). This highlights the need
460 for further field investigation to precise the conditions of application of this hypothesis in realistic
461 field conditions. In this study, even if not significant, LTE for *H. alpina* even seemed to increase
462 with soil pollution and reinforce positive STE leading to an increase of NE with pollution (Fig.

463 4A), in particular for NM targets (Fig. 3A). Again, this is consistent with the SGH, and the higher
464 facilitative response of less stress-tolerant targets (Liancourt et al. 2005; Nemer et al. 2022, 2023).

465 *4.4. The use of different methods for plant-plant interaction in metalliferous systems*

466 Combining both observational and removal methods is possible and allows important insight
467 regarding the different effects at stake in plant-plant interactions. Randé et al. (2023) successfully
468 applied both methods in a metal polluted environment. However, the study site was a slag heap
469 with homogeneous soil conditions and texture, limiting the potential bias observed here for *A.*
470 *multicaulis*. Scrutinizing site configuration and patches of *A. multicaulis* and *H. alpina* (Fig. 1,
471 Supplementary Fig. S1), we did not expect such strong soil heterogeneities within our 1 m^2
472 experimental plots, and between two close locations like the patches of an *A. multicaulis*
473 individuals and a close open microsite. However, our measurements of soil depth and humidity
474 revealed this unexpected fine-scale heterogeneity. Metalliferous systems are often found in
475 degraded areas (Ernst, 1990), and in disturbed stony areas (Faucon et al. 2009). In such context,
476 the observational method must be used with care. The problem raised by habitat heterogeneity has
477 already been expressed in the literature (Steinbauer et al. 2016). This study reopens the debate on
478 the methods to be used to quantify plant-plant interactions (Maestre et al. 2005). Michalet et al.
479 (2023b) argued that "microhabitat heterogeneity may lead to facilitation overestimation" because
480 habitats with nurse plants may be more favourable which can also benefit companion species and
481 transplanted targets. In our paper the opposite case happens, as *A. multicaulis* was located in areas
482 that appeared less favourable (shallower and drier soils) compared to the nearby opens. However,
483 as we did not measure soil metal availability at such fine-scale to compare open and nurse plant
484 canopy microsites, we cannot formally conclude about the better conditions for transplanted targets
485 in the open areas, and we cannot conclude regarding LTE for this nurse species. Steinbauer et al.

(2016) proposed three solutions to avoid this bias. The first solution consists of transplanting nurse plants in a homogeneous environment (as in Monge and Gornish, 2015) to avoid habitat heterogeneity. However, on site studies along environmental gradients are still required to reach realistic results regarding ecological processes. The removal method (second solution) avoid this bias, but it impedes studies of LTE, while such effects either positive or negative are preponderant in plant-plant interactions in metal-polluted environments (Frérot et al. 2006; Randé et al. 2023). The most appropriate solution in our case would have been to randomly sample the Open modality locations (third solution), without pairing them with close neighbouring nurse plants. It would have avoided the potential confounding effects of differences in soil depth and humidity.

5. Conclusion

In summary, this study showed that facilitation, mainly driven by short-term effects, and likely due to micro-climatic improvement, seems to be a preponderant interaction in the most polluted areas (Cuevas et al. 2013; Yang et al. 2015). These are promising results from a restoration point of view. This study provided additional knowledge regarding these positive short-term effects, Non-Metallicolous targets being the most likely to benefit from them. However, more work is needed to better understand the evolution of LTE along metal gradients, especially concerning the application conditions of the Elemental allelopathy Hypothesis, where micro-environmental heterogeneity must be meticulously controlled. This study highlighted the difficulty to disentangle the different kinds of effects at stake in plant-plant interactions along metal pollution gradients. In addition, their relative importance along these gradients is still largely unknown. This lack of knowledge reveals the need to develop more field experiments, along more diverse metal-pollution gradients and including different nurse species than what has already been done, potentially in more diverse bio-climatic contexts.

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686 **Figure legends**

687 **Fig. 1** Presentation of the experimental design. a) UAV-aerial image of the study site, with the
688 position of the four blocks and plots (red for the highly polluted plots and green for the least
689 polluted plots); b) zinc kriging pollution map of the study site, with the position of the plots; c)
690 schematic representation of the two types of plots in our experimentation.

691 **Fig. 2** Changes in short-term interactions and target Survival Performance along the whole metal
692 pollution gradient. A) variation in Short-term Relative Interaction Index ($RII_{Short\text{-}term}$) for both
693 Metallicolous (M) and Non-Metallicolous (NM) targets; B) variation in Survival Performances in
694 two Neighbouring modalities (Control and Removal) for Metallicolous (M, left panel) and Non-
695 Metallicolous (NM, right panel) targets. Note that metal pollution increased to the right with the
696 PCA_{MPG} metric. The shaded areas represent the most polluted areas and plots. Full lines represent
697 relationship with slopes significantly different from 0 (**, P<0.01, *P<0.05), dashed lines represent
698 relationships with slopes marginally different from 0 (P < 0.1).

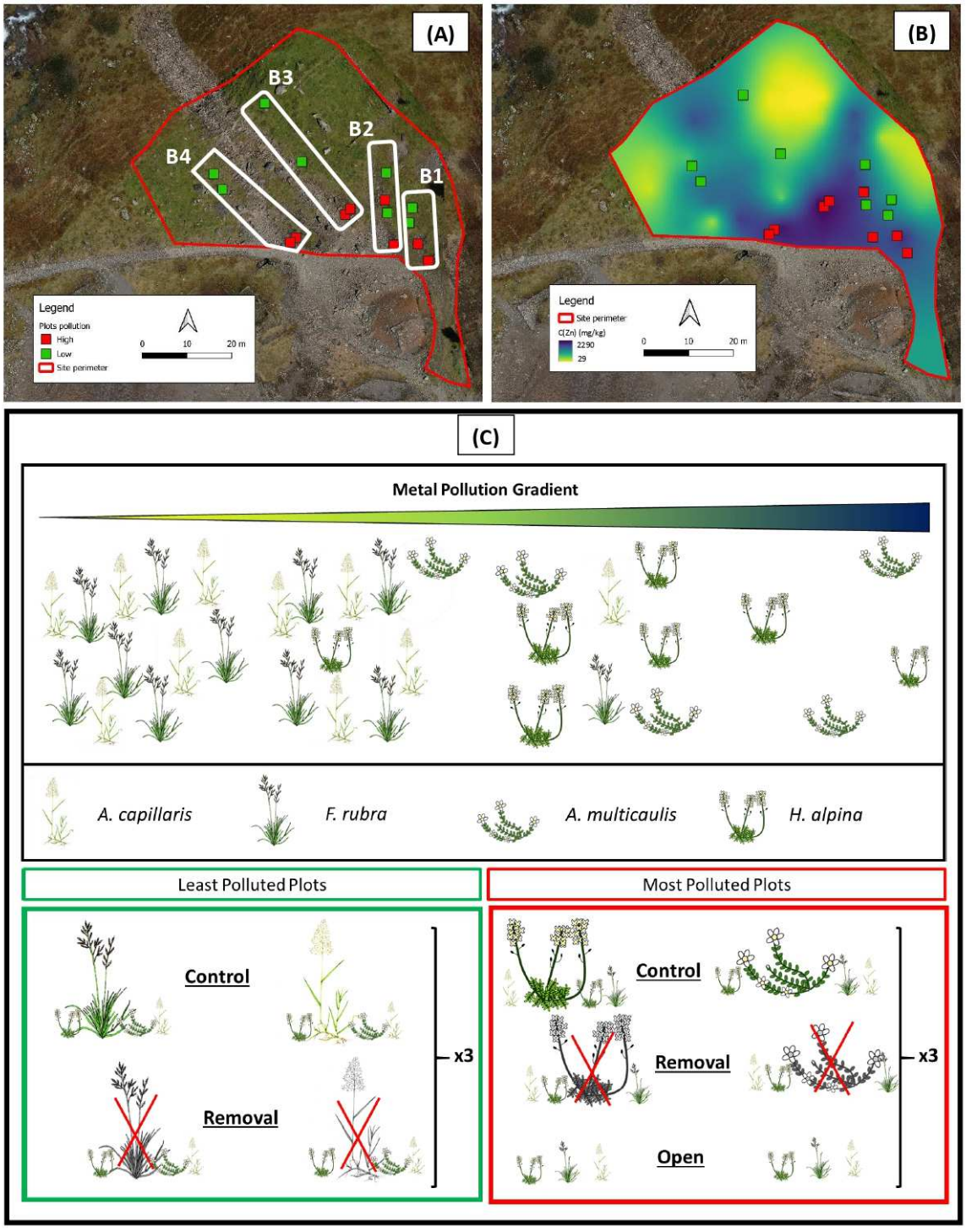
699 **Fig. 3** Changes in Net Relative Interaction Index (RII_{Net}) with increasing pollution in the most
700 polluted plots for the two types of targets. A) In *H. alpina* as nurse; B) In *A. multicaulis* as nurse.
701 Full lines represent relationships with slopes significantly different from 0 (**, P<0.01), dashed
702 lines represent relationships with slopes marginally different from 0 (P < 0.1).

703 **Fig. 4** Variation in Survival Performance and corresponding Relative Interaction Index in both
704 nurse species along the metal pollution gradient in most polluted plots. A,B) variation in $RII_{Long\text{-}}$
705 $_{term}$, $RII_{Short\text{-}term\ and}$ RII_{Net} for the two nurse species *H. alpina* (A) and *A. multicaulis* (B). C,D)
706 variation in Survival Performance in the three neighbouring modalities for the two nurse species
707 *H. alpina* (C) and *A. multicaulis* (D). Full lines represent relationships with slopes significantly

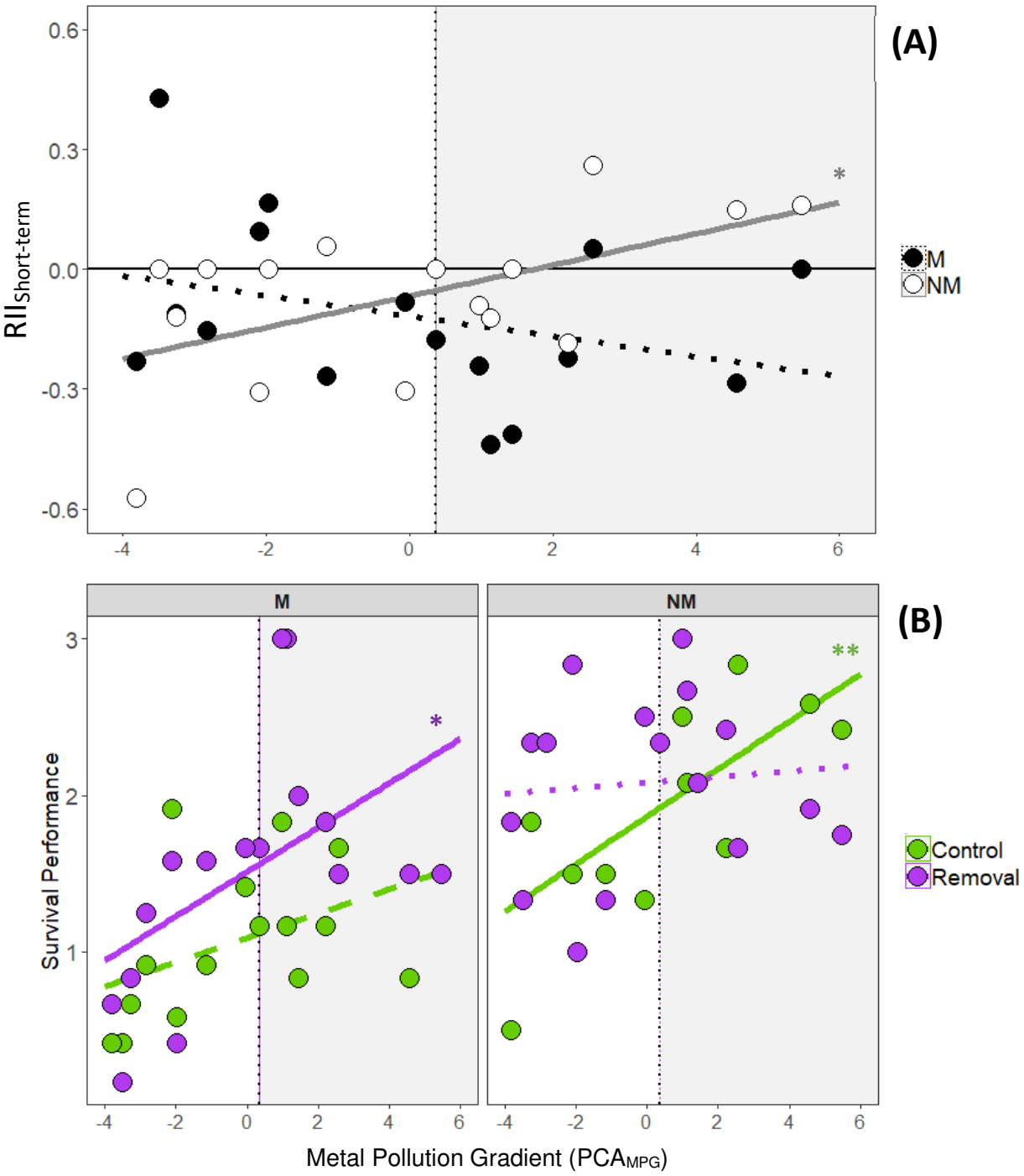
different from 0 (**, P<0.01, *P<0.05), dashed lines represent relationships with slopes marginally different from 0 (P < 0.1). Note that RII_{Net} result from the combination of $RII_{Long\text{-}term}$ and $RII_{Short\text{-}term}$. For ease of visualisation and understanding of the complex interplay between LTE, STE and NE, we also represented here the non-significant relationships along the MPG gradient with dotted lines.

Fig. 5 Variation in soil conditions in both nurse species along metal pollution gradients in most polluted plots. A,B) soil depth for *H. alpina* (A) and *A. multicaulis* (B); C,D) soil moisture for *H. alpina* (C) and *A. multicaulis* (D). . Full lines represent relationships with slopes significantly different from 0 (**, P<0.01), dashed lines represent slopes marginally different from 0 (P < 0.1).

718 **Figures**

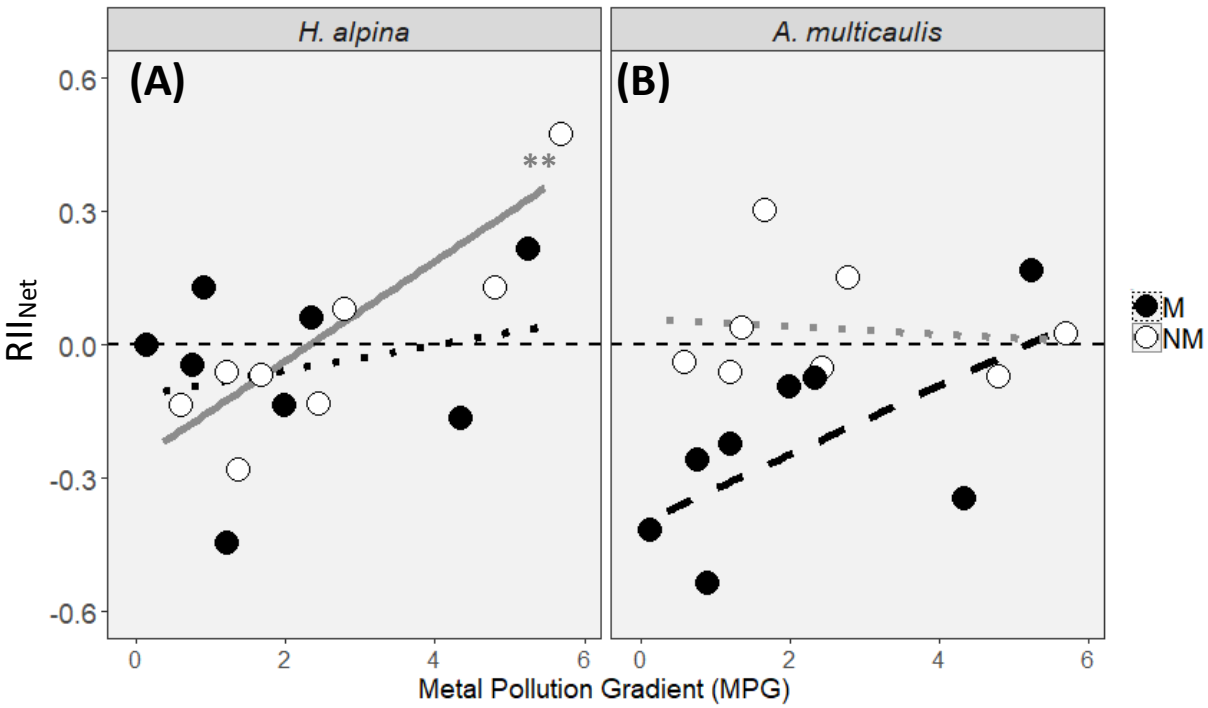


719 **Fig. 1**


(A)
RIIShort-term
0.6
0.3
0.0
-0.3
-0.6
-4
-2
0
2
4
6
*
M
NM
(B)
M
NM
Survival Performance
3
2
1
*
**
Control
Removal
Metal Pollution Gradient (PCAMPG)

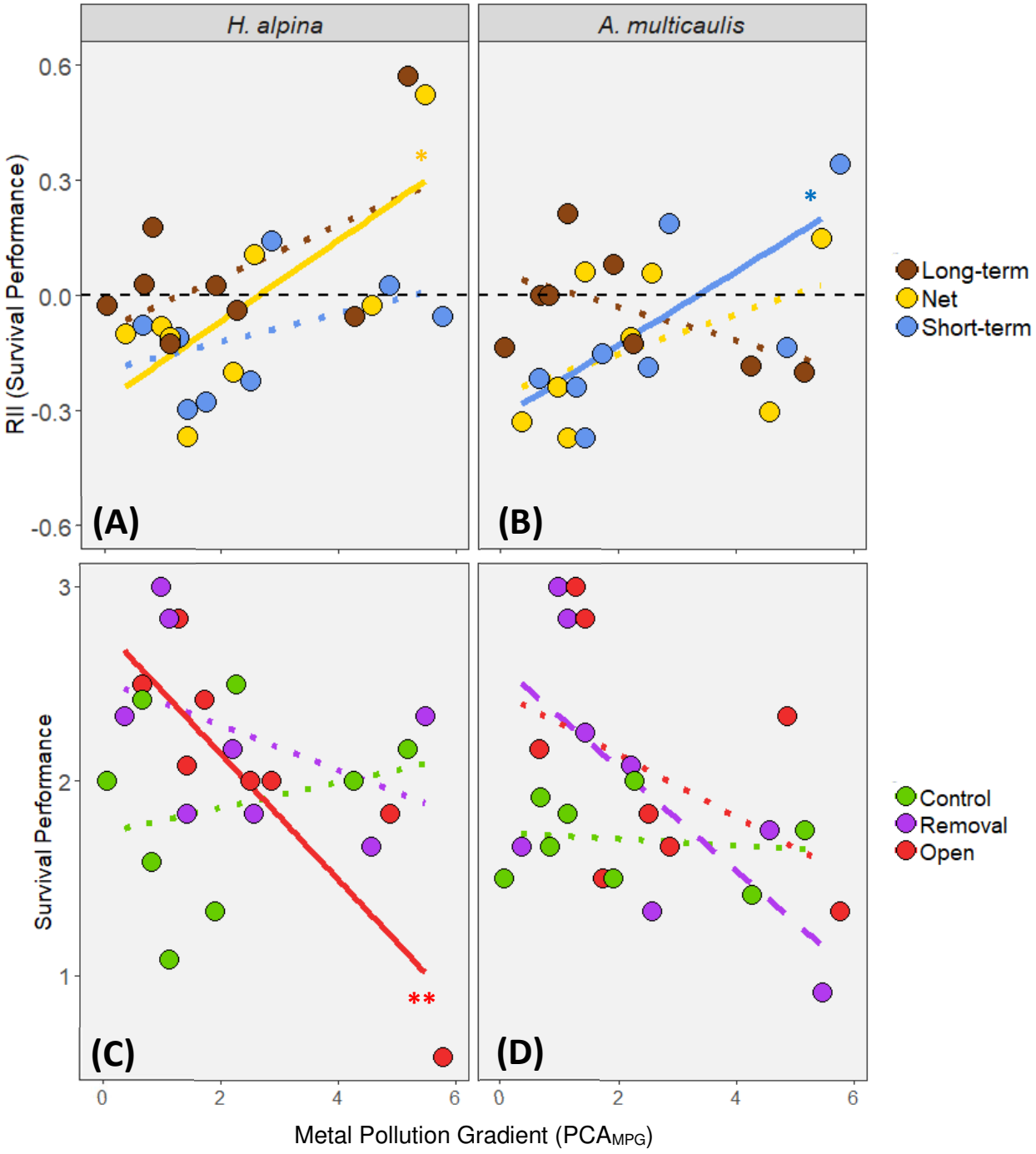
720 **Fig. 2**

721


H. alpina
A. multicaulis
(A)
(B)
**
0.6
0.3
0.0
-0.3
-0.6
RII Net
0
2
4
6
Metal Pollution Gradient (MPG)
M
NM

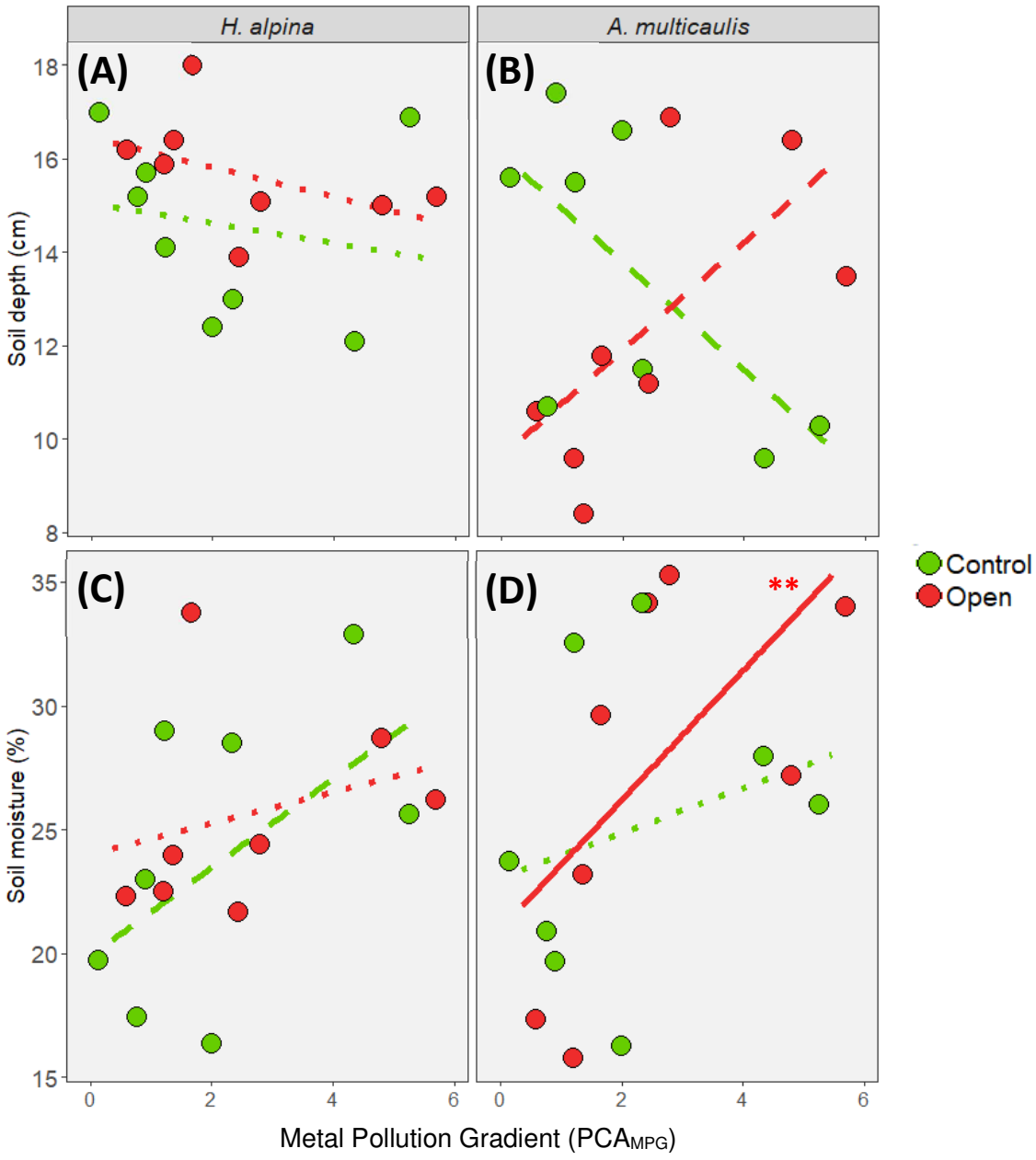
722 **Fig. 3**

723


H. alpina
A. multicaulis
RII (Survival Performance)
0.6
0.3
0.0
-0.3
-0.6
(A)
(B)
*
*
Long-term
Net
Short-term
Survival Performance
3
2
1
**
(C)
(D)
0
2
4
6
Control
Removal
Open
Metal Pollution Gradient (PCA$_{MPG}$)

724

725 **Fig. 4**



726

727 **Fig. 5**

728

729 **Table 1** Results of the linear mixed model for the effects of the Metal Pollution Gradient
730 (PCA_{MPG}), Target tolerance (M or NM) and their interactions on $RII_{Short\text{-}term}$ along the whole
731 gradient.

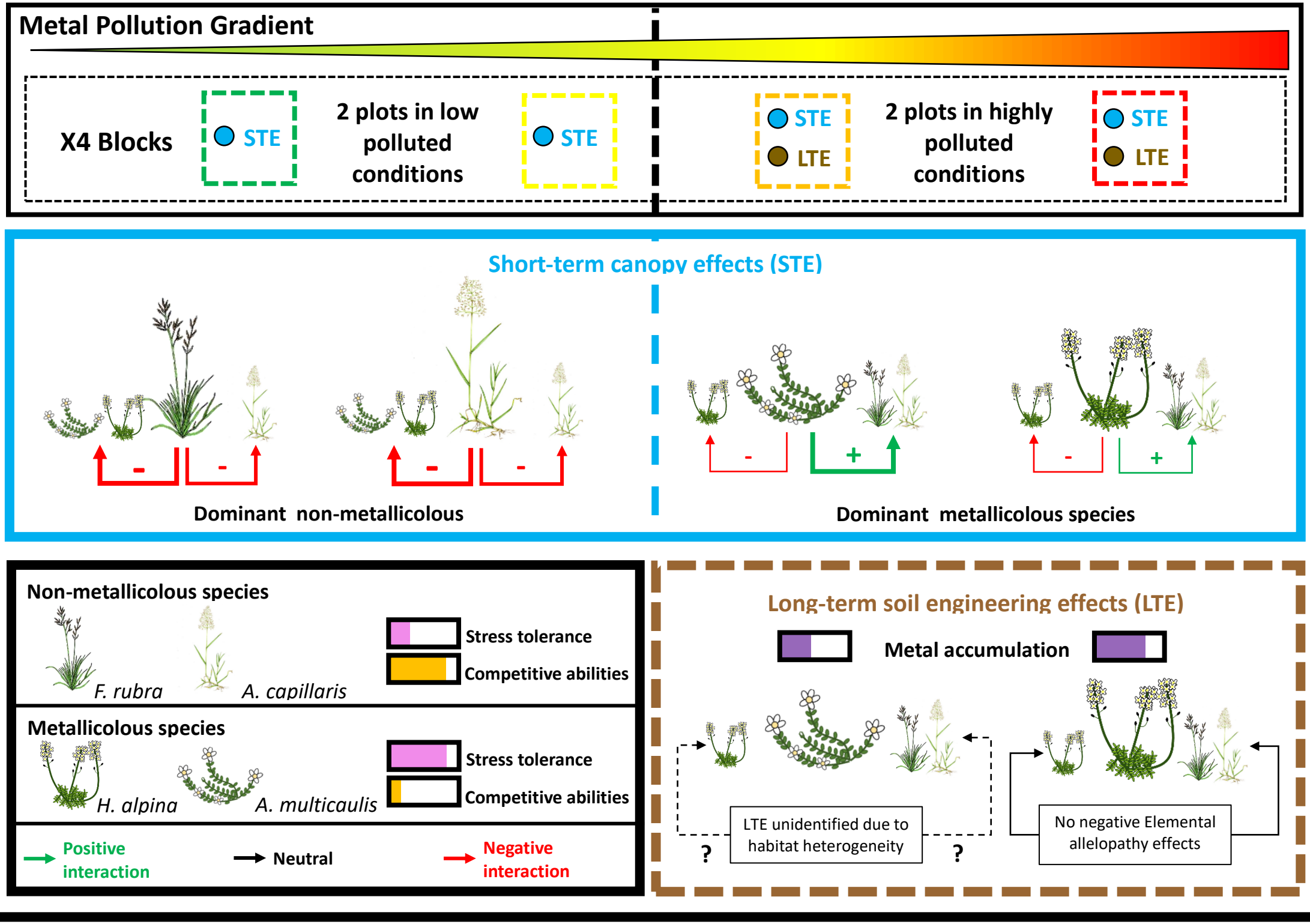
	$RII_{Short\text{-}term}$		
Factors	dF	F	P
PCA_{MPG}	1,14	0.21	0.65
Target tolerance	1,14	0.80	0.39
PCA_{MPG} X Target tolerance	1,14	9.99	**0.007 ****

732

733 **Table 2** Results of the linear mixed model for the effect of Metal Pollution Gradient (PCA_{MPG}),
734 Nurse species, Target tolerance, Effect type and their interactions on different kind of RIIs (STE,
735 LTE and NE) in highly polluted plots**.**

	RII (SP)		
Factors	dF	F	P
PCA_{MPG}	1,6	6.602	**0.04***
Nurse species	1,66	0.038	0.85
Target tolerance	1,66	4.249	**0.04***
Effect type	2,66	4.642	**0.01***
PCA_{MPG} X Nurse species	1,66	1.822	0.18
PCA_{MPG} X Target tolerance	1,66	0.002	0.96
Nurse species X Target tolerance	1,66	15.698	**<0.001****
PCA_{MPG} X Effect type	2,66	2.355	0.10
Nurse species X Effect type	2,66	1.045	0.36
Target tolerance X Effect type	2,66	0.918	0.40
PCA_{MPG} X Nurse species X Target tolerance	1,66	10.213	**0.002****
PCA_{MPG} X Nurse species X Effect type	2,66	3.522	**0.04***
PCA_{MPG} X Target tolerance X Effect type	2,66	0.117	0.89
Nurse species X Target tolerance X Effect type	2,66	1.097	0.34
PCA_{MPG} X Nurse species X Target tolerance X Effect type	2,66	0.638	0.53

736
737
738


Metal Pollution Gradient
X4 Blocks
STE
2 plots in low polluted conditions
STE
STE
LTE
2 plots in highly polluted conditions
STE
LTE
Short-term canopy effects (STE)
- - - -
Dominant non-metallicolous
- +
- +
Dominant metallicolous species
Non-metallicolous species
F. rubra
A. capillaris
Stress tolerance
Competitive abilities
Metallicolous species
H. alpina
A. multicaulis
Stress tolerance
Competitive abilities
Positive interaction
Neutral
Negative interaction
Long-term soil engineering effects (LTE)
Metal accumulation
LTE unidentified due to habitat heterogeneity
? ?
No negative Elemental allelopathy effects