

## Heavy metal tolerance strategies in metallicolous and non-metallicolous populations of mosses: Insights of $\gamma$ + $\beta$ -tocopherol regulatory role

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### ABSTRACT

As bryophytes present the capacity to colonize a wide range of habitats, including extreme environments (e.g., mine areas), we first checked the global response of bryophytes to heavy metals (HM) by a systematic review. This analysis found i) the lack of studies in a polluted environment and ii) resulted in a global trend of photochemical efficiency decrease when bryophytes faced heavy metals. Secondly, we characterized the photoprotective responses of four metallicolous populations of mosses (*Lewinskya rupestris*, *Polytrichum commune*, *Ptychostomum compactum* and *Rhynchostegium confertum*) naturally growing in an abandoned mine area by an experimental field approach. As a result, we found markedly species-specific tolerance patterns: i) less tolerant species, presenting high pollutants content accompanied by marked dissipative and antioxidative strategies. This strategy was depicted by the species *R. confertum* that result to be a good sentinel species due to the high pollutant content and high physiological sensitivity.; ii) intermediate tolerant species presenting a medium content of pollutants and less marked photoprotective mechanisms, and iii) most tolerant species minimizing pollutants content and consequently presenting no changes in their physiological performance. This evidence support that species' bryological attributes have a key role in determining species' tolerance towards environmental heavy metals, and should be taken into consideration in future studies. The findings of this study also pointed out that the higher levels of  $\gamma$  +  $\beta$ -tocopherol in metallicolous populations could play a regulatory role in metal stress tolerance in mosses and it could be a suitable functional responsive trait for environmental response prediction to heavy metals in polluted environments.

### 1. Introduction

Abandoned mining areas, as impoverished heaps and tailing dumps, represent a serious environmental problem due to the high presence of inorganic pollutants, such as heavy metals (HM) and metalloids, even when extraction has long ceased to be active (Vacca and Vacca, 2001; Bacchetta et al., 2015). These compounds can reach high concentration levels and bioavailability in soils and even in soil organisms, as they do not undergo ecosystem degradation (Barrutia et al., 2011). These

anthropogenic soil matrices result to be scarcely colonized by plants due to the challenge to live under the abiotic stresses that these polluted environments present such as poorly consolidated growth substrate, shortage of organic matter and nutrients, phytotoxicity of soil pollutants and high sun irradiation due to the absence of a vegetal canopy (Barrutia et al., 2011; Jiménez et al., 2011; Bacchetta et al., 2018; De Agostini et al., 2020a). More particularly, non-adapted plants can suffer a strong detrimental HM-related phytotoxic effect on physiology and life cycle, negatively interfering with essential processes as photosynthesis,

**Abbreviations:** A, antheraxanthin;  $\beta$ -carot,  $\beta$ -carotene; Chl, chlorophyll; CB, contaminated bryophytes; DW, dry weight; E1, major biome; E2, the eastern limit of distribution; FW, fresh weight; F, moisture; HM, heavy metals; HMT, tolerance to HM; L, light; Len, gametophores size; LF1, life-form; Lut, lutein; MP, metallicolous population; NCB, non-contaminated bryophytes; NMP, non-metallicolous population; N, nitrogen; Neox, neoxanthin; NPQ, non-photochemical quenching; PCA, principal component analysis; Per, type of perennation; R, reaction to environmental acidity; ROS, reactive oxygen species; Sex, distribution of gametangia in gametophore; TAC, total antioxidant capacity; V, violaxanthin; Z, zeaxanthin.

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respiration, nutrient uptake, gene expression and membrane integrity (Baek et al., 2012). Moreover, some metals (Fe, Cu, Mo, etc.) can interact with cellular oxygen to form reactive oxygen species (ROS), change cell redox status, and eventually replace metallic cofactors in functioning enzymes inhibiting their functionality (Baek et al., 2012). Consequently, vascular plants own several mechanisms to tolerate HM stress and survive in such polluted environments and, as a result, these metalicolous populations benefit from the reduced competence of other plants in these hostile environments (Shefferson et al., 2008; Faucon et al., 2011; De Agostini et al., 2020a). The mechanisms of tolerance of vascular plants are widely studied (Baek et al., 2012; Chandra and Kang, 2015; Singh et al., 2015). On the contrary, the mechanisms involved in the response to HM in non-vascular plants, as bryophytes, are not so well characterized.

Bryophytes are embryophyte plants that differ from tracheophytes in several anatomical and physiological traits: they lack a root system (rhizoids mainly function consist in anchoring individuals to the substrate), as well as an efficient conduction system (hydroids and leptoids described in *Polytrichum* spp., *Spagnum* spp., etc. not as efficient as the conduction systems in vascular flora; Ligrone et al., 2000). Those features imply that bryophytes nutrients' acquisition into gametophytes relies almost exclusively on dry and wet deposition and secondly, on absorption from the substratum via capillarity (Chakraborty and Paratkar, 2006). To optimize this form of nutrient acquisition, bryophytes are characterized by a great surface-to-volume ratio. Phylloids indeed are generally constituted by a single layer of cells and they do not present any kind of physical, chemical, or cellular barrier preventing water entrance or loss such as epidermis, cuticles, or stomata. As a result, bryophytes are characterized by a great permeability to water and elements dissolved in it (Chakraborty and Paratkar, 2006; De Agostini et al., 2020b). Despite the disadvantages linked to poor control over nutrient uptake and water loss, bryophytes are present in every continent and are well adapted to live in several ecological contexts, including the extreme ones, in which they generally act as early colonizers (Holyoak, 2009). For example, bryophytes are often described as colonizing HM polluted sites, such as abandoned mining areas (Holyoak, 2009; Holyoak and Lockhart, 2011; Karakaya et al., 2015; Angelovska et al., 2016) and some moss species, such as *Scopelophila cataractae* (Mitt.) Broth., are considered to be metal-tolerant species (Shaw, 1987; Konno et al., 2010; Yagura et al., 2019).

Due to the physiological and morphological features of bryophytes and their high exposure to dry and wet deposition, this group is largely used in environmental pollutants' biomonitoring (Ares et al., 2014; Chakraborty and Paratkar, 2006; Cortis et al., 2016; De Agostini et al., 2020a; Esposito et al., 2018; Maresca et al., 2020, 2018). Nevertheless, besides a recent review article (Stanković et al., 2018), studies of environmental HM pollution's effects on ecology, morphology, and physiology on bryophytes are not so frequent. Most of the scientific literature on the topic reports that: i) bryophytes can accumulate pollutants on their surface, adsorb them on cells' walls and, actively or passively, transport them into cells (Chakraborty and Paratkar, 2006; Basile et al., 2012); ii) physiochemical features of pollutants (particulate matter size, chemical nature of pollutants, as well as their solubility and charge) strongly influence their accumulation in bryophytes (Chakraborty and Paratkar, 2006; Basile et al., 2012; Capozzi et al., 2020); iii) several effects, often dose-dependent, on bryophytes' physiology have been related to environmental pollution, the most relevant negatively influencing photosynthetic parameters, pigment content, cellular ultrastructural organization, protein conformation and biosynthesis rates, and membrane integrity (Shakya et al., 2008; Basile et al., 2012; Cortis et al., 2016; Stanković et al., 2018; Chen et al., 2019), and iv) intra-specific differences and even sexual dimorphism were observed to influence tolerance to environmental HM in bryophytes (Boquete et al., 2021).

As regards the bryophytes adaptations to HM polluted environments, they have been described to consist in: i) accumulation of toxic elements

in cell walls (Krzesłowska et al., 2009; Konno et al., 2010; Karakaya et al., 2015) and vacuoles (Basile et al., 1994; Carginale et al., 2004); ii) excretion of HM salts from gametophyte (Rao, 1982; Tyler, 1990); iii) spores protection from pollutants (Basile et al., 2001); and iv) production of antioxidants (Bellini et al., 2020; Maresca et al., 2020; Vuotto et al., 1991, 2002; Ielpo et al., 1998) and heat shock proteins and phytochelatins (Basile et al., 2004; Carginale et al., 2004). Nevertheless, the role of photoprotective mechanisms of bryophytes to HM is very poorly studied, especially in metalicolous populations of mining areas.

We hypothesized that photoprotection and antioxidant mechanisms (specially pigments and tocopherols) play a key role in bryophytes' adaptation to HM stress, allowing this component of the flora to cope with HM soil pollution. Bryophytes as pioneer plants species could play an important ecological service in the restoration of soil fertility, increasing soil organic matter and favouring vascular plants and microbes' colonization and biodiversity in such hostile environments as metalliferous sites are (Szarek-Lukaszewska, 2009; Barrutia et al., 2011; Holyoak and Lockhart, 2011; Callaghan, 2018). In this study, therefore, we first checked the global response of bryophytes to HM by a data compilation from literature (from 1999 to 2019), and secondly, we compared the relevant ecological/physiological traits that evolved to colonize mining environments in four moss species (with contrasting bryological attributes) growing naturally on metalliferous and non-metalliferous areas. We analysed the physiological response by the following traits: physiological performance (photosynthetic efficiency) and biochemical parameters (chlorophylls, carotenoids, tocopherols, total antioxidant capacity, and anthocyanins content, as well as foliar pH), together with the main bryophyte ecological attributes. We aimed to answer the following questions: i) Do traits tendency obtained from the analysis of data from literature mirror the trait tendency obtained in the field? ii) Is HM tolerance species-related showing each species-specific tolerance trait? iii) Do photoprotective compounds play an important role in bryophytes tolerance to HM toxicity? This study will provide important clues regarding ecophysiological traits activated in bryophytes metalicolous populations of mining areas.

## 2. Materials and methods

i) We first analysed literature data on the photoprotective response of mosses and ii) second, we performed an experimental field approach comparing metalicolous and non-metalicolous populations. See the details below.

### 2.1. Analysis of data compilation from the literature

#### 2.1.1. Literature search and inclusion criteria

Data were collected exclusively from peer-reviewed journals published in the two decades from 1999 to 2019, using the resource "ISI Web of Knowledge". Queries were made using Boolean operators (AND, OR and NOT) with combinations of the words: "bryophyte", "moss", "pigment", "carotenoid", "tocopherol", "chlorophyll", "antioxidant", "photosynthesis", "heavy metal", "soil pollution" and "stress". As a result of this search criterion, a total of 561 articles were found. After this first search, a final database was created for the analysis phase applying the following inclusion criteria: papers dealing with stress factors differing from HM (e.g., pesticides, herbicides, ammonium, etc.) were not considered; articles without mention of bryophytes or considering other taxa, eg. lichens, Spanish moss (*Tillandsia usneoides* L.), etc., were excluded; articles considering only ultrastructural effects or antioxidant enzymes, e.g., superoxide dismutase, peroxidase, etc., were not taken into account; review-type articles and methodological articles were also excluded. In this way, articles suitable to be used in the analytical phase resulted to be in the number of 35 (listed in Table 1S), including 38 different species of bryophytes (among those 6 belonging to the *Marchantiophyta* division), divided into 19 families and 13 orders (Table 2S).

### 2.1.2. Database creation and analytical procedure

Data were extracted from tables and figures of the 35 selected articles with the program ImageJ version 1.52a (Schneider et al., 2012). To delve into the differences in photoprotective mechanisms due to HM, two categories were created: *i*) contaminated bryophytes (hereinafter CB), considered those exposed to the most intense or prolonged HM stress, and *ii*) non-contaminated bryophytes (hereinafter NCB), considered those indicated so in the original studies. When these values lacked in the original article, bryophytes growing under the minor grade of HM stress were considered as NCB. The following parameters were included in the database: the maximal photochemical efficiency of photosystem II ( $F_V/F_M$ ), the non-photochemical quenching (NPQ), chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl a+b), chlorophyll a to b ratio (Chl a/b), total carotenoids, carotenoids to Chl a+b ratio (carotenoids/Chl), anthocyanins and ascorbate. When any of these data were missing, and whenever possible, they were calculated from the original data from the paper. In some cases, units were converted. As this conversion was impossible in many cases, to standardize the diversity of data units extracted from the different articles, we expressed the final values as variations in percentage with respect to NCB values, calculated as  $(CB/NCB-1) \times 100$ .

## 2.2. Experimental field approach

### 2.2.1. Field sites description

We selected two sites, an abandoned mining area and a control area, both located in the municipality of Carranza (Basque Country, Spain) a temperate region in Northern Spain (Atlantic region), in which the annual rainfall reaches 1400 mm and the annual mean temperature ranges between 11 and 15 °C (Galende et al., 2014). The metalliferous site (43°13'39.75' N, 3°25'50.75' W), an abandoned lead (Pb) and zinc (Zn) mine was chosen as it presents open pits, waste rocks, and HM polluted tailings dams (Barrutia et al., 2011), and it is colonized by several bryophyte species. Mean total concentrations of Zn, Pb and Cd in the soil were 30,850, 12,300 and 26 mg/kg respectively. The extractable fraction (CaCl<sub>2</sub> extractable) was 656, 21 and 2 mg/kg for Zn, Pb and Cd respectively (Barrutia et al., 2011). Mining activity in the area ceased in the 1970 s and nowadays the site appears naturally colonized by a wide range of metalicolous populations of, among others, *Noccaea caerulescens* J.Presl & C.Presl F.K.Mey., *Ulex europaeus* Savi, *Agrostis capillaris* L. and *Festuca rubra* L. (Barrutia et al., 2011). The non-metalliferous site (43°13'57.79' N, 3°26'24.49' W) was chosen in an area non affected by mine activities close to the metalliferous site ( $\approx 1$  km from the metalliferous site) and presenting similar edaphoclimatic conditions, except for the presence of metals in the soil.

### 2.2.2. Sample collection, identification and storage

The sampling of plant material was performed during May 24th, 2019 (a non-stressful month) in the abandoned mining area and the close control area. We randomly collected at midday several bryophytes carpets (1 cm<sup>2</sup>) from three separated locations in the abandoned mining area and the control area, paying attention to preserving the natural populations and pooling them to obtain a homogenous sample of each growth condition of each species. The collected material was stored in the dark in zip-locked polypropylene bags with humidity kept at high levels and transported to the laboratory in a portable icebox (temperature below 5 °C) (Fernández-Marín et al., 2018). Once in the laboratory, samples were kept in darkness in a saturated atmosphere for 12 h to provide comparable conditions (Tausz et al., 2003). Healthy green apices (1–3 cm long, depending on the species) were cut and repeatedly cleaned from any debris and washed with demineralized water until the complete removal of any rest of soil and particulate matter before further analysis. Identification of the collected species was carried out on air-dried samples in the laboratory, following the most recent and adequate identification dichotomous keys (Casas et al., 2006). The species were identified as *Lewinskya rupestris* (Schleich. ex Schwägr.) F.

Lara, Garilleti & Goffinet (Orthotrichaceae), *Polytrichum commune* Hedw. (Polytrichaceae), *Ptychostomum compactum* Hornsch. (Bryaceae), *Rhynchostegium confertum* (Dicks.) Schimp. (Brachytheciaceae), as reported in Hodgetts and Lockhart (2020) and Hodgetts et al. (2020). For each sampled species, 3–5 replicates of about 20 mg fresh weight (FW) of photosynthetic tissue for each population from the metalliferous and non-metalliferous sites were dried at –80 °C for 48 h or frozen with liquid nitrogen (depending on the analysis to be performed) and stored until further use. As traits in bryophytes will depend on the hydration status, we calculated the samples' dry weight (DW).

### 2.2.3. Species' bryological attributes and autecology

Bryological attributes and autecology of each moss species were described (Table 1) using the BRYOATT database following Hill and Preston (1998) and Hill et al. (2007) to compile for each species traits information. These species were classified according to life-form (LF1), type of perennation (Per), distribution of gametangia in gametophore (Sex), gametophores size (Len), major biome (E1) and eastern limit of distribution (E2). The following indices, each one representing different ecological species' requirements, were also determined: light (L), moisture (F), reaction to environmental acidity (R), nitrogen values (N) and tolerance to HM (HMT). The description of the indices is listed below. E1, ranges from 1 (Arctic, montane biome) to 9 (Mediterranean, Atlantic biome). E2, goes from 0 (hyperoceanic) to 6 (circumpolar). LF1 is described by the following categories: Ac, aquatic colonial; At, aquatic trailing; Cu, cushion; De, dendroid; Fa, fan; Le, lemnoid; Mr, mat rough; Ms, mat smooth; Mt, mat thalloid; Sc, solitary creeping; St, solitary

**Table 1**

List of species, families, orders, and divisions appearing in the selected papers for the meta-analytical approach. The number of times each *taxon* appears among the selected articles is reported in the right column.

Species (38)	Families (19)	
<i>Barbula vinealis</i>	1	<i>Hypnaceae</i> 6
<i>Brachythecium salebrosum</i>	1	<i>Pottiaceae</i> 5
<i>Bryum cellulare</i>	1	<i>Thuidiaceae</i> 4
<i>Dicranum undulatum</i>	1	<i>Brachytheciaceae</i> 3
<i>Eurhynchium eustegium</i>	1	<i>Hylocomiaceae</i> 3
<i>Eurohypnum leptothallum</i>	1	<i>Grimmiaceae</i> 2
<i>Fontinalis antipyretica</i>	1	<i>Polytrichaceae</i> 2
<i>Grimmia anodon</i>	1	<i>Sematophyllaceae</i> 2
<i>Haplocladium angustifolium</i>	1	<i>Aneuraceae</i> 1
<i>Haplocladium microphyllum</i>	1	<i>Aytoniaceae</i> 1
<i>Hylocomium splendens</i>	1	<i>Bryaceae</i> 1
<i>Hypnum cupressiforme</i>	1	<i>Dicranaceae</i> 1
<i>Hypnum plumaeforme</i>	1	<i>Fontinalaceae</i> 1
<i>Jungermannia exsertifolia</i> subsp. <i>cordifolia</i>	1	<i>Funariaceae</i> 1
<i>Lunularia cruciata</i>	1	<i>Jungermanniaceae</i> 1
<i>Marchantia polymorpha</i>	1	<i>Lejeuneaceae</i> 1
<i>Physcomitrella patens</i>	1	<i>Lunulariaceae</i> 1
<i>Plagiochasma appendiculatum</i>	1	<i>Marchantiaceae</i> 1
<i>Pleurochaete squarrosa</i>	1	<i>Sphagnaceae</i> 1
<i>Pleurozium schreberi</i>	1	
<i>Polytrichum commune</i>	1	<b>Orders (13)</b>
<i>Polytrichum piliferum</i>	1	<i>Hypnales</i> 19
<i>Pseudoscleropodium purum</i>	1	<i>Pottiales</i> 5
<i>Ptilium crista-castrensis</i>	1	<i>Grimmiales</i> 2
<i>Ptychanthus striatus</i>	1	<i>Marchantiales</i> 2
<i>Racomitrium lanuginosum</i>	1	<i>Polytrichales</i> 2
<i>Rhytidiadelphus squarrosus</i>	1	<i>Bryales</i> 1
<i>Riccardia pinguis</i>	1	<i>Dicranales</i> 1
<i>Scopelophila ligulata</i>	1	<i>Funariales</i> 1
<i>Sphagnum squarrosus</i>	1	<i>Jungermanniales</i> 1
<i>Taxiphyllum barbieri</i>	1	<i>Lunulariales</i> 1
<i>Taxiphyllum taxirameum</i>	1	<i>Metzgeriales</i> 1
<i>Taxithelium nepalense</i>	1	<i>Porollales</i> 1
<i>Taxithelium</i> sp.	1	<i>Sphagnales</i> 1
<i>Thuidium cymbifolium</i>	1	
<i>Thuidium delicatulum</i>	1	<b>Divisions (2)</b>
<i>Timmiella barbuloidea</i>	1	<i>Bryophyta</i> 32
<i>Tortula ruralis</i>	1	<i>Marchantiophyta</i> 6

**Table 2**

Bryological attributes of the studied species as concerns: primary life-form (LF1), where Tf stands for turf, Cu for cushion and Mr for rough mat; type of perennation (Per), where P stands for perennial; distribution of gametangia in gametophore (Sex), where M stands for monoecious and D for dioecious; gametophore's size expressed in mm (Len); major biome index (E1) values, where biomes are 3 wide boreal, 5 boreo-temperate, and 7 temperate biome; eastern limit of distribution (E2) index values, where biogeographic elements are 3 European and 6 circumpolar; Ellenberg light values (L) indicating 4 shade to semi-shade plants, 6 plants growing from semi-shady to well-lit ecological contexts, 8 light-loving plants, 9 plants growing in full light; Ellenberg moisture values (F), describing plants growing on 2 midway between extreme dry and dry sites, 5 moderately moist soils, 6 moist soils or humid places, 7 constantly moist, not permanently waterlogged substrata; reaction to environmental acidity (R) values indicate plants growing on 2 extreme acid to acid substrata, 5 moderately acid soils, 7 strongly basic substrata; nitrogen values (N) indicating plants growing on sites 2 infertile, 3 moderately infertile, 4 moderately infertile to moderately fertile, 6 moderately fertile to richly fertile; and tolerance to HM (HMT) values indicating 0 plants absent from substrata with moderate or high concentrations of HM and 1 plants rarely recorded on moderate or high concentrations of HM.

Species	LF1	Per	Sex	Len (mm)	E1	E2	L	F	R	N	HMT
<i>L. rupestris</i>	Cu	P	M	40	5	3	8	2	5	3	0
<i>P. commune</i>	Tf	P	D	250	3	6	6	7	2	2	1
<i>P. compactum</i>	Tf	P	M	15	5	6	9	6	7	4	0
<i>R. confertum</i>	Mr	P	M	30	7	3	4	5	7	6	0

thalloid; Tf, turf; Thread, thread; Tp, turf protonemal; Ts, turf scattered; Tuft, tuft; We, weft. Per describes the form of perennation with four categories: A, annual; AP, annual or perennial, more frequently annual; PA, perennial or annual, more frequently perennial; P, perennial. Distribution of sex organs is described by Sex categories: D, dioecious; M (M), normally dioecious, rarely monoecious; MD, monoecious or dioecious; M(D), normally monoecious, rarely dioecious; M, monoecious; Nil, gametangia unknown. Len values report in mm length of shoot or thallus (or diameter of the rosette). L ranges from 0 (plants growing in darkness) to 9 (plants growing in full light). F goes from 1 (plants that grow in extreme dryness) to 12 (normally submerged plants). R ranges from 1 (growth conditions of extreme acidity) to 9 (growth substrata with free calcium carbonate). N assumes values between 1 (plants growing in extremely infertile sites) and 7 (richly fertile growth conditions). Finally, HMT ranges from 0 (no tolerance to HM) to 5 (plants confined to substrata presenting moderate to a high concentration of HM).

#### 2.2.4. Fluorescence measurements

Fluorescence parameters were measured as a proxy of fitness on dark-adapted plants (30 min) at room temperature using the imaging fluorometer (Handy FluorCam FC 1000-H) and its related software FluorCam7 version 1.2.5.16. (P.S.I., Brno, Czech Republic, <http://www.psi.cz>), as described in Esteban et al. (2008). Fluorescence was detected by a high-sensitivity charge-coupled device camera equipped with a F 4.5–10 mm, 1:1.6 objective that produced 12-bit, colour images. Images of the dark-adapted fluorescence level,  $F_0$ , were determined using non-actinic measuring flashes, followed by an 800 ms duration pulse of saturating light radiation (2000  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) using 12 measuring flashes to measure the maximum fluorescence level,  $F_m$ . To improve the signal-to-noise ratio, both  $F_0$  and  $F_m$  were averaged. The maximal photochemical efficiency of photosystem II was estimated by the ratio  $F_v/F_m = (F_m - F_0)/F_m$ . Data were collected in five replicates for each of the four sampled species and to process the captured images, false-color images of leaf  $F_v/F_m$  were established based on the assumption that pixel intensity values can be related to the physiological process. In detail, fluorescence data analysis was made integrating results over the surface of the picture of the moss samples on square areas of about 200–350 pixels comprising green, photosynthetic apices to extract the most relevant information.

#### 2.2.5. Heavy metals analysis in moss tissues

The dry samples were weighed, pulverized (to obtain a representative sample) and then subjected to acid digestion for 15 min with 10 ml of concentrated  $\text{HNO}_3$  at 180 °C in a microwave (CEM, MARS One). The obtained solution was then diluted at 2%. The Cd, Pb and Zn concentrations were determined by inductively coupled plasma mass spectrometry (Agilent 7700), after generating calibration curves of emission using standard solutions of each metal (Sigma).

#### 2.2.6. Photosynthetic pigment and tocopherol content analysis

Pigments and tocopherols were extracted, analysed and quantified following the method of Esteban et al. (2014). Plant tissues were homogenized in pure acetone centrifuged at 4 °C for 20 min at 16,000  $\times$  g the supernatant being syringe-filtered by a 0.22  $\mu\text{m}$  PTFE filter (Teknokroma, Barcelona, Spain). 15  $\mu\text{l}$  of the extract of each replicate were injected on a reverse-phase C18 column HPLC system (water Spherisorb ODS1, 4.6  $\times$  250 mm, Mildford, MA, USA) as reported in García-Plazaola and Becerril (1999) and Fernández-Marín et al. (2018). This study was focused on compounds that indicate the level of photoprotection in bryophytes (Esteban et al., 2015). For this reason, in the results, the following compounds are shown: the total chlorophyll pool expressed on a dry weight basis (Chl a+b, nmol/g), the a to b chlorophyll ratio (Chl a/b, mol/mol), total carotenoids (sum of neoxanthin (Neox), lutein (Lut),  $\beta$ -carotene ( $\beta$ -carot), Violaxanthin (V), Antheraxanthin (A) and Zeaxanthin (Z)) expressed on both DW (nmol/g) and chlorophyll basis (carotenoids/Chl; mol/mol), total xanthophyll pool on DW ( $V+Z = V + A + Z$ ; nmol/g), de-epoxidation index ( $A+Z/V+A+Z$ ; mol/mol), Lut, Neox and  $\beta$ -carot on DW basis (nmol/g). Finally,  $\alpha$ -tocopherol,  $\gamma$  +  $\beta$ -tocopherols and total tocopherols (as the sum of the isomers  $\alpha$ ,  $\gamma$  +  $\beta$ ) were also expressed on a DW basis (nmol/g).

#### 2.2.7. Total antioxidant content determination

The antiradical (antioxidant) activity of moss samples (i.e., TAC), based on the scavenging activity of the stable free radical 2,2-diphenyl-2-picrylhydrazyl (DPPH) was estimated according to Clarke et al. (2013), with minor modifications. First, antioxidants were extracted from mosses tissues (50 mg) with 1.5 ml of a mixture of methanol:water:acetic acid (70:23:7). This solvent mainly extracts polar (hydrophilic) antioxidants such as ascorbate and phenols. Then, 50  $\mu\text{l}$  of the extract was added to 1.0 ml DPPH solution in methanol (0.05 mM) and the absorbance decay of the DPPH free radical at 517 nm was determined after 60 min (in the dark) at room temperature. The results were expressed as ascorbate equivalents as this antioxidant was used as a standard.

#### 2.2.8. Anthocyanin determination

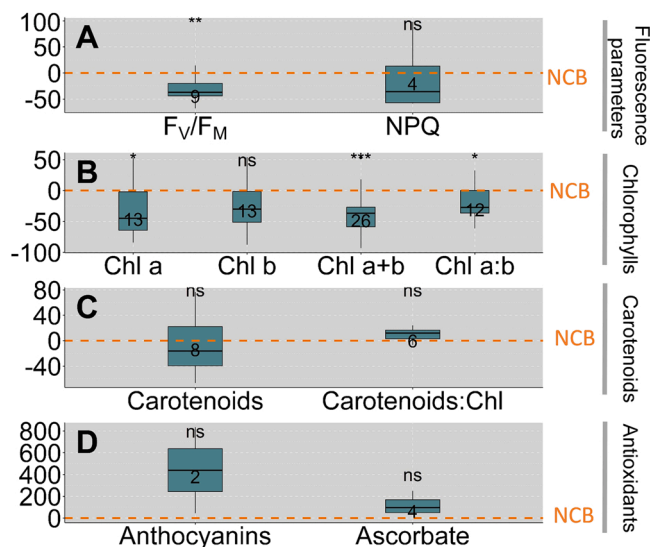
Anthocyanin determination was carried out as described in Esteban et al. (2008), analysing five replicates of each species from MP and NMP. In detail, approximately 15 mg (FW) of plant material were disrupted in liquid nitrogen and extracted in 1 ml of 3 M  $\text{HCl:H}_2\text{O: MeOH}$  (1:3:16 by vol.) using a tissue homogeniser. Extracts were centrifuged and anthocyanin levels estimated as cyanidin-3-glycoside equivalents using a molar extinction coefficient of 33,000 (Gould et al., 2000). The absorbance of anthocyanins at 524 nm was corrected by subtracting the interference by pheophytin as  $A_{524} - 0.24A_{653}$  (Murray and Hackett, 1991).

### 2.2.9. pH measurements

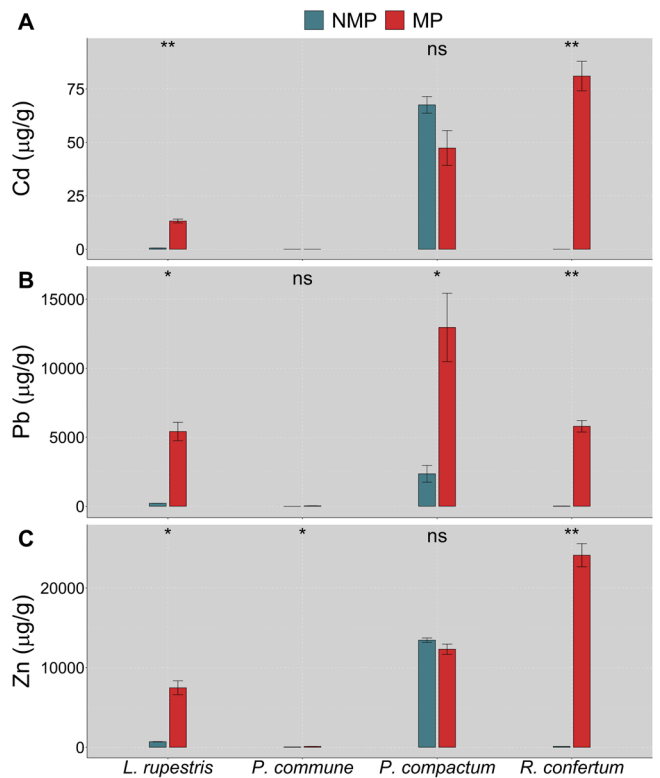
In MP and NMP five replicates of each species (~10 mg DW) were selected for pH determination. This was carried out as described in Cornelissen et al. (2006). Plant material was mixed with 1 ml of deionized water. The mixtures were agitated at 250 rpm for 1 h, then centrifuged at  $16,000 \times g$  for 5 min. The pH of the fluid was then measured using a narrow (5 mm diameter) SenTix Mic electrode connected to an Inolab Level 2 pH meter (both: WTW, Weilheim, Germany).

### 2.3. Statistical analysis

The normal distribution of data was assessed by the Shapiro-Wilk's test. Data representing the percentage of variation from control values (Fig. 1) were tested to significantly differ from a hypothetical sample of non-contaminated bryophytes (NCB) with a one-sample *t*-test or one-sample Wilcoxon test, in case of normally or non-normally distributed data, respectively. In the experimental field approach, we created a new category by averaging samples in each treatment (hereinafter *All*) that allows us to visualize whether a variation in a specific parameter due to HM (represented by boxplots) is specific to each species or if it is a global response. Wilcoxon test was used to determine if differences between metalliculous and non-metalliculous populations (NMP and MP) were significant (Figs. 2–5). The principal component analysis (PCA) to analyse heavy metals composition and species traits was performed using the packages “FactoMineR” and “factoextra”, to explain the variability of data in relation to the analysed variables and to evaluate if individuals clustered in relation to any of them. All statistical analyses



**Fig. 1.** Analysis of data compilation from the literature. Boxplots represent the percentage of variation of contaminated bryophytes with respect to non-contaminated bryophytes (NCB) values (represented by the orange dashed line) for *i*) fluorescence related parameters, i.e. photochemical efficiency ( $F_v/F_m$ ) and non-photochemical quenching (NPQ), (A), *ii*) chlorophylls, i.e. chlorophyll (Chl) a, b and Chl a+b (per weight unit), and Chl a to b ratio (Chl a/b) (B); *iii*) total carotenoids, i.e. carotenoids per weight unit and carotenoids to chlorophyll basis (carotenoids/Chl) (C); *iv*) antioxidants, i.e. anthocyanins and ascorbate; per weight unit (D). Each boxplot reports 50% of the measured values (inside the box), comprised between the first quartile value (lower side of the box) and the third quartile value (upper side of the box), the median is indicated by the black line inside the box, while whiskers join the first and third quartiles with lower and higher measured value respectively (outliers are reported by black dots). The number inside each box indicates the number of replicates. Statistical significance was tested by one-sample *t*-test or Wilcoxon test (in case of normality or non-normality of data distribution, tested by Shapiro-Wilk's test) to percentage values of variation from control values in the selected parameters and represented by asterisks when significant (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Note that ns indicates non-statistical significance.



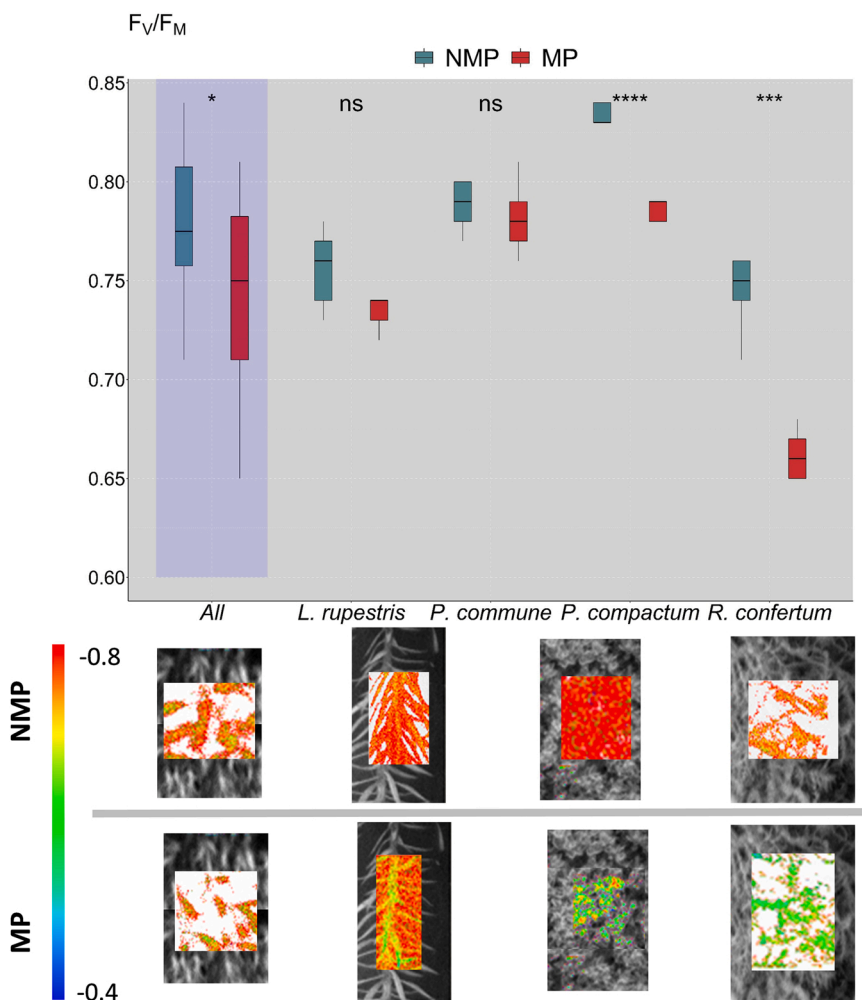
**Fig. 2.** Mean content of heavy metals in moss tissues (Cd, Pb and Zn;  $\mu\text{g/g}$ ) in metalliculous and non-metalliculous populations (NMP and MP, represented by green and red bars respectively) of the following species: *L. rupestris*, *P. commune*, *P. compactum* and *R. confertum* ( $n = 3 \pm \text{S.E.}$ ). Statistical significance of differences between NMP and MP values was tested by *t*-test and the results are represented by asterisks when significant (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Note that ns indicates non-statistical significance.

were carried with 1.2.1335 version of the R-Studio software (R Core Team, 2019). Data was considered significant at  $\alpha = 0.05$  or below.

## 3. Results

### 3.1. Data compiled from literature revealed a lack of field studies but also a global decrease trend of photochemical efficiency due to metals presence

Just 35 articles resulted suitable to be integrated into the analysis of data from the literature in the present study (Table 1S). Among them, the great majority of papers discussed results on individuals exposed to HM stress under laboratory conditions. Only 5 papers (those reported as 10, 21, 23, 26 and 32 in Table 1S) were based on field sampling and only one article (reported in Table 1S as 20) discussed data analysed on individuals naturally growing in a polluted abandoned mining area. We analysed the percentage of variation between CB and NCB of several traits implicated in photoprotective response: Fluorescence parameters ( $F_v/F_m$  ratio and NPQ; Fig. 1A), chlorophylls (Chl a, Chl b, Chl a+b and Chl a/b; Fig. 1B), carotenoids (on weight and chlorophyll basis; Fig. 1C), anthocyanins and ascorbate (Fig. 1D). We found a 32% statistically significant decrease of  $F_v/F_m$  (Fig. 1A), together with a significant decrease in Chl a (36% of decrease with respect to NCB), Chl a+b (38% decrease) and Chl a/b (10% decrease) in CB as compared with NCB mosses (Fig. 1B). However, we did not find a significant decrease in Chl b (Fig. 1B). Regarding carotenoids, no significant change was found with respect to CB (Fig. 1C). Although a 440% increase in the anthocyanins content and a 91% increase in hydrophilic antioxidants as ascorbate manifested in CB, these data resulted to be not significant, probably due to the low number of replicates ( $n = 2-4$ ) (Fig. 1D).



**Fig. 3.** Maximum photochemical efficiency ( $F_v/F_M$ ) in metallicolous and non-metallicolous populations (NMP and MP, represented by green and red bars respectively) of the following species: *L. rupestris*, *P. commune*, *P. compactum* and *R. confertum* ( $n = 5$ ). The first two boxplots (grouped in the category *All* and light blue-shaded) averaged the data of the four studied species. Each boxplot reports 50% of the measured values (inside the box), comprised between the first quartile value (lower side of the box) and the third quartile value (upper side of the box), the median is indicated by the black line inside the box, while whiskers join the first and third quartiles with lower and higher measured value respectively (outliers are reported by black dots). False color images from  $F_v/F_M$  are shown for each species, represented by the cold-to-hot color gradient reported in the scale bar at the left. Statistical significance of differences between NMP and MP values was tested by *t*-test and the results are represented by asterisks when significant (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Note that ns indicates non-statistical significance.

### 3.2. Experimental field approach highlighted the species-specific tolerance patterns

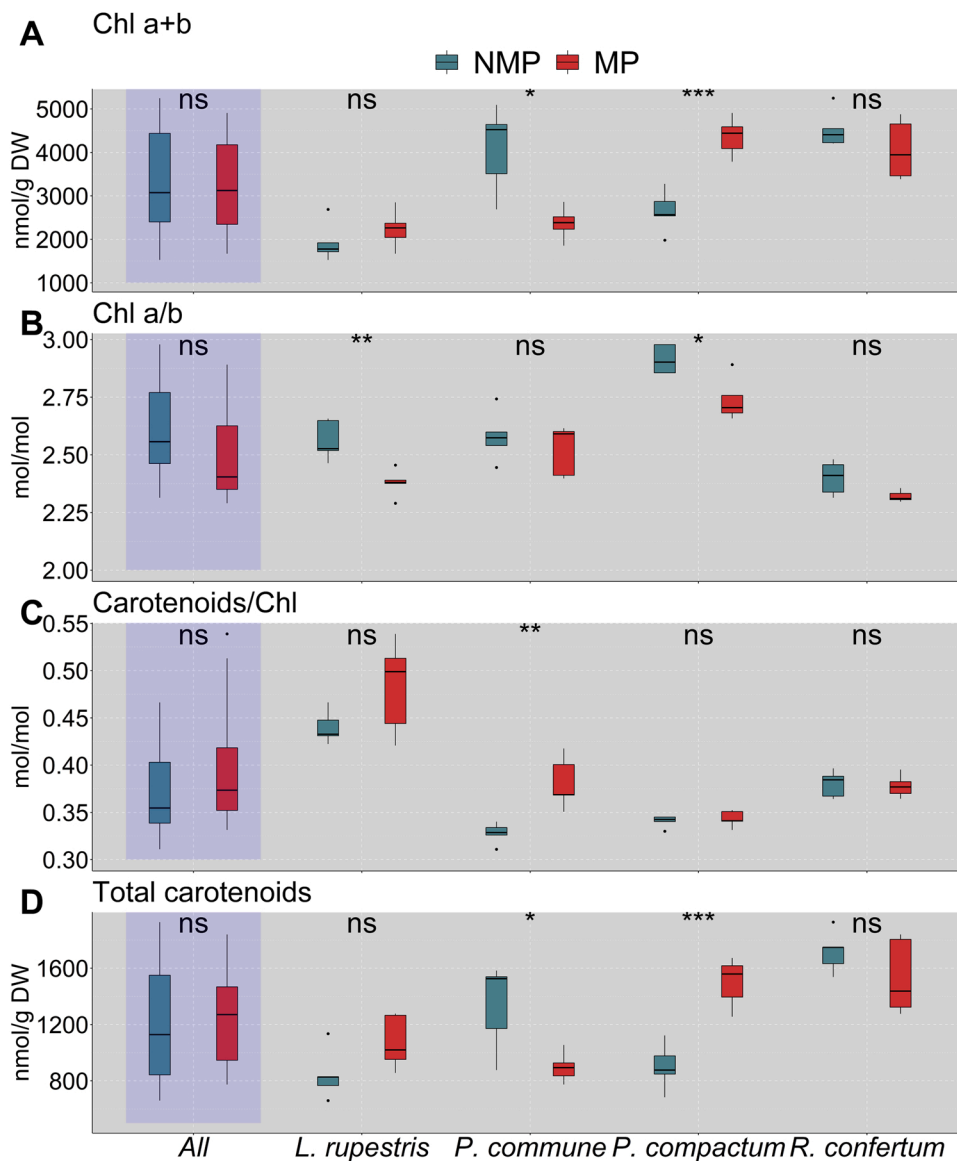
We analysed the metal composition (Cd, Pb and Zn) in both metallicolous (MP) and non-metallicolous (NMP) mosses. Zinc was the metal that reached the highest tissue concentration in all the species, most likely due to the high availability levels of this metal present in soil (see site description in material and methods) as well as to its high mobility. Besides, we found species-specific metal content (Fig. 2). In detail, *L. rupestris* and *R. confertum* metallicolous populations presented significant higher values of the three elements, whilst *P. compactum* only showed significant higher values for the element Pb. On the other hand, *P. commune* seemed to avoid HM content in tissues with both populations (MP and NMP) showing null (Cd) or very low levels (Pb and Zn).

Regarding traits induced by HM, we analysed the photoprotective responses of the metallicolous and non-metallicolous populations of mosses in this mining environment. The pattern observed for photochemical efficiency (Fig. 3) was similar to the results obtained by the analysis of data compilation from literature, with significantly lower values of  $F_v/F_M$  for *P. compactum* and *R. confertum* growing under HM (MP). However, this decrease ( $\approx 13\%$ ) was smaller than the decrease observed in the analysis of data from the literature. To be noticed, *L. rupestris* and *P. commune* did not show significant differences in  $F_v/F_M$  between both populations. If we average  $F_v/F_M$  results of the four species together, the statistically significant difference between NMP and MP was maintained (Fig. 3, category *All*), indicating a generalized and

global decrease trend of this parameter under HM presence.

Regarding pigments (Figs. 4–5), we obtained species-specificity responses, however, any statistically significant difference between MP and NMP was not maintained when the four species values were averaged (*All* boxplots, Figs. 4–5). This means that differential responses between growth conditions (MP and NMP) and species were flattered and counterbalanced when plotted together, due to opposite pigment trends responses in each species. In detail, Chl a+b of MP significantly decreased in *P. commune*, while in *P. compactum* increased and it was not affected in *L. rupestris* (low total chlorophyll pool in both populations;  $\approx 2000$  nmol/g DW) and *R. confertum* (Fig. 4A). The ratio Chl a/b (Fig. 4B) decreased significantly only in two species, *L. rupestris* and *P. compactum*. A significant increase in carotenoids to chlorophyll ratio (Fig. 4C) was measured only for MP of *P. commune*. Total carotenoids (Fig. 4D) followed the same response observed in Chl a+b content.

Analysing individuals' carotenoids content (VAZ, AZ/VAZ, total Lut, Neox, and  $\beta$ -carot; Fig. 5), we observed again that the response trend of each MP moss was different, confirming the lack of any statistically significant difference between MP and NMP when values proceedings from the four species are averaged (*All* boxplots, Fig. 5A–E). As expected, *L. rupestris* did not show any significant change in any of the carotenoids analysed, as was shown by the total carotenoid pool (Fig. 4C, D). The moss *P. commune* significantly decreased the  $\beta$ -carot content (Fig. 5E). On the other hand, *P. compactum* significantly decreased the de-epoxidation index in MP (Fig. 5B), together with a significant increase of VAZ, Lut, Neox and  $\beta$ -carot (Fig. 5A, C–E), whilst *R. confertum* exhibited a significant decrease of total xanthophyll pool, VAZ (Fig. 5A)



**Fig. 4.** Boxplots showing the total chlorophyll content (Chl a+b; nmol/g DW) (A); Chlorophyll a to b ratio (Chl a/b, mol/mol) (B); carotenoids to Chl ratio (carotenoids/Chl; mol/mol) (C); total carotenoids content (D; nmol/g DW), in metallicolous and non-metallicolous populations (NMP and MP, represented by green and red bars respectively) of the following species: *L. rupestris*, *P. commune*, *P. compactum* and *R. confertum* ( $n = 5$ ). The first two boxplots (grouped in the category All and light blue-shaded) averaged the data of the four studied species. Each boxplot reports 50% of the measured values (inside the box), comprised between the first quartile value (lower side of the box) and the third quartile value (upper side of the box), the median is indicated by the black line inside the box, while whiskers join the first and third quartiles with lower and higher measured value respectively (outliers are reported by black dots). Statistical significance of differences between NMP and MP values was tested by *t*-test and the results are represented by asterisks when significant (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Note that ns indicates non-statistical significance.

but an increase of the de-epoxidation index (Fig. 5B) in MP.

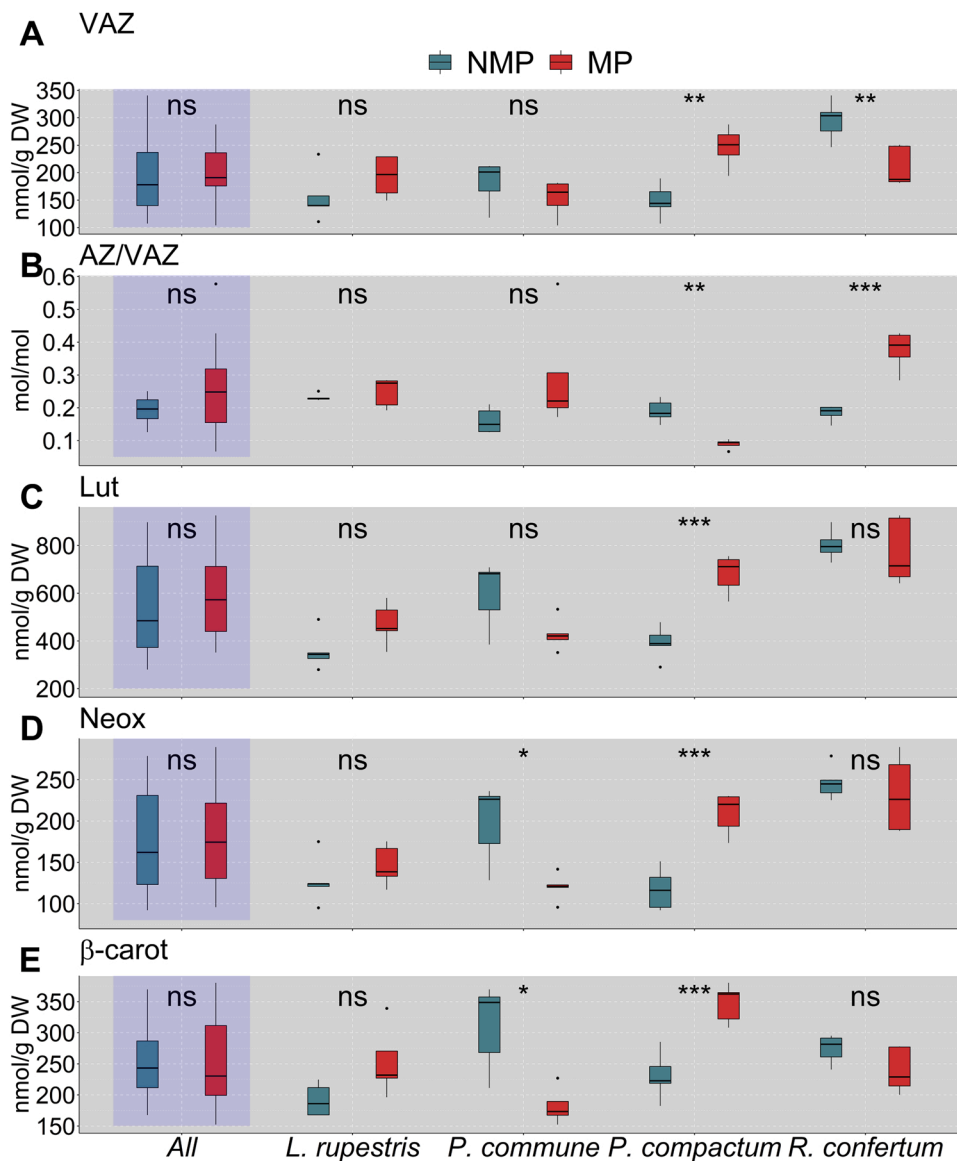
We again found a great differential response on the accumulation of antioxidants between MP and NMP depending on the moss species (Fig. 6). Nevertheless, when  $\gamma + \beta$ -tocopherol values were considered together (Fig. 6B, All boxplots), these compounds maintained a statistically significant increase in MP, meaning that the same trend is roughly shared by each species considered singularly as well. If we analyse the results individually, we found that *L. rupestris* was the only species showing a significant increase of both  $\alpha$ -tocopherol and  $\gamma + \beta$ -tocopherol (Fig. 6A, B), but with no changes in the rest of the parameters. The MP of *P. commune* also increased significantly  $\gamma + \beta$ -tocopherol, (Fig. 6B) and showed the highest basal values of total antioxidant capacity (TAC) in both MP and NMP (Fig. 6C). The level of this hydrophilic antioxidant can be related to very low pH values measured in this species (Fig. 6E), especially in MP (in which pH values significantly decreased). MP of *R. confertum* increased significantly  $\gamma + \beta$ -tocopherol (Fig. 5B) and anthocyanins (Fig. 5D). Besides, *R. confertum* MP was the only case in which pH values significantly increased (Fig. 6E). On the other hand, MP of *P. compactum* did not show any significant change in any antioxidant with respect to the NMP (Fig. 6A-D).

A principal component analysis (PCA) was performed to help

disentangle species strategies to cope with metallicolous growth conditions (Fig. 7). The 53.4% of the total variance of the analysed data was explained by two principal components. Most of the total variance of the PCA was explained by PC1, being the total carotenoids, Lut, Neox, Chl a+b, VAZ and  $\beta$ -carot the variables that better explain PC1 (that alone explains the 34.4% of the total variance of data). The PC2, that explained 18.9% of the variance, results to be very informative as it explains the influence of Cd, Pb and Zn and main antioxidants (TAC,  $\gamma + \beta$ -tocopherol and  $\alpha$ -tocopherol). Overall, the PCA confirmed the trend observed in previous Figs. (3–6). The MP and NMP of each species clustered apart occupying different regions of the PCA space as a result of different and complex strategies put in place by the different species to respond to the same ecological challenge. The species *P. compactum* and *R. confertum* presented higher content of pollutants and resulted to be heavily influenced in their physiological parameters in MP, while *L. rupestris* and *P. commune* presented lower/minimum contents of pollutants being less influenced in their physiological traits.

#### 4. Discussion

Anthropocene activities and especially mining activity caused local and diffuse accumulation of heavy metals that might threaten the

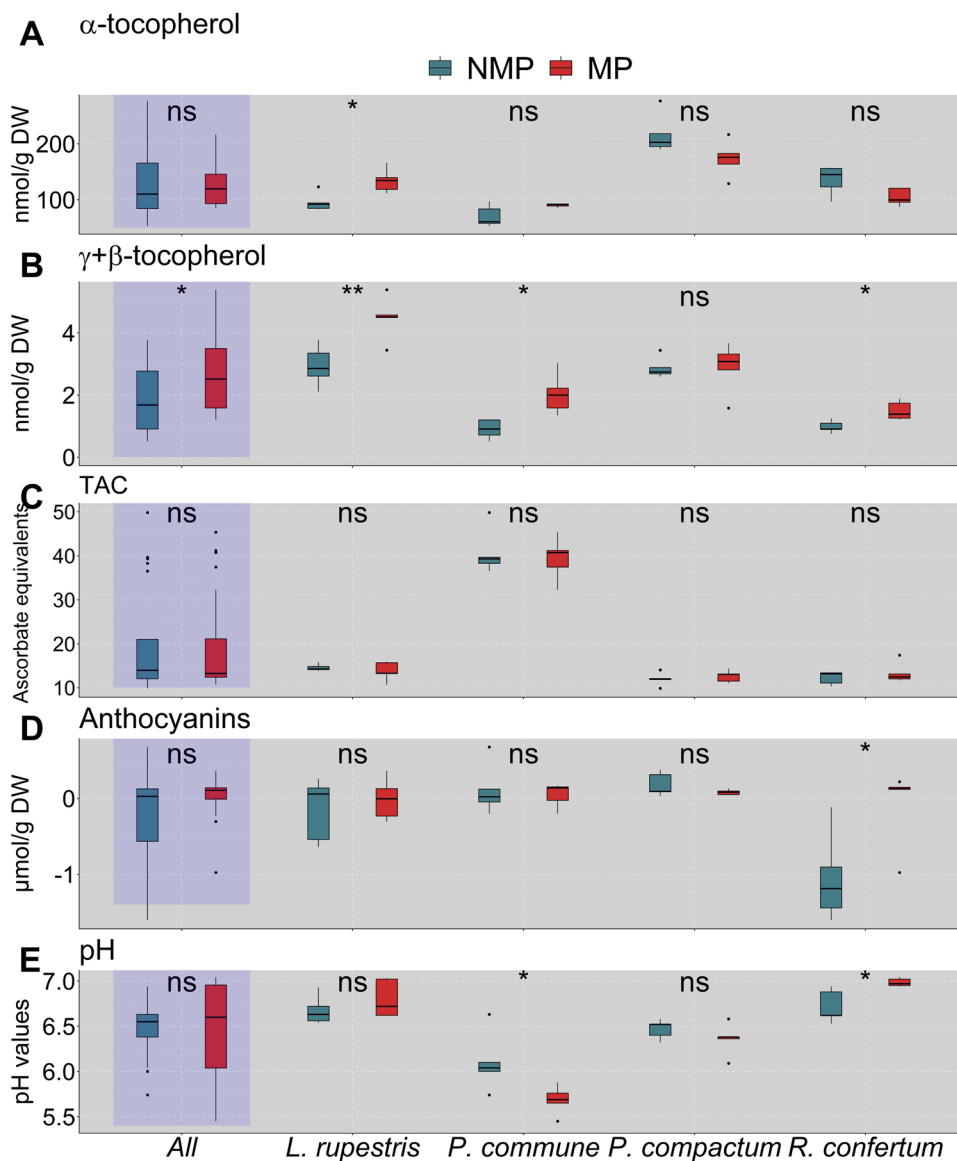


**Fig. 5.** Boxplots showing total xanthophyll content (VAZ, nmol/ g DW) (A), deepoxidation ratio (AZ/VAZ, mol/ mol) (B); lutein content (Lut, nmol/ gDW) (C); neoxanthin content (Neox, nmol/ gDW) (D);  $\beta$ -carotene content ( $\beta$ -carot, nmol/g DW) (E), in metalloicolous and non-metalloicolous populations (NMP and MP, represented by green and red bars respectively) of the following species: *L. rupestris*, *P. commune*, *P. compactum* and *R. confertum* ( $n = 5$ ). The first two boxplots (grouped in the category All and light blue-shaded) averaged the data of the four studied species. Each boxplot reports 50% of the measured values (inside the box), comprised between the first quartile value (lower side of the box) and the third quartile value (upper side of the box), the median is indicated by the black line inside the box, while whiskers join the first and third quartiles with lower and higher measured value respectively (outliers are reported by black dots). Statistical significance of differences between NMP and MP values was tested by *t*-test and the results are represented by asterisks when significant (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Note that ns indicates non-statistical significance.

environment and human health (Stewart, 2019). Ongoing mining activity is estimated on 57,277 km<sup>2</sup> of planet surface (Maus et al., 2020), however larger areas remain still affected nowadays by the consequence of past and ceased mining activities. These habitats with past mining activities history are characterized by a combination of abiotic stresses (e.g., high irradiation, soil scarcity, aridity and HM-polluted growth substrate) and are usually colonized by bryophytes that adapt to these metalloicolous conditions (due to their early land colonizers ability; e.g., Holyoak and Lockhart, 2011; Graham et al., 2014; Soriano et al., 2019). Nevertheless, the photoprotective mechanisms involved in this group of plants in the adaptation and tolerance to HM polluted ecosystems, such as abandoned mining areas, are far to be deeply understood. We confirmed this gap of knowledge by the analysis of data compilation from literature (Fig. 1, Table S1). Most of the articles consisted indeed of non-preadapted species or individuals suddenly exposed to HM treatments under laboratory conditions. Despite the great contribution that this approach provided to the knowledge on bryophytes behaviour and tolerance to HM stress, this resulted in addressing the relationship between bryophytes and HM with an acclimation-focused perspective rather than by a more adaptive-oriented view. Indeed, among the 35 articles selected, only 6 were focused on field-sampled individuals. This was the reason why we only found three parameters affected in

contaminated bryophytes: a decreasing trend of photochemical efficiency (Fig. 1) concomitant with total chlorophylls and chlorophyll a to b ratio decrease (Fig. 1B) under a contaminated environment. This response was due to chlorophyll degradation (Panda and Choudhury, 2005; Ares et al., 2018; Chen et al., 2018; Liang et al., 2018), antenna size changes, mainly by chlorophyll a degradation (Shakya et al., 2008; Aydođan et al., 2017; Nakajima and Itoh, 2017); and probably to photoinhibition due to HM phytotoxicity expressing at a chloroplast level (Chen et al., 2015). Regarding carotenoids, we did not find a consistent response, possibly because different strategies existed regarding HM-stress response, and the overall effects were diluted (e.g., opposite pigments trends). This dilution effect was also evident when averaging the four species data in the experimental field approach (All boxplot, Figs. 3–6), indicating high species-specificity and the possible role of different bryological attributes in the studied species in determining the variety of responses to this stress. Indeed, Cornelissen et al. (2007) discussed how different cryptogam traits could relate and influence ecosystems' properties and highlighted the cruciality of deepening this knowledge to understand important ecosystems functions and so predict their changes. Nowadays, there is a lack of general trait patterns that could be applied to all bryophyte species globally. More HM-tolerance-oriented studies for non-vascular plants, together with a





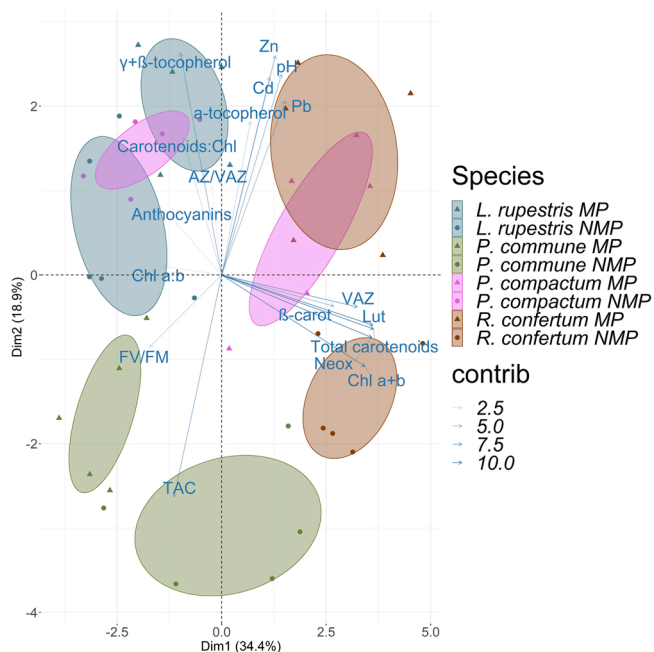
**Fig. 6.** Boxplots showing  $\alpha$ -tocopherol content (nmol/g DW, A),  $\gamma$  +  $\beta$ -tocopherol content (nmol/g DW, B); total antioxidant content (TAC, expressed as ascorbate equivalents; C); anthocyanins, expressed as cyanidin-3-glycoside equivalents content (D); pH (E) in metalicolous and non-metalicolous populations (NMP and MP, represented by green and red bars respectively) of the following species: *L. rupestris*, *P. commune*, *P. compactum* and *R. confertum* (n = 5). The first two boxplots (grouped in the category *All* and light blue-shaded) averaged the data of the four studied species. Each boxplot reports 50% of the measured values (inside the box), comprised between the first quartile value (lower side of the box) and the third quartile value (upper side of the box), the median is indicated by the black line inside the box, while whiskers join the first and third quartiles with lower and higher measured value respectively (outliers are reported by black dots). Statistical significance of differences between NMP and MP values was tested by *t*-test and the results are represented by asterisks (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001). Note that ns indicates non-statistical significance.

global scale meta-analysis considering species bryological attributes, are needed to reveal the photoprotective responses activated living in a metalicolous environment.

In the experimental field approach, we studied bryophytes living in a mine. Therefore, the results should be interpreted as adaptive mechanisms gradually developed by bryophytes in their struggle to colonize the metalliferous niche and cope with the HM stress and related abiotic stresses associated with these environments. In general, we found two important events: *i*) the metal content found in bryophytes resulted to be much higher than those of vascular plants growing in the same study site (Barrutia et al., 2009); *ii*) all the species analysed were adapted to grow and live in the metalicolous site. This was indicated by the low reduction of the photosynthetic efficiency between MP and NMP (Fig. 3), which indicates downregulation of the photosystems more than a photoinhibition process (Chen et al., 2015, 2018).

We also found large adaptive interspecific differences between the metalicolous bryophyte community (Fig. 8), with three main strategies activated to cope with the abiotic stresses present in the mining site, outlined by the photosynthetic, pigments and antioxidant responses together (Figs. 3–7) with the ecological attributes and growing habits of the studied species helping in explaining of the observed variability (Table 1):

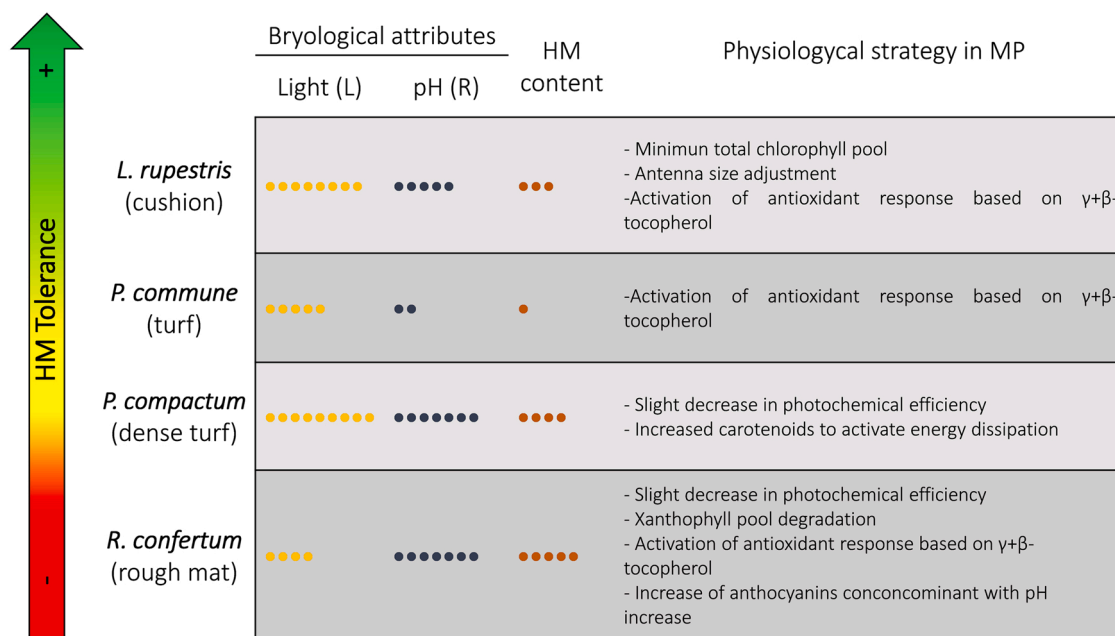
- Sensitive strategy depicted by the species *R. confertum*. This species is a pleurocarpous moss that forms rough, loose mats (Table 1, LF1 index), so this growth habit obliges the bryophyte to live in strict relation with the polluted soil having a great surface exposed to both wet and dry atmospheric pollutants deposition. Pleurocarpous (prostrate) mosses seem more metal-sensitive than other life forms as acrocarpous (erect) mosses (Širka et al., 2019). This may explain the fact that *R. confertum* was the species that accumulated most pollutants in MP (Fig. 2) and the increase of its internal pH values (Fig. 6E). We interpreted this as a consequence of the basic cations' uptake in the metalliferous site (a base-rich environment) (Cornelissen et al., 2011), considering the intimate relation of the prostrate moss with the soil and its elements (Širka et al., 2019). All this caused a significantly lower photochemical efficiency in MP than in NMP (Fig. 3) indicating that HM negatively affected its photosynthetic yield. To cope with this photosynthetic activity decrease, the species put in place the photoprotective mechanisms in MP marked by a significant decrease of total xanthophyll pool, probably due to carotenoids degradation (Esteban et al., 2015), and a significant increase in AZ/VAZ (Fig. 5 A and B), showing the activation of the violaxanthin cycle (Esteban et al., 2014). The photoprotective strategy



**Fig. 7.** Principal component analysis biplot for each species (*L. rupestris*, *P. commune*, *P. compactum* and *R. confertum*) represented by different colours (blue, green, pink and brown respectively) and growing conditions (metallicolous and non-metallicolous populations; MP and NMP) represented by different symbols (triangle and circle respectively). The contribution of each considered variable to each principal component is represented by the transparency of the associated vector. Confidence concentration ellipses are drawn around groups' mean points to better identify clustered individuals.

was accompanied by increased production of antioxidants as  $\alpha$ -tocopherol and/or  $\gamma + \beta$ -tocopherol (Fernández-Marín et al., 2017; Cotado and Munné-Bosch, 2020) together with enhanced anthocyanins in MP (Chen et al., 2015, 2019), likely to mitigate oxidative stress and ROS production (Foyer et al., 2016) or as part

- of signalling mechanisms in imparting tolerance to heavy metal stress, as discussed at the end of this section.
- ii. Medium tolerance strategy depicted by *P. compactum*. This species was sampled sunlit, forming dense turfs in direct contact with the soil (Table 1, LF1 index). Its close relationship with the polluted soil and the tendency to form felt-like dense turfs is probably the cause for the high concentration of Cd, Zn, and Pb in MP (Fig. 2). Curiously, both NMP and MP accumulated high pools of Cd and Zn. This is explained by their life form, bryological attributes, and the presence of the studied mining area nearby. These bryophytes usually grow deprived of a vegetal canopy. Thus, the soil microparticles and airborne pollutants reach the individuals, being these metals entrapped in plant tissues. This species showed a slight but significant decrease in the photochemical efficiency (Fig. 3) together with a significant increase of total carotenoids (Fig. 4D) in MP, adopting an energy-dissipative strategy (Fig. 5B) (Esteban et al., 2015) to prevent photo-inhibition in highly irradiated and polluted individuals to compensate the oxidative stress generated due to HM.
- iii. Tolerant strategy represented by low pollutants content and little changes in biochemical composition and physiological response depicted by *L. rupestris* and *P. commune*. The latter is a perennial moss that was sampled in shaded turfs growing on deep, moist soils in both MP and NMP (in line with its F and L values in Table 1). Interestingly, *P. commune*, despite being the only species among the four studied presenting hydroids and leptoids, managed to avoid the metal content in its tissues (Cd, Pb) with only, low levels Zn (high mobility element). If we pay attention to the Ellenberg's indicator (R and HM values in Table 1), *P. commune* is depicted to tolerate the lowest soil pH and the highest HM content in the soil. These concomitant features led us to hypothesize that the species' tolerance for acidic substrata could be involved in its tolerance toward HM soil pollution. Indeed, acidophilous bryophytes taxa are described in Büscher et al. (1990) to present lower cation-exchange capacity, since soil acidity often leads plants to suffer metal uptake-linked stress. So plants that thrive in acidic environments may develop metal exclusion mechanisms and antioxidative responses at ready to



**Fig. 8.** Species physiological strategy depiction to metallicolous soils based on their life form, bryological attributes (each dot indicating one unit in the light (L) and pH (R) indexes; based on Table 2), metal content (HM, based on Fig. 2) and physiological strategy (based on Figs. 3–7) The range of colours of the arrow indicates the adaptation degree of each bryophyte (red to green gradient specifies increasing adaptation degree in species).

counteract metal-linked oxidative stress (Guo et al., 2004; Kochian et al., 2004). This is reflected in the present study by the lowest pollutant content and highest TAC measured in *P. commune*. As a result of the low pollutant content in the species,  $F_V/F_M$  ratio between MP and NMP did not change, indicating any down-regulation of photosynthetic activity. Therefore, the decrease in pigments (total chlorophylls (Fig. 4A), total carotenoids (Fig. 4D), and Neox (Fig. 5D) in MP should be attributed to HM-independent and controlled mechanism put in place by the species to avoid photoinhibition in MP rather than an effect of HM phytotoxicity. The pH of *P. commune* was the lowest among the four species, further decreasing in MP. This could be related to the role of organic acids in mitigating abiotic stresses linked to soil acidity in tolerant plants (Kochian et al., 2004) and to the highest levels of TAC measured in the species. This also needs to be interpreted as a native feature of the acidophilic moss. Finally, the moss *L. rupestris*, which is a perennial epilithic moss (Casas et al., 2006), was sampled growing in cushions upon massive calcareous boulders and appears to be the more tolerant species of this study. This morphology favours high exposure to sun irradiation and fast dehydration (coherently with species' L and F indexes values reported in Table 1), limiting, therefore, the pollutant content to the sole atmospheric deposition of soil particles carried by the wind. This explicates the low levels of Cd, Pb and Zn measured in its tissues (Fig. 2) if compared with those of the other species growing in close contact with the polluted soil (e.g., *R. confertum*, *P. compactum*). This low HM content in *L. rupestris* reflected in its physiological parameters and photoprotective responses that remain largely unaffected by metal-liferous growth conditions (Figs. 3–6). Indeed, the only traits that significantly differed between MP and NMP in this species were the ratio Chl a/b (decreasing in MP; Fig. 3C), indicating antenna size adjustment (Croce, 2012; Morosinotto and Bassi, 2012) and induction of both  $\alpha$ -tocopherol and  $\gamma + \beta$ -tocopherol in MP (Fig. 6A–B). This, together with its basal chlorophyll content (Fig. 4A) avoided photoinhibition in the species (Fig. 3). No phytotoxicity symptoms in response to the pollutants is an inherent characteristic of tolerant plants (Barrutia et al., 2011; Grijalbo et al., 2016) indicating that their metabolic equilibrium is not damaged by an increased metal uptake (Adriano, 2001; Širka et al., 2019). Future metal-liferous individuals sampling at different seasons and ecological conditions may give light to the relationship between the physiology of the heavy metal stress in bryophytes and other elements such as season, wet deposition, growth condition and age of samples.

The interspecific traits variation (mainly metabolites) presented here could be a tool for understanding and even predicting bryophytes' responses to environmental conditions (Cornelissen et al., 2007). Indeed, metabolites are key components in both biochemical and ecological processes (van Dam and van der Meijden, 2018; Matich et al., 2019). We have observed a general trend of increase in the levels of  $\gamma + \beta$ -tocopherol in moss species from MP. Although  $\alpha$ -tocopherol was the most abundant form in the analysed species of this study (i.e., 100–200 nmol/g DW), as in most plant species (Esteban et al., 2009), we only found its induction in *L. rupestris* (Fig. 6). This antioxidant protects photosystem II from photoinhibitory damage (Munné-Bosch, 2005) and seems the most powerful antioxidant form, however other factors as mobility, accessibility, distribution and interaction with membrane components can modify its antioxidant properties and other potential functions (Falk and Munné-Bosch, 2010). Recent evidence suggests specific functions for different tocopherol homologs, specially related to abiotic stress responses (Fritsche et al., 2017), as  $\gamma + \beta$ -tocopherol that could exert a specific role in plant resistance to salinity, osmotic stress, and/or desiccation tolerance (Abbasi et al., 2007; Cela et al., 2011). Moreover, several studies have shown a preferential increase of

$\gamma + \beta$ -tocopherol in response to pollutants including metals (Collin et al., 2008; Grijalbo et al., 2016; Lacalle et al., 2018). Indeed these results could be relevant in mosses due to particular tolerance to desiccation of bryophytes (Proctor, 2000). An intriguing fact to be noted is that the levels of  $\gamma + \beta$ -tocopherol were very low (1.5–4.5 nmol g<sup>-1</sup> DW) as compared with those of  $\alpha$ -tocopherol (Fig. 6 A, B), and the observed significant accumulation of  $\gamma$ -tocopherol could be more related to stress signals to cope with metal stress, rather than merely role as antioxidant or precursor of  $\alpha$ -tocopherol. Several studies indicate the potential role of  $\gamma$ - and other tocopherols in stress sensing and signalling (Fritsche et al., 2017; Munné-Bosch, 2019). In salt-stressed mutants that accumulate  $\gamma$ -tocopherol, jasmonic acid- and ethylene-signaling genes were downregulated (Cela et al., 2011). A similar response leading to gene regulation under metal adaptation to metalliferous soils could occur on mosses, as ethylene and jasmonic acid production have been demonstrated in bryophytes (Záveská Drábková et al., 2015; Sabovljević et al., 2014). Phytohormones crosstalk may have an important impact on photoprotection due to their role in scavenging ROS (Müller and Munné-Bosch, 2021). Bryophytes can be interesting model organisms to unravel the role of tocopherols in stress sensing and its relationship with phytohormones, a very unknown issue.

## 5. Conclusions

The species *R. confertum* appeared as a suitable sentinel species due to its high pollutant content and its ability to colonize polluted ecosystems. However, the four studied species can colonize and cope with the complexity of abiotic stress linked to the metalliferous site by the specific-species dependant response. This answer may be attributed partly to the modulation of the metabolites' basal levels (i.e., those measured in NMP) and partly to the diversity of species attributes (Fig. 8), some advantaging (e.g., epilithism of *L. rupestris* or tolerance to soil acidity of *P. commune*), some other disadvantaging (e.g., the strict relation with the soil of *R. confertum*). Among the induced response to pollutants,  $\gamma + \beta$ -tocopherol content emerged to be suitable as a plant functional trait biomarker and could play an important role in stress sensing and signalling in bryophytes adapted to HM polluted habitats. This overlooked group of plants that are equally important to terrestrial ecosystems represents peculiar biodiversity that should be better characterized in its adaptation to extreme metalliferous environments to preserve them as models to study the complex signalling response that integrates isoprenoids and phytohormones.

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## CRedit authorship contribution statement

**Antonio De Agostini:** Research conception and design, Field experimental, Data collection and laboratory analysis, Analysis and interpretation of the data, drafting of the article, Critical revision of the article. **Annalena Cogoni:** Research conception and design, Critical revision of the article. **Pierluigi Cortis:** Research conception and design, Critical revision of the article. **Andrea Vacca:** Research conception and design, Critical revision of the article. **José María Becerril:** Research conception and design, Analysis and interpretation of the data, Critical revision of the article. **Antonio Hernández:** Research conception and design, Field experimental, Data collection and laboratory analysis, analysis and interpretation of the data, Critical revision of the article, work supervision, **Raquel Esteban:** Research conception and design, Analysis and interpretation of the data, Drafting of the article, Critical

revision of the article, Work supervision.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.envexpbot.2021.104738](https://doi.org/10.1016/j.envexpbot.2021.104738).

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