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Ecophysiological responses of bryophytes and orchids to environmental pollution: an integrated approach

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Index

ABSTRACT

Environmental pollution is one of the largest threats to human societies. To deepen the relationships between plants and environmental pollution it is crucial to profit from plant biodiversity in the control and mitigation of environmental pollution whilst simultaneously preserving such biodiversity from the adverse effects of pollution. Plant–pollutant interactions are widely studied in the scientific literature. However, studies focusing on natural populations facing polluted environments are rare. In this scenario, mosses and orchids (the two groups discussed in the present thesis) are even less studied in their adaptive mechanisms for coping with environmental pollution. Mosses and orchids respectively belong to the Bryophyta (*sensu lato*) and Angiospermae *taxa*, the former being non-vascular cryptogams whilst the latter are vascular flowering plants. The two plants belong to phylogenetically distant groups (at the antipodes of the evolutionary path of land plants) and their ecophysiological relationships with environmental inorganic pollutants (*i.e.* heavy metals) and their survival strategies were studied herein. The two groups considered were found to occur naturally in environments featured by severe heavy metal pollution due to previous mining activity. The metallicolous populations of orchids and mosses were characterised by several morphometric, physiological and ecological parameters and then compared with unpolluted control populations. The studied metallicolous populations were found to be different from control populations due to the adaptive process that allowed the colonisation of such extreme environments. The observed differences between metallicolous and control populations are as follows: *i*) varying dimensions of adult individuals and seeds (orchids), *ii*) alterations in basal levels of metabolites and photosynthetic efficiency (mosses and orchids) and *iii*) differential early seed development. The present thesis also focused on the use of plants as biomonitors of environmental pollution. The reliability of the moss bag technique was evaluated by analysing accumulation values measured during an extensive biomonitoring campaign conducted in the

vicinities of an oil refinery. Obtained results not only confirmed the reliability of the biomonitor species and the adopted biomonitoring technique but also provided evidence regarding the influence of several experiments and ecology-related factors on accumulation values. Both focal points of the present thesis (the study of naturally occurring metallicolous populations and the biomonitoring of environmental pollution) reveal that plants are extremely useful tools in the control of environmental pollution and the study of the biosphere–pollutant interaction at the individual and ecological levels.

Introduction

Environmental pollution is defined as the introduction of matter or energy originating from human activities (defined as pollutants) in the environment, resulting in deleterious effects on the ecosystems and/or compromising the quality of the atmosphere, water and soil for any subsequent use (Russell, 1974). Hazardous elements and compounds could have a natural geogenic source, however, the majority of pollutants that threaten human health and ecosystems could be defined as anthropogenic, which means originating from human activities (Lee et al., 2020; Rodríguez-Eugenio et al., 2018).

The Lancet Commission on pollution and health defined pollution as one of the remarkable existential challenges of the Anthropocene epoch because it 'endangers the stability of the Earth's support systems and threatens the continuing survival of human societies' (Landrigan et al., 2018). Pollution endangers the health of billions of people, the integrity of ecosystems and the economic security of nations; consequently, pollution is responsible for an enormous amount of diseases, disabilities and premature deaths (Landrigan et al., 2018). In 2012, unhealthy environments were responsible alone for 23% of the total global mortality and 26% of deaths of children under five years of age (Neira and Prüss-Ustün, 2016). Moreover, pollution 'threatens fundamental human rights: the rights to life, to health and to well-being, jeopardises the rights of the child, the right to safe work and the protection of the most vulnerable' (Landrigan et al., 2018). Therefore, controlling and reducing the levels of environmental pollution is a crucial issue involving public health, ecology and social justice. Pollution control is also directly involved in several targets of the Sustainable Development Goals established by the United Nation to direct human development in the 21st century. Environmental pollution is a complex issue (Philp et al., 2014; Russell, 1974); thus, extensive and diverse research is needed to understand pollution and scaffold pollution control policies (Landrigan et al., 2018).

Plant-pollution relationships should be central in the above-mentioned research due to the following. *i*) Plants are a valid tool in mitigating the detrimental effects of pollution from ecological and public health viewpoints (Diener and Mudu, 2021; Lee et al., 2020; Meena et al., 2021). *ii*) In some cases, plants can naturally thrive in polluted habitats (De Agostini et al., 2020a; Faucon et al., 2011; Shaw, 1987; Shefferson et al., 2008; Whiting et al., 2004), thus constituting an excellent case study of the adaptive mechanisms of plants to cope with abiotic stress. *iii*) Plant biodiversity is seriously threatened by environmental pollution; thus, to deepen understanding of environmental pollution–plant relationship is crucial to preserve such biodiversity (Baek et al., 2012; Colpaert et al., 2011; Zhu, 2016). *iv*) Similarly, plants that thrive in heavily polluted environments (*e.g.* metallicolous and metal-tolerant plants) are peculiar biodiversity that should be preserved in case of remediation of the polluted environments where they settle (Baker et al., 2010; De Agostini et al., 2020a; Faucon et al., 2011; Holyoak, 2008; Whiting et al., 2004).

Despite the heterogeneity and diversification of environmental pollution, the present work focuses on the relationships of plants with inorganic pollutants, which are generally defined as heavy metals. The term ‘heavy metals’ historically defined metals and metalloids that are associated with toxicity or ecotoxicity (Duffus, 2002). This term is currently discussed for its scientific soundness (Duffus, 2002) and the use of the definition ‘potentially toxic elements’ is currently increasing. Nevertheless, the term ‘heavy metals’ will still be used in the present work because it is still predominant and commonly accepted in the scientific literature on public health and environmental fields of study. Many metal elements (Cu, Fe, Mn and Ni) are essential micronutrients for plant metabolism, whilst others (As, Cd and Pb) are considered non-essential metals known only for their toxicity exerted on plants (phytotoxicity) (Hodson, 2012; Rascio and Navari-Izzo, 2011; Singh et al., 2015). Essential metals could also prejudice plant metabolism at supra-optimal levels, that is, excessive abundance in the growth substratum (Hodson, 2012; Rascio and Navari-Izzo, 2011). Non-essential metals or supra-optimal levels of essential metals are phytotoxic to plants and are legitimately considered as a form of abiotic stress, which is generally defined as ‘heavy metal stress’ and is considered to be one of the most prejudicial to plant life and crop productivity (Singh et al., 2015). Heavy metal-related phytotoxicity affects plants in different ways, resulting in foliar chlorosis, root damage, reduced biomass production and ultimately in death (Rascio and Navari-Izzo, 2011; Singh et al., 2015; Vickers et al., 2009). Heavy metals directly or indirectly affect photosynthesis, protein structure, enzyme activity, reactive oxygen species (ROS) accumulation and nutrient uptake (Singh et al., 2015). Considering photosynthesis, the prejudicial effects of heavy metals could lead to the dismantling of chlorophyll molecules and altered chloroplast and thylakoid membrane, resulting in a decreased photochemical efficiency (Hodson, 2012; Rascio and Navari-Izzo, 2011; Singh et al., 2015). Heavy metals can also bind with proteins or even substitute some of their functional groups or essential elements due to resemblance or high affinity (Hodson, 2012; Rascio and Navari-Izzo, 2011; Singh et al., 2015). The interaction of heavy metals with biological molecules and proteins alters their structures and their functions in the cell. This alteration could negatively affect the activity of enzymes as well as the integrity of biological membranes, compromising embryo development, seed germination and plant growth (Rascio and Navari-Izzo, 2011; Singh et al., 2015). Stress elicits generic responses in plants, whilst stress-specific responses are rare (Vickers et al., 2009). Excessive ROS production is a common response in plants facing stress and cellular ROS accumulation is also one of the adverse effects of heavy metal stress. Heavy metals are divided into redox-active and inactive elements: the former actively leads to ROS formation via redox reactions, whilst the latter interferes with the electron transport chain and indirectly accelerates ROS generation at a cellular level (Singh et al., 2015). In normal conditions, ROS are important signalling molecules the cellular balance of which is maintained under rigid control. However, ROS balance is broken in the presence of stress, thus initiating a defensive response (Vickers et al., 2009). Despite their essential role in stress defence, different severe damages occur when the control over ROS cellular level is lost and they are overproduced (Vickers et al., 2009). ROS overproduction directly damages plant cells by oxidising essential macromolecules and biological components, such as nucleic acids, proteins and lipids (Vickers et al., 2009). The resulting oxidative stress will lead to membrane dismantling (due to lipid peroxidation), inhibition of the reparation of photo-damaged photosynthetic centres, ion leakage and DNA cleavage (Hodson, 2012; Rascio and Navari-Izzo, 2011; Singh et al., 2015). ROS overproduction may also ultimately trigger a chain reaction that can lead to even higher ROS production and accumulation, thus resulting in programmed cell death (Vickers et al., 2009).

Stress from heavy metals would influence plants beyond the individual level, affecting their distribution, biodiversity and ecological traits (Laghlimi et al., 2015; Vickers et al., 2009). This fact is particularly true in a climate change scenario where extreme temperatures, drought and pollution often occur simultaneously, wherein one exasperates the deleterious effects of the other (Lee et al., 2020; Vickers et al., 2009). Metalliferous environments (*i.e.* growth contexts featured by the presence of high amounts of single or multiple metals in the growth substratum) are remarkably restrictive habitats for plants. These environments exert a strong selective pressure (Baker et al., 2010) that could result via microevolutionary processes, in the speciation of metal-tolerant plant species or ecotypes that can thrive in environments far too toxic for non-adapted *taxa* (Baker et al., 2010).

The present work focuses on the relationships between environmental heavy metals and two groups of plants, namely mosses and orchids. Mosses and orchids are two remarkably different and phylogenetically distant groups of plants: the former are ancient, non-vascular cryptogams whilst the latter are recent and diversified flowering monocots. Herein, both plants were characterised on the basis of their response to environmental heavy metals to explore how the two edges of terrestrial plant evolution handle this abiotic stress. Detrimental effects of inorganic pollutants on plants are generally studied in laboratory conditions, applying heavy metal treatments on model species and consequently measuring their response following the mutated homeostasis. This approach provided considerable contributions to the knowledge on plant–heavy metal interaction mechanisms, however, rather than the adaptation of plants to inorganic pollutants, the acclimation response of non-preadapted species is studied by this way. The research activity behind the present thesis work was conducted entirely in the field to fill the aforementioned gap, studying naturally occurring metallicolous populations. Thus, the topic was addressed from an adaptive-oriented viewpoint, thereby focusing on the outcomes of a long-standing natural selection process acting on mosses and orchids colonising such harsh environments.

Mosses (Bryophyta Schimp.) are a large group of ancient non-vascular embryophytes. They lack conduction systems and roots because they are non-vascular plants. These features result in nutrient and water acquisition limited to dry and wet depositions from the atmosphere and uptake from the growth substratum via capillarity, respectively (Chakrabortty and Paratkar, 2006). This form of nutrient acquisition is optimised by a large surface-to-volume ratio and substantial permeability to water and elements in it (Chakrabortty and Paratkar, 2006; De Agostini et al., 2020b). Bryophytes are extensively used in environmental pollution studies due to these morphological adaptations, especially in the biomonitoring of environmental pollutants under their natural presence in an environment to be monitored or artificial exposure as transplants or moss bags (Bargagli et al., 2002; Capozzi et al., 2018; Chakrabortty and Paratkar, 2006, among the other). Bryophytes also led land colonisation, being amongst the first plants to leave water around 470–515 million years ago (Chen et al., 2018; Li et al., 2020). Consequently, bryophytes had to face extreme biotic and abiotic stressors linked to the new terrestrial habitat (*e.g.* competition with other colonisers, enhanced solar radiation, soil scarcity and dehydration) (Chen et al., 2018; Plackett and Coates, 2016). Such an evolutive path led bryophytes to the virtual colonisation of all terrestrial ecosystems (Shaw et al., 2011) and transformed them as excellent early land colonisers that can settle on extreme growth contexts unavailable otherwise for the considerable majority of vascular plants (Arróniz-Crespo et al., 2014; Gypser et al., 2016; Holyoak and Lockhart, 2011). Severely polluted environments are amongst the extreme growth contexts in which bryophytes can settle (Holyoak and Lockhart, 2009; Karakaya et al., 2015; Liu et al., 2018; Yagura et al., 2019). Bryophytes evolved several protective and detoxicating mechanisms that can be resumed as follows: *i*) accumulation of toxic elements in cell walls (Karakaya et al., 2015; Konno et al., 2010; Krzesłowska et al., 2009) and vacuoles (Basile et al., 1994; Carginale et al., 2004), *ii*) excretion from gametophytes of heavy metals in the form of salts (Rao, 1982; Tyler, 1990), *iii*) spores protection from pollutants (Basile et al., 2001) and *iv*) production of antioxidants (Stanković et al., 2018) and heat-shock proteins and phytochelatins (Carginale et al., 2004).

By contrast, orchids (Orchidaceae Juss.) constitute a recent plant group compared with mosses. This group comprises herbaceous plants currently experiencing substantial adaptive radiation that started in the Tertiary (Ramírez et al., 2007), in which a variety of ecological adaptations and strategies are explored. In orchids, individuals rely on complex networks of ecological interactions to complete their life cycle. Symbiosis with soil fungi allows orchids to germinate and uptake water and nutrients (Rasmussen et al., 2015; Tešitelová et al., 2012), whilst refined plant–pollinator relationships guarantee orchid reproduction (Scopece et al., 2010). Despite the burden of such a web of ecological interactions to be established, orchids also managed to settle on harsh and unfavourable environments, including heavily polluted ones. Thus, orchids colonised roadsides, cemeteries, industrial dumps and mining tailings (De

Agostini et al., 2020a; Fekete et al., 2017; Jurkiewicz et al., 2001; Löki et al., 2019; Shefferson et al., 2008; Szarek-Łukaszewska, 2009). Orchids are slower early land colonisers than mosses, but their native genetic plasticity, together with their complex network of ecological interactions, allow them to thrive in the most diverse environments. For example, orchids are amongst the first colonisers of recently-formed volcanic islands (Arditti and Ghani, 2000). Orchid–fungus symbiotic interactions are mandatory in early developmental stages because they provide nutrients to endosperm-lacking seeds until individuals can autonomously sustain themselves via photosynthesis (Rasmussen et al., 2015; Tešitelová et al., 2012). However, symbiosis with soil fungi is maintained during the entire life cycle of orchids because it provides several advantages to the host (Rasmussen et al., 2015; Tešitelová et al., 2012). Consequently, the occurrence of fungal partners of orchids (mycobionts) shapes the germination niches of seeds as well as the spatial distribution of adult individuals (Bell, 2021; Jacquemyn et al., 2007; Rasmussen et al., 2015; Tešitelová et al., 2012). Fungal symbiosis could also help orchids tolerate heavy metals. Fungal cells produce pigments and other organic substances that can chelate and detoxify heavy metals (Baker et al., 2010; Galli et al., 1994; González-Chávez et al., 2004; Jurkiewicz et al., 2001; Turnau et al., 2006).

The assessment of the effects of environmental pollution on orchids and mosses was conducted *in-situ* in abandoned mines, whilst the moss-mediated biomonitoring approach was performed in the vicinity of an active oil refinery.

Regarding mining sites, depending on their extraction activity, they may continue to represent important sources of environmental pollution that degrade and jeopardise surrounding lands, water bodies and air even when the extraction activity ceases (Beane et al., 2016; De Giudici et al., 2019; Mannu et al., 2020; Rodríguez-Eugenio et al., 2018). More precisely, by-products of the extraction activity of metal sulphides are rich in heavy metals. Pollutants easily disperse from abandoned mines carried out by wind or water as suspended particulate matter or in solution and represent a considerable ecological and public health issue (Cidu et al., 2014; Landrigan et al., 2018; Rodríguez-Eugenio et al., 2018; Stewart, 2020; Varrica et al., 2014). The oxidative dissolution of the remaining metal sulphide minerals in extraction by-products could generate the so-called acid mine drainage, which is characterised by low pH and high concentrations of inorganic pollutants (De Giudici et al., 2019). Acid mine drainages represent a significant route for inorganic pollutants to disperse in the environment, especially if they reach the surface or underground waters (Cidu et al., 2014; De Giudici et al., 2019; Varrica et al., 2014). Metals could in fact persist in solution for several kilometres from the source of contamination despite natural attenuation processes (Cidu et al., 2018, 2014). Sardinia Island (where the main part of the thesis fieldwork and plant sampling were conducted) hosted over several centuries an intense mining activity that reached its peak between the 19th and 20th centuries (De Giudici et al., 2019; Varrica et al., 2014). By-products of this intense activity were often improperly managed. Consequently, numerous abandoned impoverished heaps and tailing dumps endanger ecosystems and human health in their surroundings due to the aforementioned dispersion mechanisms (Cidu et al., 2018; De Giudici et al., 2019; Varrica et al., 2014).

Metallophytes and metal-tolerant plants settle in metalliferous environments because they rely on soil heavy metals to survive (obligate metallophytes) (Baker et al., 2010) or profit from the lack of competition (Baker et al., 2010; Barrutia et al., 2011). In one case or another, metalliferous environments as abandoned mines could promote the differentiation of metal-tolerant and metallophyte *taxa*, which can settle regardless of the extreme growth conditions (Baker et al., 2010). Metallophyte distribution can be often considered locally endemic (Baker et al., 2010; Whiting et al., 2004). Therefore, abandoned mines, similarly to naturally metalliferous environments, could host peculiar and unique biodiversity that should be preserved (Baker et al., 2010; Faucon et al., 2011; Holyoak and Lockhart, 2009; Whiting et al., 2004). In the context described thus far, the following ecological issue to be addressed in the future years delineates: the urgency for reclamation of polluted abandoned mines would clash with the conservation of the peculiar metallicolous biodiversity developed there, its genetic heritage and its potential as an evolutionary model.

The use of plants (mosses) as biomonitors of environmental pollution, particularly of atmospheric pollution and air quality, was explored in the present thesis work. Biomonitoring is the practice of using living organisms or biological tissues to measure a certain ecological condition (Wolterbeek, 2002). Mosses were recently proven to be effective in the biomonitoring of water quality (Capozzi et al., 2018; Debén et al., 2020, 2018, 2016) and are commonly used as biomonitors of air quality since the second half of the 20th century (Wolterbeek, 2002). Mosses are indeed ideal tools in the biomonitoring of environmental pollution because their physio-anatomical features enable the easy entrapment and accumulation of inorganic and organic pollutants that come in contact with them. More precisely, mosses possess a high surface-to-volume ratio, lack well-developed epidermis or cuticle and their cell walls are featured by the presence of abundant ion-binding sites. Consequently, mosses present high permeability to water and the elements dissolved in it as well as relevant adsorption capacity (Chakrabortty and Paratkar, 2006; De Agostini et al., 2020b; González and Pokrovsky, 2014). Atmospheric pollutants interact with mosses in the form of solid particles, in aqueous solutions or as gas. Once in contact with the moss, pollutants adhere to cell walls via ion exchange or enter the cells via passive or active transport (Chakrabortty and Paratkar, 2006; Wolterbeek, 2002). Mosses are used as biomonitors when naturally occurring in the area to be monitored or purposely placed in the area of interest as transplants or moss bags. Amongst the moss-based biomonitoring techniques, the ‘moss bag technique’ is the most frequently used; this technique exposes mesh bags containing moss (generally grown in the absence of contamination) to assess air quality and the presence of airborne pollutants in a certain area (Ares et al., 2012). Several factors can influence pollutant uptake and accumulation when biomonitoring airborne pollutants and air quality using mosses. Some of these factors are intrinsic to the moss species used (structure, number and type of cation exchange sites, cell pH, biomass productivity and age of the exposed tissue), whilst others are environmental factors (features of the particulate, temperature, rainfall, aridity, vegetal coverage, occurrence of exposed mineralisation, presence of sea salt and acidic rainfalls) (Brown and Beckett, 1983; Chakrabortty and Paratkar, 2006; Seaward, 1995; Wolterbeek, 2002). The results of an extensive moss-mediated biomonitoring campaign conducted around an oil refinery are discussed in the present thesis. Sixteen years of accumulation values could be approached due to the intensive biomonitoring effort. The accumulation data referring to such an extended time frame facilitated the evaluation of the precision of the biomonitoring technique in the long run and helped determine how ecological factors affect the effectiveness of the biomonitoring technique and the reliability of the measured accumulation values. Therefore, the impact of industrial activity on air quality was confirmed. Moreover, the strong influence on accumulation values of environmental and anthropogenic factors (*e.g.* vicinity of the sea, further human activities in addition to the one monitored) was also indicated.

Aims and synopsis of the Ph.D. thesis

The present work studies the relationships between plants and environmental pollution in moss and orchid populations settling on heavy metal polluted environments, which are referred to as ‘metallicolous populations’ or ‘metallicolous individuals’. This thesis generally aims to describe adaptations to environmental pollution in the two plant groups. The focus on mosses constitutes the first two chapters of the thesis. The first chapter (Chapter 1) reports the results of a study conducted in the University of the Basque Country in the EKOFISKO (Ecofisiología del Estrés y la Contaminación en Plantas) research group under the scientific supervision of Antonio Hernàndez (Department of Plant Biology and Ecology, Faculty of Science and Technology, University of the Basque Country) and Raquel Esteban (Department of Plant Biology and Ecology, Faculty of Science and Technology, University of the Basque Country). In this chapter, the relationship of mosses with soil pollution was studied in the field, more precisely in the abandoned mine Mina Clara in the Basque Country (Spain). The photoprotective response of the metallicolous populations of four moss species (*Lewinskya rupestris*, *Polytrichum commune*, *Ptychostomum compactum* and *Rhynchostegium confertum*) colonising heavy metal polluted mine wastes was studied and compared with those of control, non-metallicolous populations. Consequently, markedly species-specific tolerance patterns, which were strongly determined by the bryological attributes of species, were identified. Additional details are as follows: *i*) less tolerant species presented high pollutant uptake accompanied by marked dissipative and antioxidative strategies; *ii*) intermediate tolerant species presented medium accumulation of pollutants and less marked photoprotective mechanisms; *iii*) most tolerant species reduced their pollutant uptake, consequently revealing the absence of changes in their physiological performance. γ + β-tocopherol induction in metallicolous populations resulted to be a functional responsive trait of the studied bryophytes facing heavy metal polluted environments and could play a regulatory role in their response and tolerance to heavy metal stress. The results of this research are reported as a published paper that constitutes Chapter 1 of the present thesis.

The second chapter discusses the results of a long-standing and currently ongoing biomonitoring campaign conducted via moss bags around an oil refinery in South Sardinia (Italy). Effects of rainfalls and distance from the source of contamination on the accumulation values of 14 trace elements measured over 16 years were evaluated. Statistical elaboration of accumulation values demonstrated the following: *i*) the influences of the vicinity of the contamination source as well as rainfalls on element content in the biomonitor depending on the considered element and the exposure condition; *ii*) provision of relatively stable measurements by the monitor species *Hypnum cupressiforme* exposed in the form of moss bags during the 16-year time frame and *iii*) acquisition of stable accumulation values through similar exposure conditions. This research is a published paper that appears in the present thesis as the second chapter (Chapter 2).

Orchid tolerance to environmental heavy metals was explored from an ecophysiological viewpoint in the third chapter of the present thesis work (Chapter 3). This exploration was possible because a large population of the orchid *Epipactis tremolsii* (*E. helleborine* subsp. *tremolsii* for some authors) was found colonising a heavily polluted tailing dump (Barraxiutta abandoned mine, South Sardinia, Italy) during the Ph.D. fieldwork. In this framework, the uptake of soil pollutants was measured in the orchids colonising the tailing dump together with their morphometric parameters and photosynthetic efficiencies. Results were compared with those measured in individuals of the same species growing in the absence of soil pollution (control population). Observing the central role of orchid symbiosis with soil fungi, fungi associated with the roots of contaminated and control individuals were isolated, propagated and then identified by a DNA barcoding approach. Plants from the contaminated sites were found to tolerate heavy metals from the soil and accumulate and translocate them into their roots and epigeal organs. Polluted individuals are smaller and presented markedly reduced photosynthetic efficiency compared with control plants. Fungi belonging to the genus *Ilyonectria* (Ascomycota) were found in contaminated and control sites, whilst an unidentified fungus was isolated from roots in the contaminated site only. This research represented the first explorative investigation on the *E. tremolsii* population colonising the Barraxiutta abandoned tailing dump and is reported in the present thesis work as a published article. The studies on the metallicolous orchid population continued by focusing on the morphometry of its seed. Seed morphology analysis is indeed a widely used approach in ecological and taxonomic studies. In this context, intraspecific variability considering seed morphology (size, weight and density) was assessed between the polluted *E. tremolsii* population and a control population located nearby sharing the same ecological conditions, except for the soil pollution. Larger and heavier seeds than those produced by the control population were found in plants growing on the heavy metal polluted site, whilst no differences in seed density emerged between the studied populations. Coats and embryos of seeds covaried in the control population, whilst coats with varying sizes independent from embryos were described in the polluted site. These results were published and are reported in the present thesis in chapter four (Chapter 4). The differences observed in several measured parameters suggest intraspecific seed variability in the case study and its ecological significance could consist in seed coats shielding embryos from toxic pollutants of the tailing dump and/or in favouring seed infection by their fungal symbiont.

A reclamation of the abandoned mine hosting the metallicolous *E. tremolsii* population is becoming increasingly imminent. Nevertheless, such an intervention in the tailing dump could seriously undermine the perpetuation of the metallicolous orchid population. A pilot study, in which the suitability of the seed packet technique in the study area was evaluated, was performed to explore different conservation strategies for the endangered metallicolous orchid population. Four different sowing treatments (full-crossed experimental design) were set up in the tailing dump and a nearby unpolluted site (control site). The field phase of the experiment lasted 12 months in which the experimental seed bank preservation and incipient seed development were observed and tested to be significant in five retrieval campaigns. The experimental seed bank remained unaltered during the pilot study, incipient and differential site-specific seed development was observed and interactions between seeds and soil fungi were also observed. Consequently, the suitability of the seed packet technique (even in extreme environments, such as polluted mine wastes) was proven to be a valuable tool to evaluate the best seed-based conservation option for the metallicolous *E. tremolsii* population. The research results are submitted for publication and constitute the final chapter of the thesis (Chapter 5).

Obtained results integrate the state of art on plant adaptation to extreme environments and heavy metal stress. The study was performed in mosses and orchids, two groups of plants often neglected in the *in-situ* ecophysiological approaches. Moreover, the obtained knowledge could be profitable for the management of abandoned mines and in the remediation approaches to similar environments.

Considering the biomonitoring-focused part of the thesis, obtained results also contribute to the awareness on the correct interpretation of accumulation values and biomonitoring campaign outputs to profit from this green technology effectively in the control of atmospheric pollution and air quality.

Chapter 1: Heavy metal tolerance strategies in metallicolous and non-metallicolous populations of mosses: Insights of γ+β-tocopherol regulatory role

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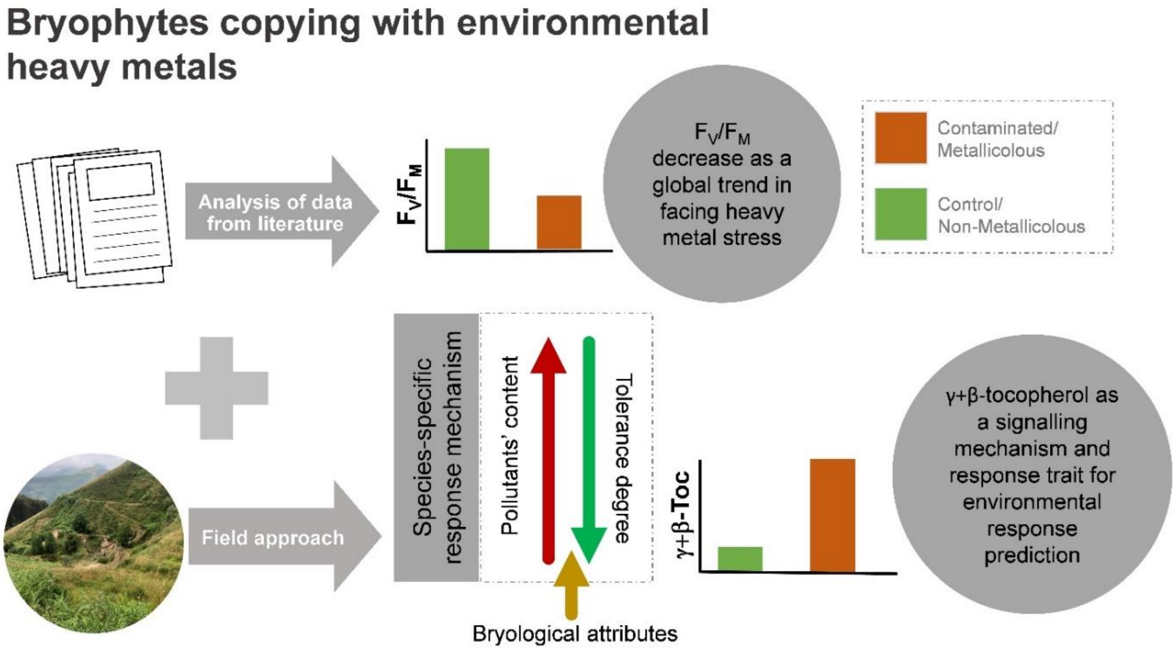
ABSTRACT

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 supports 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 γ + β-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.

Keywords: *Bryological attributes; Mining soils; Bryophytes; Pigments; Tocopherols; Tolerance.*

Abbreviations: A, antheraxanthin; β-carot, β-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.



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, 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 metallicolous 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 (Chakrabortty 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 (Chakrabortty 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; Chakrabortty 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 (Chakrabortty 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 (Chakrabortty 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*) intraspecific 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 metallicolous 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-Łukaszewska, 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 metallicolous 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 metallicolous and non-metallicolous 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*, *e.g.*, 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 1).

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) x 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 ($CaCl_2$ 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 1970s and nowadays the site appears naturally colonized by a wide range of metallicolous populations of, among others, *Noccaea caerulescens* J.Presl & C.Presl F.K.Mey., *Ulex europaeus* Savi, *Agrostis capillar*is 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 (≈ 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^2) 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 Schw¨agr.) 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 2) 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 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; D (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).

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)**	
Barbula vinealis	1	*Hypnaceae*	6
Brachythecium salebrosum	1	*Pottiaceae*	5
Bryum cellulare	1	*Thuidiaceae*	4
Dicranum undulatum	1	*Brachytheciaceae*	3
Eurhynchium eustegium	1	*Hylocomiaceae*	3
Eurohypnum leptothallum	1	*Grimmiaceae*	2
Fontinalis antipyretica	1	*Polytrichaceae*	2
Grimmia anodon	1	*Sematophyllaceae*	2
Haplocladium angustifolium	1	*Aneuraceae*	1
Haplocladium microphyllum	1	*Aytoniaceae*	1
Hylocomium splendens	1	*Bryaceae*	1
Hypnum cupressiforme	1	*Dicranaceae*	1
Hypnum plumaeforme	1	*Fontinalaceae*	1
Jungermannia exsertifolia subsp. *cordifolia*	1	*Funariaceae*	1
Lunularia cruciata	1	*Jungermanniaceae*	1
Marchantia polymorpha	1	*Lejeuneaceae*	1
Physcomitrella patens	1	*Lunulariaceae*	1
Plagiochasma appendiculatum	1	*Marchantiaceae*	1
Pleurochaete squarrosa	1	*Sphagnaceae*	1
Pleurozium schreberi	1		
Polytrichum commune	1	**Orders (13)**	
Polytrichum piliferum	1	*Hypnales*	19
Pseudoscleropodium purum	1	*Pottiales*	5
Ptilium crista-castrensis	1	*Grimmiales*	2
Ptychanthus striatus	1	*Marchantiales*	2
Racomitrium lanuginosum	1	*Polytrichales*	2
Rhytidiadelphus squarrosus	1	*Bryales*	1
Riccardia pinguis	1	*Dicranales*	1
Scopelophila ligulata	1	*Funariales*	1
Sphagnum squarrosum	1	*Jungermanniales*	1
Taxiphyllum barbieri	1	*Lunulariales*	1
Taxiphyllum taxirameum	1	*Metzgeriales*	1
Taxithelium nepalense	1	*Porellales*	1
Taxithelium sp.	1	*Sphagnales*	1
Thuidium cymbifolium	1		
Thuidium delicatulum	1	**Divisions (2)**	
Timmiella barbuloides	1	*Bryophyta*	32
Tortula ruralis	1	*Marchantiophyta*	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 substrates 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**
L. rupestris	Cu	P	M	40	5	3	8	2	5	3	0
P. commune	Tf	P	D	250	3	6	6	7	2	2	1
P. compactum	Tf	P	M	15	5	6	9	6	7	4	0
R. confertum	Mr	P	M	30	7	3	4	5	7	6	0

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 μmol photon $m^{-2}s^{-1}$) 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 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 × *g* the supernatant being syringe-filtered by a 0.22 µm PTFE filter (Teknokroma, Barcelona, Spain). 15 µl of the extract of each replicate were injected on a reverse-phase C18 column HPLC system (water Spherisorb ODS1, 4.6 × 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), β-carotene (β-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 (VAZ = V+ A+ Z; nmol/g), de-epoxidation index (A+Z/V+A+Z; mol/mol), Lut, Neox and β-carot on DW basis (nmol/g). Finally, α-tocopherol, γ + β-tocopherols and total tocopherols (as the sum of the isomers α, γ + β) 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 µl 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 $HCl:H_2O:MeOH$ (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 A524–0.24A653 (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 × *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 significatively 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 is specific to each species or if it is a global response. Wilcoxon test was used to determine if differences between metallicolous and non-metallicolous populations (NMP and MP) were significant (Figs. 2–5). Obtained results are presented as boxplots. 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). If present, the number inside each box indicates the number of replicates. When significant, statistical significance is reported by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Note that ns indicates non-statistical significance. 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 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).

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 significative higher values of the three elements, whilst *P. compactum* only showed significative 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 (≈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; ≈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. 4 C) 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 β-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. 4 C, D). The moss *P. commune* significantly decreased the β-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 β-carot (Fig. 5A, C-E), whilst *R. confertum* exhibited a significant decrease of total xanthophyll pool, VAZ (Fig. 5 A) 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 γ + β-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 α-tocopherol and γ + β-tocopherol (Fig. 6A, B), but with no changes in the rest of the parameters. The MP of *P. commune* also increased significantly γ + β-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 γ + β-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 β-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, γ + β-tocopherol and α-tocopherol). Overall, the PCA confirmed the trend observed in previous Figures (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.

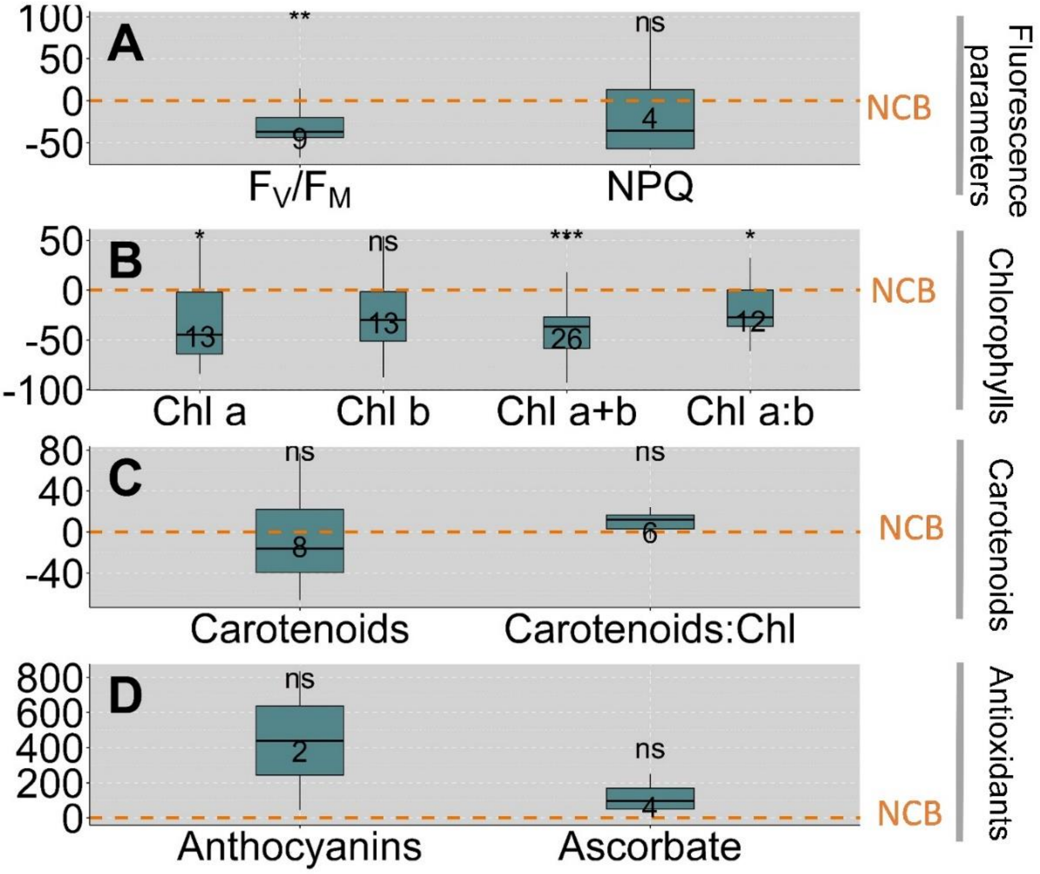


Fig. 1: Analysis of data compilation from the literature. Boxplots represent the percentage of variation of contaminated bryophytes with respect to non-contaminated bryophytes (NCB) values (represented by the orange dashed line) for *i*) fluorescence related parameters, *i.e.*, photochemical efficiency (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). 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 NCB values in the selected parameters and represented by asterisks when significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

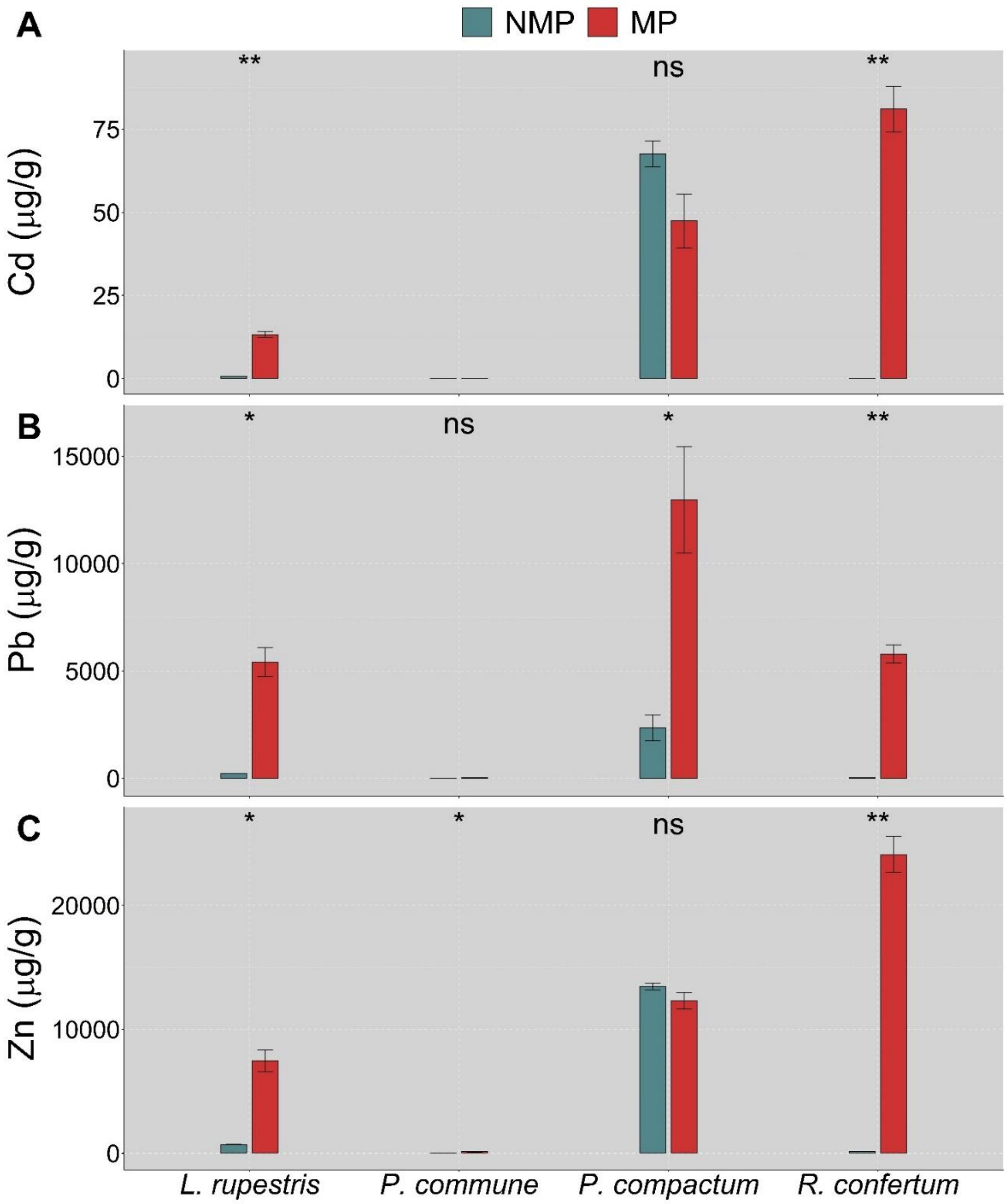


Fig. 2: Mean content of heavy metals in moss tissues (Cd, Pb and Zn; µg/g) in metallicolous and non-metallicolous populations (NMP and MP, represented by green and red bars respectively) of the following species: *L. rupestris*, *P. commune*, *P. compactum* and *R. confertum* (n = 3). Statistical significance of differences between NMP and MP values when significant is reported by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Note that ns indicates non-statistical significance.

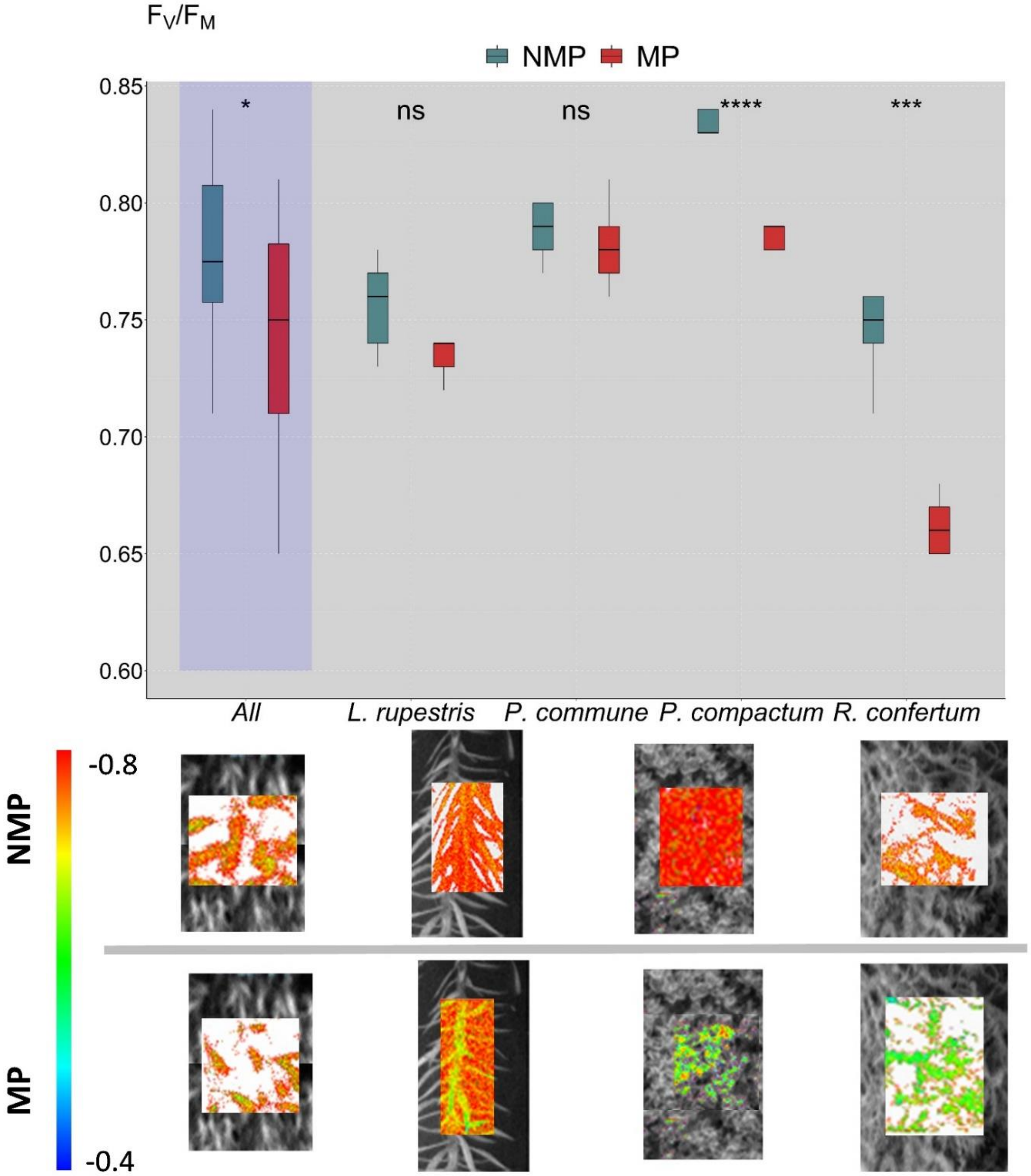


Fig. 3: Maximum photochemical efficiency (F_V/F_M) in metallicolous and non-metallicolous populations (NMP and MP, represented by green and red bars respectively) of the following species: *L. rupestris*, *P. commune*, *P. compactum* and *R. confertum* (n = 5). The first two boxplots (grouped in the category *All* and light blue-shaded) averaged the data of the four studied species. False colors 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 when significant is reported by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Note that ns indicates non-statistical significance.

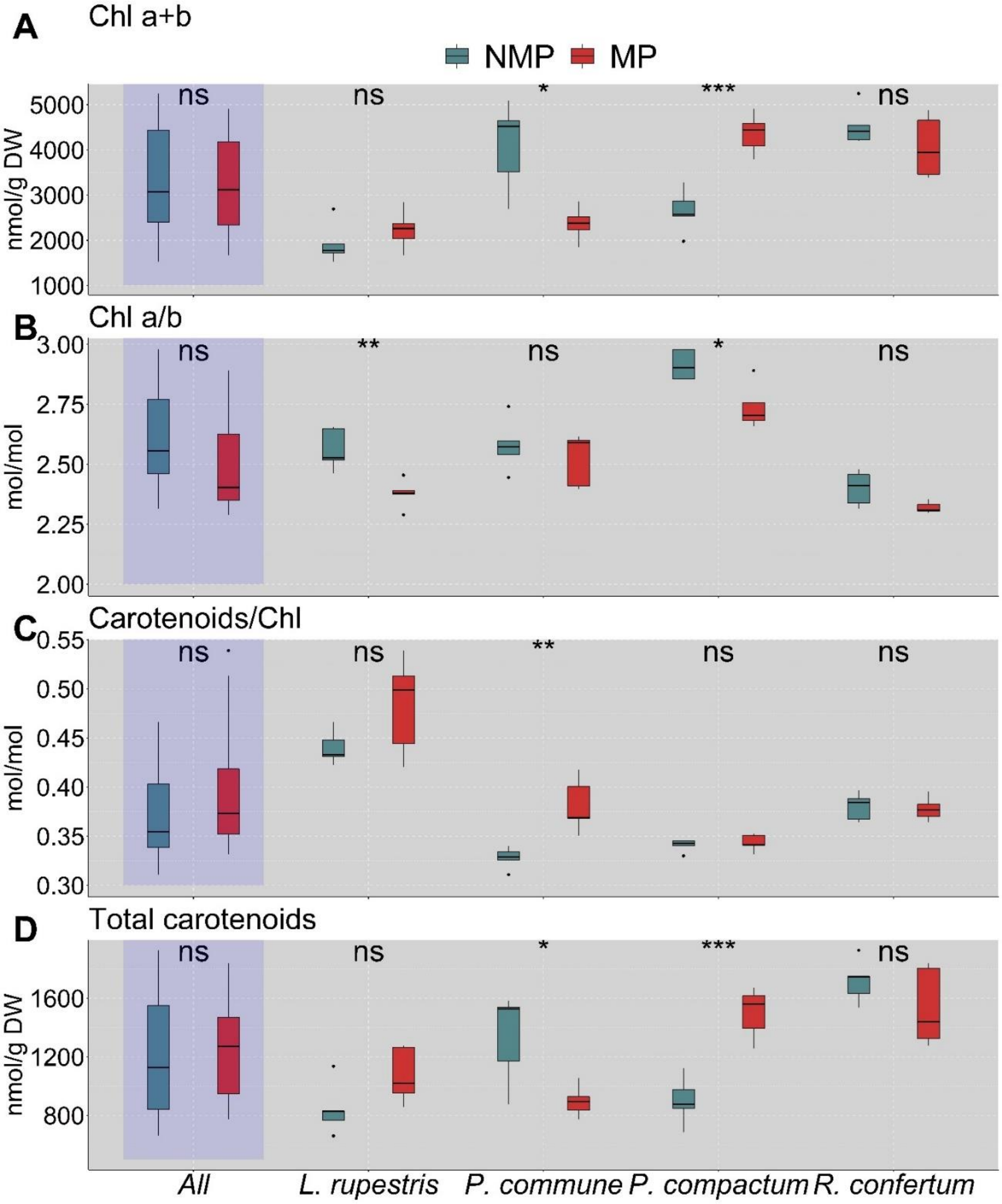


Fig. 4: Boxplots showing the total chlorophyll content (Chl a+b; nmol/g DW) (A); Chlorophyll a to b ratio (Chl a/b, mol/mol) (B); carotenoids to Chl ratio (carotenoids/Chl; mol/mol) (C); total carotenoids content (D; nmol/g DW), in metallicolous and non-metallicolous populations (NMP and MP, represented by green and red bars respectively) of the following species: *L. rupestris*, *P. commune*, *P. compactum* and *R. confertum* (n = 5). Statistical significance of differences between NMP and MP values when significant is reported by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Note that ns indicates non-statistical significance.

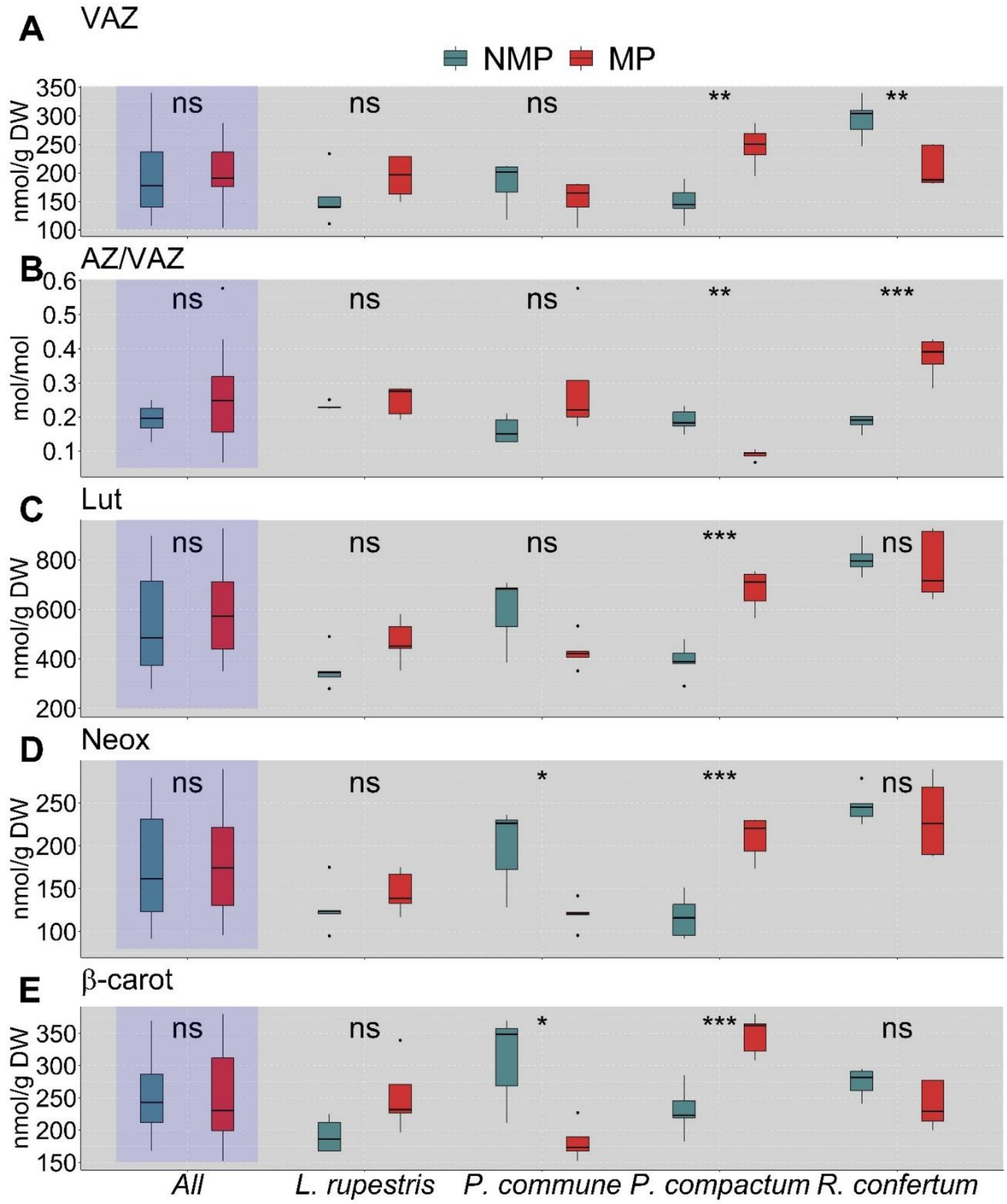


Fig. 5: Boxplots showing total xanthophyll content (VAZ, nmol/g DW) (A), deepoxidation ratio (AZ/VAZ, mol/mol) (B); lutein content (Lut, nmol/g DW) (C); neoxanthin content (Neox, nmol/g DW) (D); β-carotene content (β-carot, nmol/g DW) (E), in metallicolous and non-metallicolous populations (NMP and MP, represented by green and red bars respectively) of the following species: *L. rupestris*, *P. commune*, *P. compactum* and *R. confertum* (n = 5). Statistical significance of differences between NMP and MP values when significant is reported by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Note that ns indicates non-statistical significance.

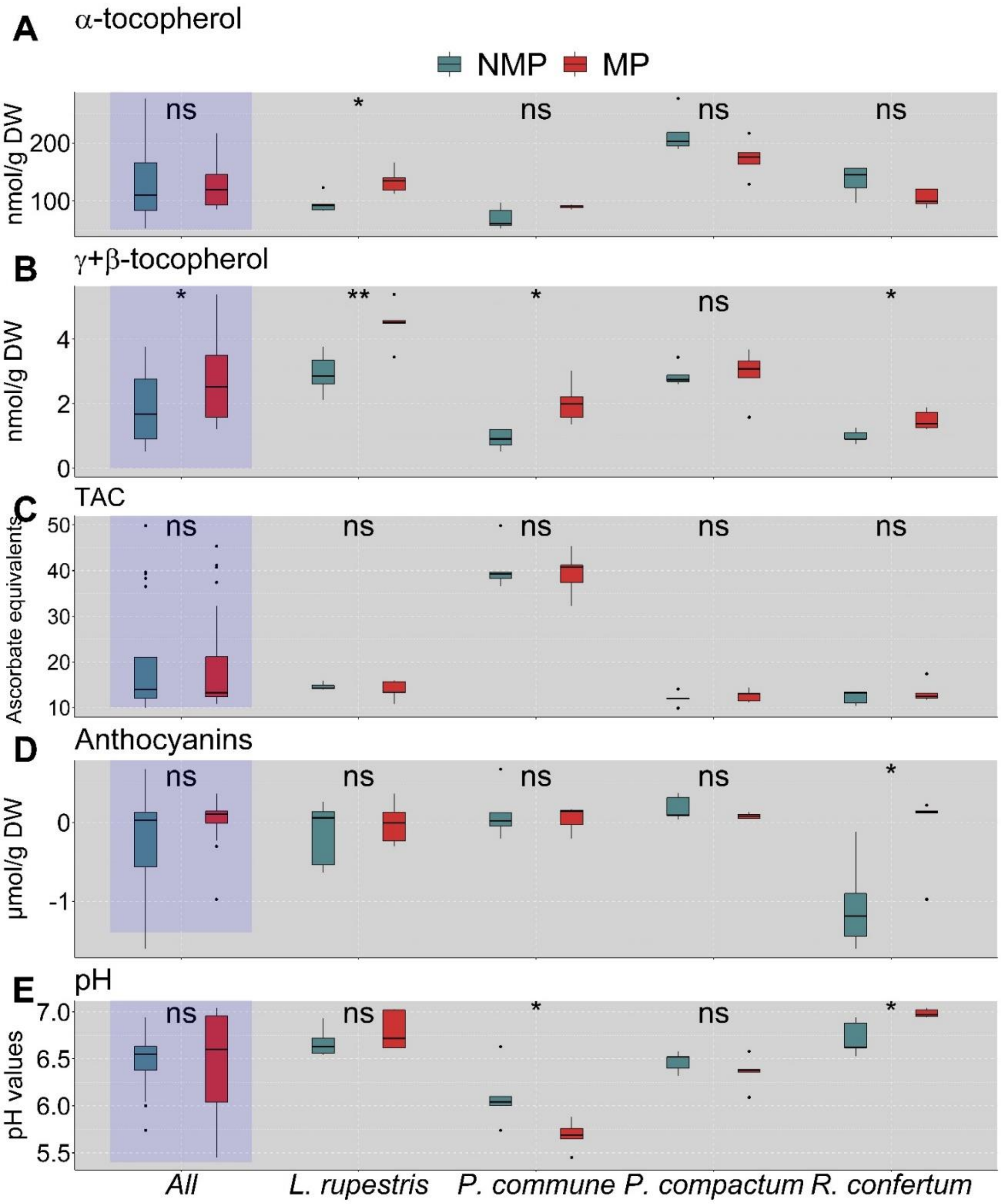


Fig. 6: Boxplots showing α-tocopherol content (nmol/g DW, A), γ + β-tocopherol content (nmol/g DW, B); total antioxidant content (TAC, expressed as ascorbate equivalents; C); anthocyanins, expressed as cyanidin-3-glycoside equivalents content (D); pH (E) in metallicolous and non-metallicolous populations (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). Statistical significance of differences between NMP and MP values when significant is reported by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Note that ns indicates non-statistical significance.

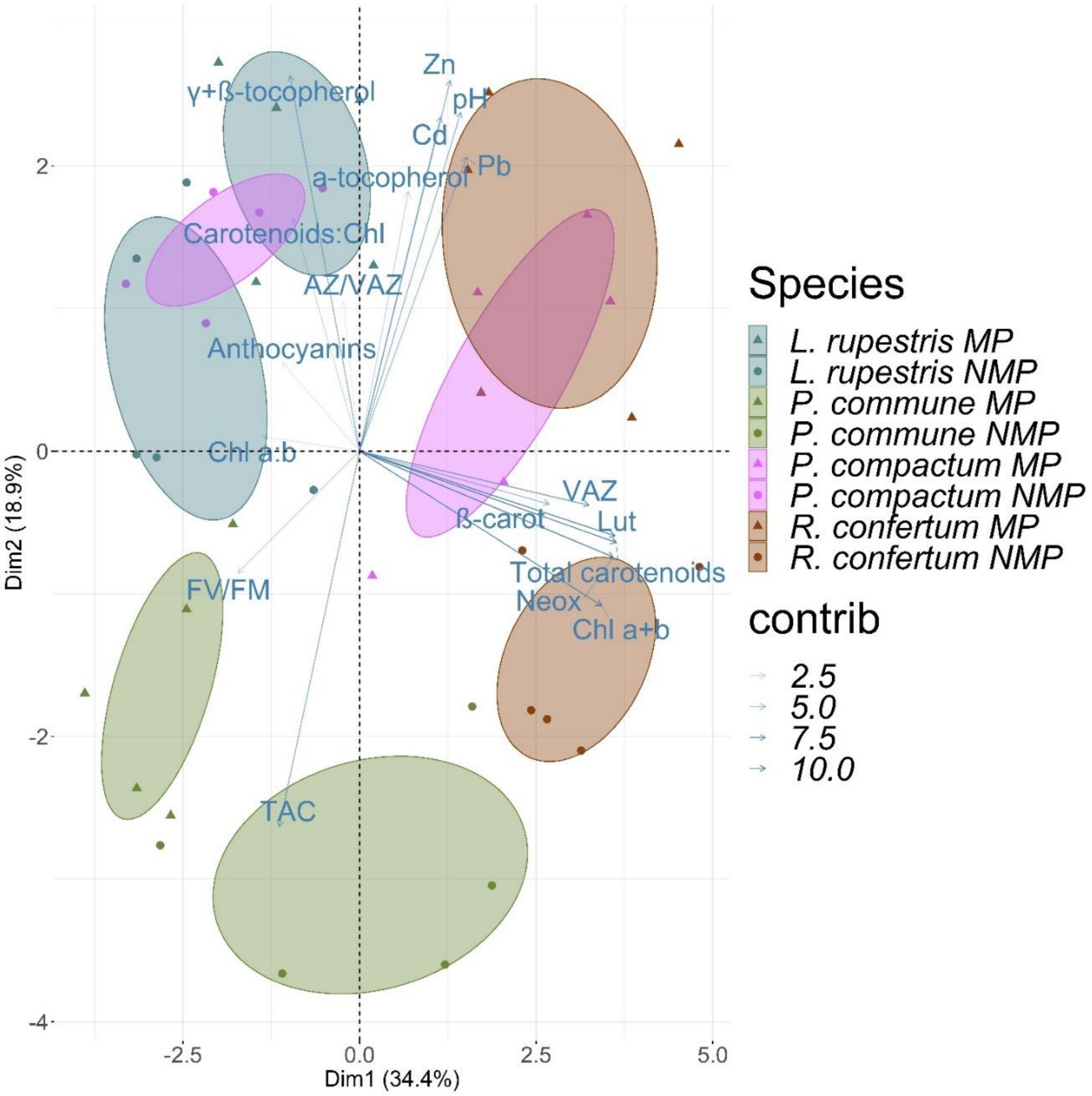


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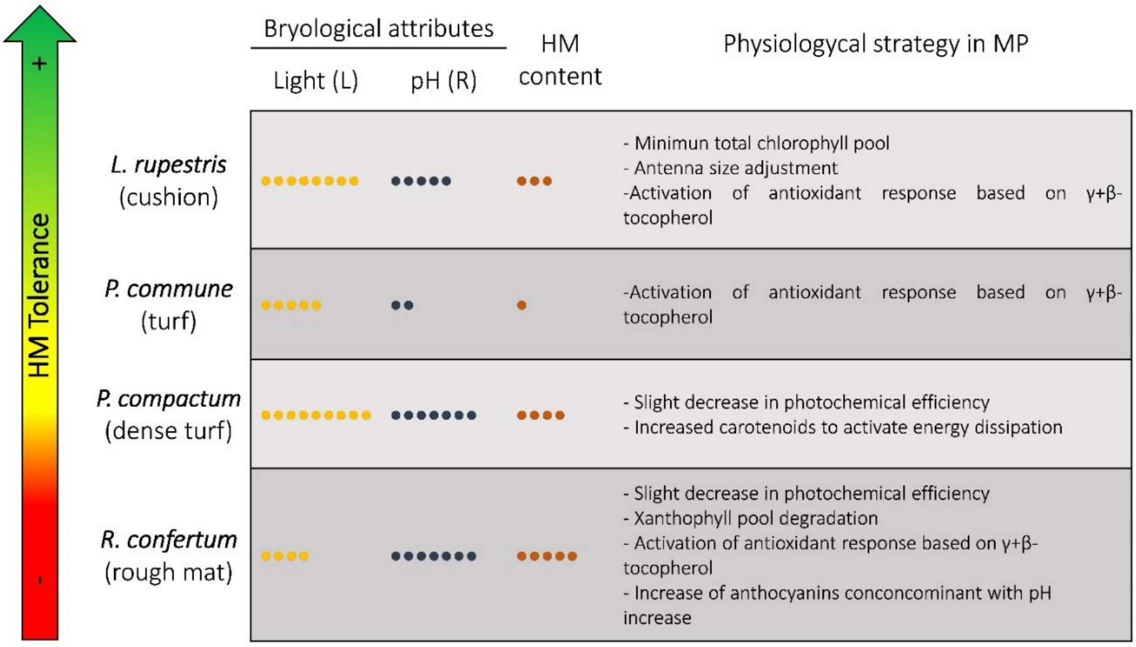
Fig. 7: Principal component analysis biplot for each species (*L. rupestris, P. commune, P. compactum and R. confertum*) represented by different colours (blue, green, pink and brown respectively) and growing conditions (metallicolous and non-metallicolous populations; MP and NMP) represented by different 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.

4. Discussion



	Bryological attributes		HM content	Physiologycal strategy in MP
	Light (L)	pH (R)		
L. rupestris (cushion)	●●●●●●●●	●●●●●	●●●	- Minimun total chlorophyll pool - Antenna size adjustment -Activation of antioxidant response based on γ+β-tocopherol
P. commune (turf)	●●●●●	●●	●	-Activation of antioxidant response based on γ+β-tocopherol
P. compactum (dense turf)	●●●●●●●●●	●●●●●●●	●●●●	- Slight decrease in photochemical efficiency - Increased carotenoids to activate energy dissipation
R. confertum (rough mat)	●●●●	●●●●●●●	●●●●●	- Slight decrease in photochemical efficiency - Xanthophyll pool degradation - Activation of antioxidant response based on γ+β-tocopherol - Increase of anthocyanins conconcominant with pH increase

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).

Anthropocene activities and especially mining activity caused local and diffuse accumulation of heavy metals that might threaten the environment and human health (Stewart, 2019). Ongoing mining activity is estimated on 57,277 km^2 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 metallicolous 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. 1A) 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; Aydoğan 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 global scale meta-analysis considering species bryological attributes, are needed to reveal the photoprotective responses activated living in a metallicolous 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 metallicolous 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 metallicolous 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 2):

i. Sensitive strategy depicted by the species *R. confertum*. This species is a pleurocarpous moss that forms rough, loose mats (Table 2, 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 was accompanied by increased production of antioxidants as α-tocopherol and/or γ + β-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 2, 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 photoinhibition 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 2). 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 2), *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 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 2), 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 metallicolous 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 α-tocopherol and γ + β-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 metallicolous 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 γ + β-tocopherol in moss species from MP. Although α-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 γ + β-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 γ + β-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 γ + β-tocopherol were very low (1.5–4.5 nmol g^{-1} DW) as compared with those of α-tocopherol (Fig. 6 A, B), and the observed significant accumulation of γ-tocopherol could be more related to stress signals to cope with metal stress, rather than merely role as antioxidant or precursor of α-tocopherol. Several studies indicate the potential role of γ- and other tocopherols in stress sensing and signalling (Fritsche et al., 2017; Munné-Bosch, 2019). In salt-stressed mutants that accumulate γ-tocopherol, jasmonic acid- and ethylene-signalling 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 Drábkováet 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, γ + β-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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Supplementary material

Table 1S: List of articles employed in the analysis of data compilation part of the study.

N.	Title	Year	Authors	Journal	Volume and pages
1	Ag nanoparticles inhibit the growth of the bryophyte, *Physcomitrella patens*.	2018	Liang, L. et al.	Ecotox. Environ. Safe.	164, 739-748
2	Antioxidant enzyme activities as affected by trivalent and hexavalent chromium species in *Fontinalis antipyretica* Hedw.	2008	Dazy, M. et al.	Chemosphere	73, 281-290
3	Antioxidative responses related to H_2O_2 depletion in *Hypnum plumaeforme* under the combined stress induced by Pb and Ni.	2010	Sun, S.Q. et al.	Environ. Monit. Assess.	163, 303-312
4	Bioaccumulation and glutathione-mediated detoxification of copper and cadmium in *Sphagnum squarrosum* Crome Samml.	2012	Saxena, A. and Saxena A.	Environ. Monit. Assess.	184, 4097-4103
5	Bioaccumulation and oxidative stress impact of Pb, Ni, Cu, and Cr heavy metals in two bryophyte species, *Pleurochaete squarrosa* and *Timmiella barbuloides*.	2017	Aydogan, S. et al.	Turk. J. Bot.	41, 464-475
6	Biochemical responses of the aquatic moss *Fontinalis antipyretica* to Cd, Cu, Pb and Zn determined by chlorophyll fluorescence and protein levels.	2007	Rau, S. et al.	Environ. Exp. Bot.	59, 299-306
7	Biomonitoring chromium III or VI soluble pollution by moss chlorophyll fluorescence.	2018	Chen, Y.E. et al.	Chemosphere	194, 220-228
8	Biomonitoring heavy metal contaminations by moss visible parameters.	2015	Chen, Y.E. et al.	J. Hazar. Mater.	296, 201-209
9	Changes in nitrate reductase activity and oxidative stress response in the moss *Polytrichum commune* subjected to chromium, copper and zinc phytotoxicity.	2005	Panda S.K. and Choudhury S.	Braz. J. Plant Physiol.	17, 191-197
10	Chlorophyll a fluorescence measurement for validating the tolerant bryophytes for heavy metal (Pb) biomapping.	2010	Tuba, Z. et al.	Curr. Sci.	98, 1505-1508
11	Comparison of vascular and non-vascular aquatic plant as indicators of cadmium toxicity.	2017	Kováčik, J. et al.	Chemosphere	180, 86-92

12	Differential Metal Tolerance and Accumulation Patterns of Cd, Cu, Pb and Zn in the Liverwort *Marchantia polymorpha* L.	2018	Ares, A. et al.	B. Environ. Contam. Tox.	100, 444-450
13	Effects of cadmium and enhanced UV radiation on the physiology and the concentration of UV-absorbing compounds of the aquatic liverwort *Jungermannia exsertifolia* subsp. *cordifolia*.	2006	Otero, S. et al.	Photochem. Photobiol. Sci.	5, 760-769
14	Exaggeration of desiccation stress by heavy metal pollution in *Tortula ruralis*: a pilot study.	2001	Zoltán, T. et al.	Plant Growth Regul.	35, 157-160
15	Field and Laboratory Exposures of Two Moss Species to Low Level Metal Pollution.	2004	Anja, H.T. et al.	J. Atmos. Chem.	49, 111-120
16	Heavy-metal phytotoxicity induces oxidative stress in a moss, *Taxithellium* sp.	2003	Panda, S.K.	Curr. Sci.	84, 631-633
17	Impact of Heavy Metals (Copper, Zinc, and Lead) on the Chlorophyll Content of Some Mosses.	2008	Shakya, K. et al.	Arch. Environ. Contam. Toxicol.	54, 412-421
18	Induction of oxidative stress and ultrastructural changes in moss *Taxithelium nepalense* (Schwaegr.) Broth. under lead and arsenic phytotoxicity.	2004	Choudhury, S. and Panda, S.K.	Curr. Sci.	87, 342-348
19	Influences of Cu and Cr stress on antioxidant system and chlorophyll fluorescence in terrestrial moss *Taxiphyllum taxirameum*.	2015	Chen, Y. et al.	Fresenius Environ. Bull.	10
20	Physiological adaptations in the lichens *Peltigera rufescens* and *Cladina arbuscula* var. *mitis*, and the moss *Racomitrium lanuginosum* to copper-rich substrate.	2009	Bačkor, M. et al.	Chemosphere	76, 1340-1343
21	Relationship between metal and pigment concentrations in the Fe-hyperaccumulator moss *Scopelophila ligulata*.	2017	Nakajima, H. and Itoh, K.	J. Plant. Res.	130, 135-141
22	Response mechanisms of antioxidants in bryophyte (*Hypnum plumaeforme*) under the stress of single or combined Pb and/or Ni.	2009	Sun, S.Q. et al.	Environ. Monit. Assess.	49, 291-302
23	The biological response chain to pollution: a case study from the "Italian Triangle of Death" assessed with the liverwort *Lunularia cruciata*.	2017	Basile, A. et al.	Environ. Sci. Pollut. Res.	24, 26185-26193

24	The influence of glutathione on physiological effects of lead and its accumulation in moss *Sphagnum squarrosum*.	2003	Saxena, A. et al.	Water Air Soil Poll.	143, 351-361
25	Toxic effects, oxidative stress and ultrastructural changes in moss *Taxithelium nepalense* (Schwaegr.) Broth. Under chromium and lead phytotoxicity.	2005	Choudhury, S. and Panda, S.K.	Water Air Soil Poll.	167, 73-90
26	Decreased photosynthetic efficiency in plant species exposed to multiple airborne pollutants along the Russian–Norwegian border.	2000	Odasz-Albrigtsen, A.M. et al.	Can. J. Bot.	78, 1021-1033
27	Effects of air pollution from road transport on growth and physiology of six transplanted bryophyte species.	2008	Bignal, K.L. et al.	Environ. Pollut.	156, 332-340
28	Effects of the urban environmental conditions on the physiology of lichen and moss.	2016	Sujetovienė, G. and Galinytė, V.	Atmos. Pollut. Res.	7, 611-618
29	Lichen and moss bags as monitoring devices in urban areas. Part I: Influence of exposure on sample vitality.	2007	Tretiach, M. et al.	Environ. Pollut.	146, 380-391
30	*Marchantia polymorpha* L.: A Bioaccumulator.	2007	Sharma, S.	Aerobiologia	23, 181-187
31	Physiological and Growth Responses of Transplants of the Moss *Pseudoscleropodium purum* to Atmospheric Pollutants.	2013	Varela, Z. et al.	Water Air Soil Pollut.	224, 1753-1763
32	Physiological and isotopic signals in epilithic mosses for indicating anthropogenic sulfur on the urban–rural scale.	2011	Liu, X.Y. et al.	Ecol. Indic.	11, 1245-1250
33	Physiological responses to atmospheric fluorine pollution in transplants of *Pseudoscleropodium purum*.	2008	Aboal, J.R. et al.	Environ. Pollut.	153, 602-609
34	Seasonal pattern of metal bioaccumulation and their toxicity on *Sphagnum squarrosum*.	2006	Saxena, A.	J. Environ. Biol.	27, 71-75
35	Urban background of air pollution: Evaluation through moss bag biomonitoring of trace elements in Botanical Garden.	2017	Urošević, M.A. et al.	Urban For Urban Gree	25, 1-10

Chapter 2: Monitoring of air pollution by moss bags around an oil refinery: a critical evaluation over 16 years

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ABSTRACT

The present study analyses the results of a biomonitoring campaign, carried out by means of *Hypnum cupressiforme* Hedw. moss bags around an oil refinery, located in the southwestern part of Sardinia island (Italy). This work focuses mainly on the effects of rainfall and distance from the source of contamination on the content of 14 trace elements measured over 16 years. In addition, to point out any increasing or decreasing trends, as well as any peak in presence of airborne pollutants in the area, annual elements' concentration values are plotted and discussed. Coefficients of variation were also calculated on accumulation values in order to evaluate stability of measurements across the years and to evaluate if similar exposure conditions, *i.e.*, humidity and distance from contamination source, resulted in more uniform accumulation values. In conclusion, *i*) the vicinity of the source of contamination as well as rainfall influenced element content in the biomonitor in the case study differently, depending on the considered element and on the exposure condition, *ii*) *H. cupressiforme* moss bags provided relatively stable measurements during the 16-year time frame (observed variations in elements content can be attributed to environmental inputs in the area), *iii*) similar conditions of exposure determined less variable accumulation values.

Keywords: *air pollution; biomonitoring; airborne pollutants; bryophytes.*

1. Introduction

Biomonitoring, intended as the use of living organisms or biological tissues to measure a certain ecological condition [1], is a simple, efficient and cost-effective way to characterize (qualitatively and quantitatively) the presence of airborne pollutants, known to represent a threat to ecosystems and human health [1–5]. Bryophytes and lichens, due to their structural and physiological features – they lack a root system, therefore, their supply of nutrients relies mainly on wet and dry atmospheric deposition [3] – are a commonly used taxa in the monitoring of air quality and deposition of airborne pollutants. The systematic use of bryophytes in the biomonitoring of atmospheric pollutants started in the 1950s, to characterize industrial emissions of fluorine [6], while from the 1980s they also began to be considered as a valid tool to study the deposition of organic airborne pollutants [7]. *Taxa* belonging to the bryophytes generally present an elevated surface to volume ratio, lack well developed epidermis or cuticle, and possess high permeability to water and to elements dissolved in it [8], moreover, ion-binding sites are abundant in bryophytes' cell walls, so the adsorption capacity of their tissues is relevant [3]. The above-mentioned features enable bryophytes, and especially mosses, to accumulate airborne pollutants coming in contact with them. Atmospheric pollutants could interact with mosses in three forms: As aqueous solution, gas or solid particles and can adhere on cells' surfaces, outer walls (via ion exchange process) or be included into cells (via passive or active transport) [1,9]. Accumulation of atmospheric pollutants can be influenced by the features of the particulate, of the biomonitor and by ecological parameters. Particles' size, as well as the chemical nature of the pollutants greatly influence their accumulation in tissues—generally, uptake efficiency follows the following order, lead (Pb) > cobalt (Co), chromium (Cr) > copper (Cu), cadmium (Cd), molybdenum (Mo), nickel (Ni), vanadium (V) > zinc (Zn) > arsenic (As) [3,10,11]. Surface structure (and its number and type of cation exchange sites), cells' pH, biomass productivity and age of the tissue are biomonitor-constitutive factors also affecting bioaccumulation of particles. Finally, temperature, precipitations, aridity [12], vegetal coverage [13], occurrence of exposed mineralization and presence of sea salt [10,14,15] and acidic precipitations [16,17] are ecological factors that affect accumulation and concentration of elements in mosses [9], and consequently their reliability in biomonitoring anthropogenic airborne pollutants. Among the several moss-based biomonitoring approaches, the “moss bag technique” is one of the most frequently used and it consists in exposing mesh bags containing moss samples (generally grown in absence of contamination) to assess air quality and the presence of airborne pollutants in a certain area [18]. The present study is based on and discusses a subset of data resulting from a biomonitoring activity which started in the 1990s and is currently ongoing. The biomonitor used in the survey is the moss *Hypnum cupressiforme* Hedw., which is exposed in the form of subspherical moss bags, to monitor air quality in the vicinities of an industrial plant (an oil refinery) located in the southwestern part of Sardinia island (Italy). According to the sampling design, the moss bags were exposed for 9 weeks at different distances from the contamination source (oil refinery) for three, non-consecutive times a year. This sampling design was conceived following Castello et al. 1999 [19], in order to monitor airborne pollutants in the area around the source of contamination—in the immediate vicinities and in more remote areas—and to test if topography and meteorological condition of exposure (essentially rainfall) affects moss uptake.

2. Materials and Methods

2.1. Study Area

The present study was carried out in Sardinia (Italy) (Figure 1a), the second largest island of the Mediterranean Sea. Located in a central position in the sea basin, it presents a typical Mediterranean climate: Warm summers (temperatures can easily exceed 40 °C) with high levels of humidity and generally an absence of precipitation, mild winters (temperatures rarely drop below 0 °C), and rainfall occurs mainly in autumn and spring, presenting annual mean values of about 400 mm. Dominant winds in the island come from NW and E-SE. The monitored industrial area is located in the southeastern portion of the island (Figure 1b), on magmatic (leucogranites) and metamorphic (schists) rocks, surrounded by typical Mediterranean vegetation presenting *Quercus suber* L., *Quercus ilex* L., *Juniperus communis* L., *Phillyrea latifolia* L., etc. [3]. The main industrial activities of the island are carried out in the study area, where the oil refinery reaches 10 mln tons of products per year.

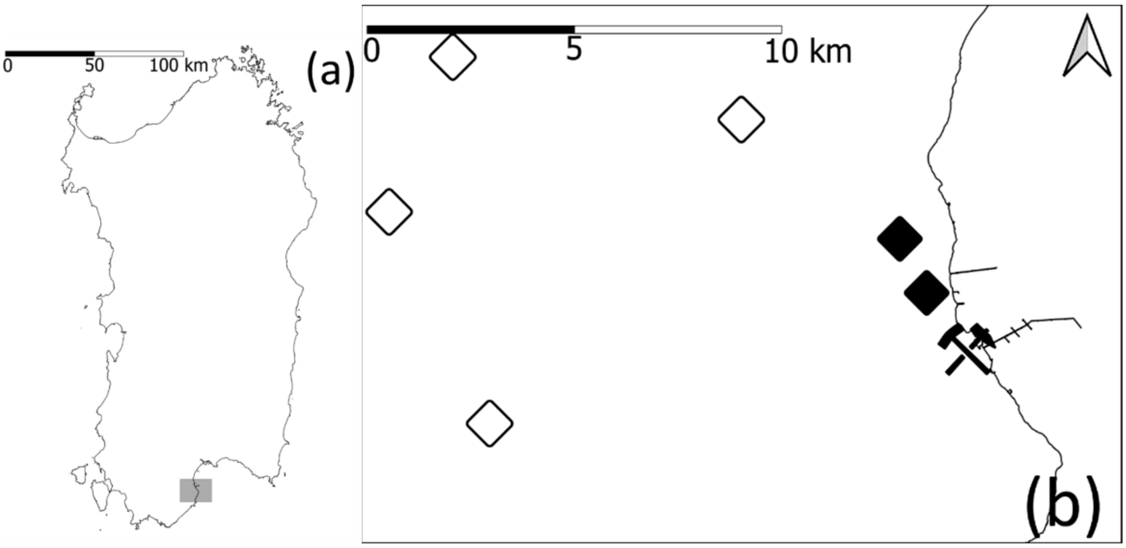


Fig. 1: (a) the study area; (b) the oil refinery is represented by the crossed hammers. Black and white marks indicate exposure sites near and far from the source of contamination.

2.2. Moss Bags

Moss bags were prepared using the moss *H. cupressiforme* collected using plastic gloves to avoid contamination. The collection area is well-preserved, unpolluted and natural (coordinates 39°17’9.87’’ N and 9°23’12,87’’ E), and presents meteorological and geographical features similar to the investigated area. Each subspherical bag contains 1.4 g of moss, carefully washed in distilled water (washings were repeated until every residual debris in the moss was eliminated), air-dried under laboratory conditions and packed in a 15 × 15 cm nylon net (2 mm mesh size) [19].

2.3. Sampling Design

Bags were spatially distributed in the study area paying attention to avoid proximity to isolated houses, urban areas, and communication routes in order to minimize influences of sources of air pollution other than the refinery in the final content of elements in moss bags [18]. Dominant winds and geomorphology of the area were considered at the time of choosing the disposition of the bags, in fact bags were exposed downwind to the refinery, considering the dominant winds, and avoiding the interposition of relevant reliefs between the source of contamination and the exposure sites. Consistent with these principles, a total of 6 sites of exposure were selected: 4 distant, about 15 kms from the centre of contamination (from now on identified as “far” sites), and 2 distanced less than 1 km from the refinery (from now on identified as “near” sites of exposure). In each site, three bags were exposed in order to obtain the elements’ measurements in triplicate. Each year, bags were exposed three, non-consecutives times, for 9 weeks (consistent with Capozzi et al. [20], who suggested a period of exposure longer than 6 weeks), distributed throughout the year: From March to May, from July to September and from October to December. In this way, it was possible to obtain 189 days of monitoring (half a year) distributed evenly along the year with the intention to better represent climatological variability of the region. In the present work, autumn (from October to December) and spring (from March to May) were considered together and identified as “humid” conditions of exposure, as rainfall occurs mostly during these months in the region [21], while summer (from July to September) was considered as a “dry” condition of exposure, due to the aridity occurring in the region during this season. After each exposure, period bags were gathered and the concentration of 14 elements As, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Na, Ni, Pb, V, Zn assessed. During each exposure, three period bags were kept unexposed in the laboratory, carefully preserved from any kind of contamination by storing them in a sealed thermostat, in order to calculate background levels of contamination moss.

Background levels of contamination were subtracted from the final elements' concentration values of the exposed bags, as in [3].

2.4. Chemical Analysis

Elements' content was measured as undertaken in [3], through the use of plasma optical emission spectrometry (Thermo iCAP 6500 duo). Of each one of the three moss bag exposed in each site, 0.5 g of the content was air-dried using a homogenizer, and then digested for 45 min in a microwave digester (MILESTONE ETHOS 1) with 7 mL of HNO3 65% (Sigma-Aldrich) and 1 mL of H_2O_2 30% (Sigma-Aldrich) at 200 °C. The digested samples were then diluted with distilled water to a total volume of 50 mL. To obtain calibration curves, a stock solution (Merck) containing 1.000 µg/mL of each of the 13 elements of interest was used to prepare intermediate, multi-element standard solutions with the following concentrations: 50, 20, 1, 0.1, 0.01, and 0.005 µg/mL. Quality control was performed using the standard reference material, lichen-336 (IAEA). Elements' deposition values of each site were recorded. Obtained values were used in the statistical analysis.

2.5. Data Analysis

Analyses, in the present study, were conducted on a subset of data collected over the duration of 16 consecutive years (from 2003 to 2018), in which the following trace elements were assessed: As, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Na, Ni, Pb, V, Zn. From now on, we will refer and report element content values as the enrichment value of each element measured in bags (final content of the element after the exposure period, minus the pre-exposure value for that element). Effects of rainfall and distance from the source of contamination on the accumulation of elements were estimated, calculating elements mean concentration values during the 16 years of monitoring campaign for each exposure condition, and subsequently calculating statistical significance of differences among compared sets of data by the Mann–Whitney U test. Data were plotted to represent year-by-year fluctuation in pollutants' accumulation values and their relation to rainfall and distance from the contamination source. Mean accumulation values were calculated before and after 2010 (the year in which the construction works of a great dam in the study area began) and significance of differences among compared set of data was tested by Wilcoxon test. Finally, coefficients of variation (cv) were calculated on concentration values, not considering differences in exposure conditions, and later subsetting concentration values based on a similar condition of exposure. This procedure permitted to evaluate if more uniform ecological parameters during the exposure period could reduce variation of accumulation values. Calculating the cv of various sets of data (intended as the standard deviation to mean ratio) permitted the comparison of values' variation around mean values among the different datasets considered. The explorative analysis of data (means, standard errors, and coefficients of variation) was carried out using the 1.2.1335 version of the RStudio software [22]. The same software implemented with the ggplot2 package was also used to create graphs.

3. Results

3.1. Element Accumulation in Relation to Exposure Conditions

In Tables 1–3, elements concentration values (mg kg^{-1}) are reported as the mean of the values measured during the whole 16 years in the different exposure conditions (per year: n far = 12; n near = 6; n humid = 12; n dry = 6). Elements can be divided on the basis of the magnitude of their concentration values: Elements reaching hundreds of mg per kg of moss (Na, Ca, Fe, Mg, K), reported in Table 1, and elements with concentrations levels lower than 10 mg per kg of moss (Zn, Pb, Ni, Cu, V, Cr, As, Cd, Hg), reported in Table 2. Statistical significance of differences among data are reported in Table 3 as *p*-values obtained by the Mann–Whitney U test, with significance level alfa at 0.05.

3.2. Temporal Fluctuations in Pollutant Content

Graphs describe fluctuation of accumulation values year by year, summarizing differences in pollutants content measured near and far from the source of contamination (Figure 2a–d), as well as in dry and humid exposure conditions (Figure 2e–j). In Table 4, mean accumulation values are reported for the most representative elements, before and after the start of the construction works of a great dam (year 2010) in the vicinity of the study area.

3.3. Dispersion of Accumulation Data

In Table 5, coefficients of variation of accumulation data of each element are reported, calculated without subsetting data on the basis of exposure conditions of bags (cv total), and calculated on accumulation data measured in bags exposed to the same ecological conditions.

Table 1: Element content in moss bags. Data reported as mg kg^{-1} mean values ± standard error.

Exposure condition		[Na]	[Ca]	[Fe]	[Mg]	[K]
Near	Humid	2,138.19 ± 395.37	589.75 ± 93.36	475.66 ± 61.27	312.41 ± 41.79	171.37 ± 43.51
	Dry	966.11 ± 164.58	1,518.07 ± 302.07	405.37 ± 67.29	340.52 ± 67.97	148.04 ± 51.62
Far	Humid	1,362.78 ± 229.02	707.44 ± 91.05	420.45 ± 50.38	287.94 ± 34.31	222.95 ± 43.52
	Dry	550.42 ± 67.97	1,215.39 ± 208.88	486.43 ± 52.43	297.41 ± 49.29	171.37 ± 59.67

Table 2. Element content in moss bags. Data reported as mg kg^{-1} mean values ± standard error.

Exposure condition		[Zn]	[Pb]	[Ni]	[Cu]	[V]	[Cr]	[As]	[Cd]	[Hg]
Near	Humid	12.25 ± 1.66	3.23 ± 0.47	2.32 ± 1.31	1.97 ± 0.41	1.71 ± 0.16	0.88 ± 0.11	0.13 ± 0.02	0.05 ± 0.01	0.04 ± 0.02
	Dry	16.58 ± 3.68	3.43 ± 0.99	5.05 ± 3.09	2.66 ± 0.66	2.24 ± 0.40	1.39 ± 0.45	0.15 ± 0.03	0.08 ± 0.02	0.01 ± 0.03
Far	Humid	5.40 ± 0.61	4.26 ± 0.76	0.91 ± 0.10	1.69 ± 0.27	1.11 ± 0.11	1.01 ± 0.14	0.10 ± 0.03	0.06 ± 0.01	0.06 ± 0.03
	Dry	6.60 ± 1.00	1.63 ± 0.25	4.88 ± 1.63	1.81 ± 0.36	1.72 ± 0.31	0.82 ± 0.25	0.13 ± 0.02	0.05 ± 0.01	0.03 ± 0.01

Table 3. Statistical significance between compared sets of values assessed by Mann–Whitney U test. Data reported as *p*-values (significance level alfa at 0.05).

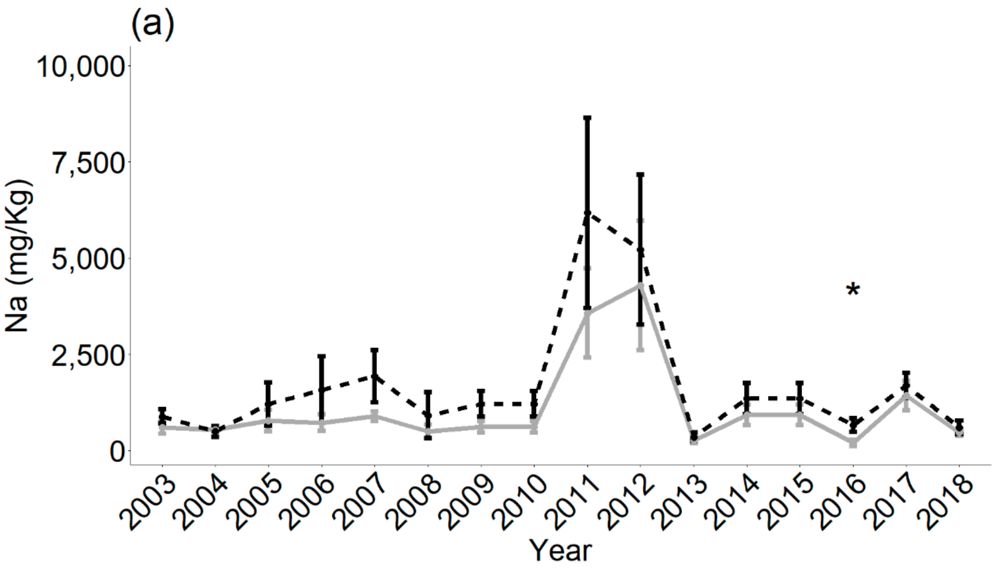
	Na	Ca	Fe	Mg	K	Zn	Pb	Ni	Cu	V	Cr	As	Cd	Hg
Near humid vs Near dry	0.008	0.14	0.45	0.32	0.82	0.5	0.72	0.36	0.55	0.57	0.9	0.7	0.13	0.07
Far humid vs Far dry	0.009	0.84	0.06	0.68	0.26	0.96	0.05	0.49	0.85	0.28	0.04	0.06	0.08	0.8
Near humid vs Far humid	0.003	0.33	0.2	0.34	0.63	>0.001	0.99	0.21	0.32	0.003	0.97	0.14	0.9	0.67
Near dry vs Far dry	0.06	0.84	0.23	0.8	0.15	0.003	0.23	0.27	0.33	0.08	0.23	0.93	0.95	0.08

Table 4. Accumulation data measured before and after the start of the construction works (2010). Data are reported as mg kg^{-1} mean values. Statistical significance of differences between compared sets of data are reported as Wilcoxon test *p*-values (significance level alfa at 0.05).

	Na	Ca	Pb	Ni
Before 2010	842.38 ± 83.59	811.46 ± 107.80	2.23 ± 0.58	1.12 ± 3.66
After 2010	1,691.11 ± 231.42	929.90 ± 100.83	4.08 ± 0.21	3.66 ± 1.12
p-value	0.10	0.93	0.35	0.01

Table 5. Coefficients of variation calculated on the totality of accumulation data (cv total) and on data measured in same condition of exposure (humid, dry, near, far).

Exposure condition		[Na]	[Ca]	[Fe]	[Mg]	[K]	[Zn]	[Pb]	[Ni]	[Cu]	[V]	[Cr]	[As]	[Cd]	[Hg]
cv total		189.84	146.38	116.32	125.86	210.02	151.23	191.52	427.49	176.30	120.09	182.31	212.27	159.32	610.67
Near	Humid	169.47	141.59	118.06	119.63	227.09	124.19	134.40	516.71	190.47	85.75	118.21	190.79	115.12	487.03
	Dry	110.40	125.85	107.58	126.25	220.52	144.06	187.43	396.48	160.59	117.07	209.87	146.62	205.47	324.13
Far	Humid	187.89	140.39	133.96	129.98	212.93	125.95	200.60	125.97	177.69	107.01	160.27	297.24	141.73	596.59
	Dry	98.79	133.13	86.23	128.37	198.97	121.40	121.32	266.89	159.67	146.41	239.93	143.14	154.08	289.70


(a)
Na (mg/Kg)
10,000
7,500
5,000
2,500
0
*
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
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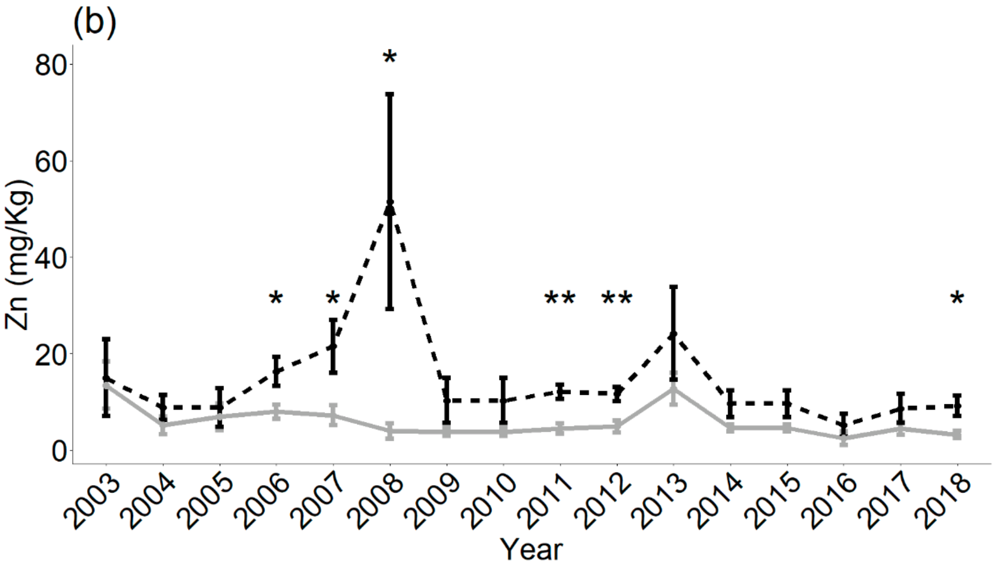
2015
2016
2017
2018
Year


(b)
Zn (mg/Kg)
80
60
40
20
0
*
*
*
**
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*
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
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2016
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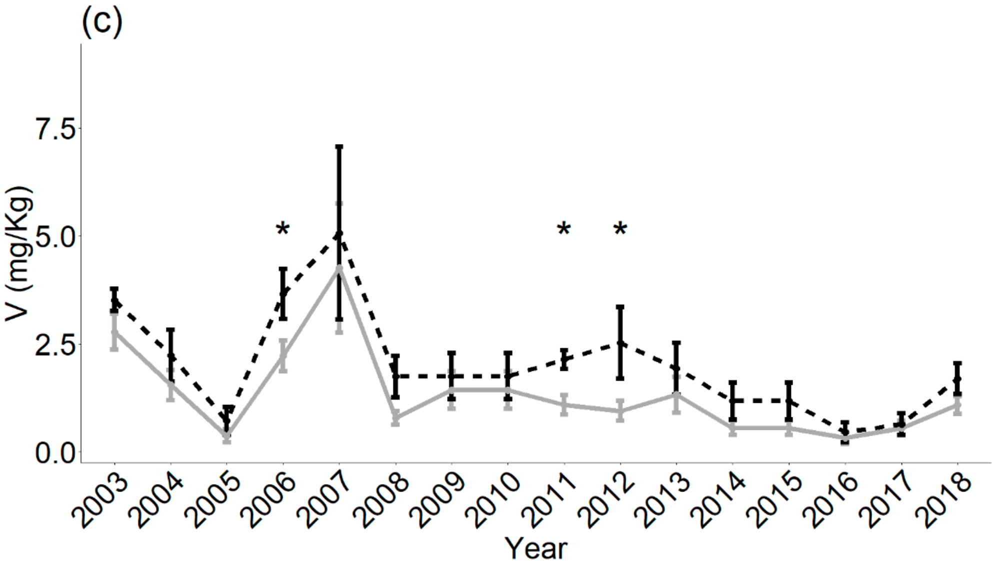
2018
Year


(c)
V (mg/Kg)
7.5
5.0
2.5
0.0
*
*
*
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
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2016
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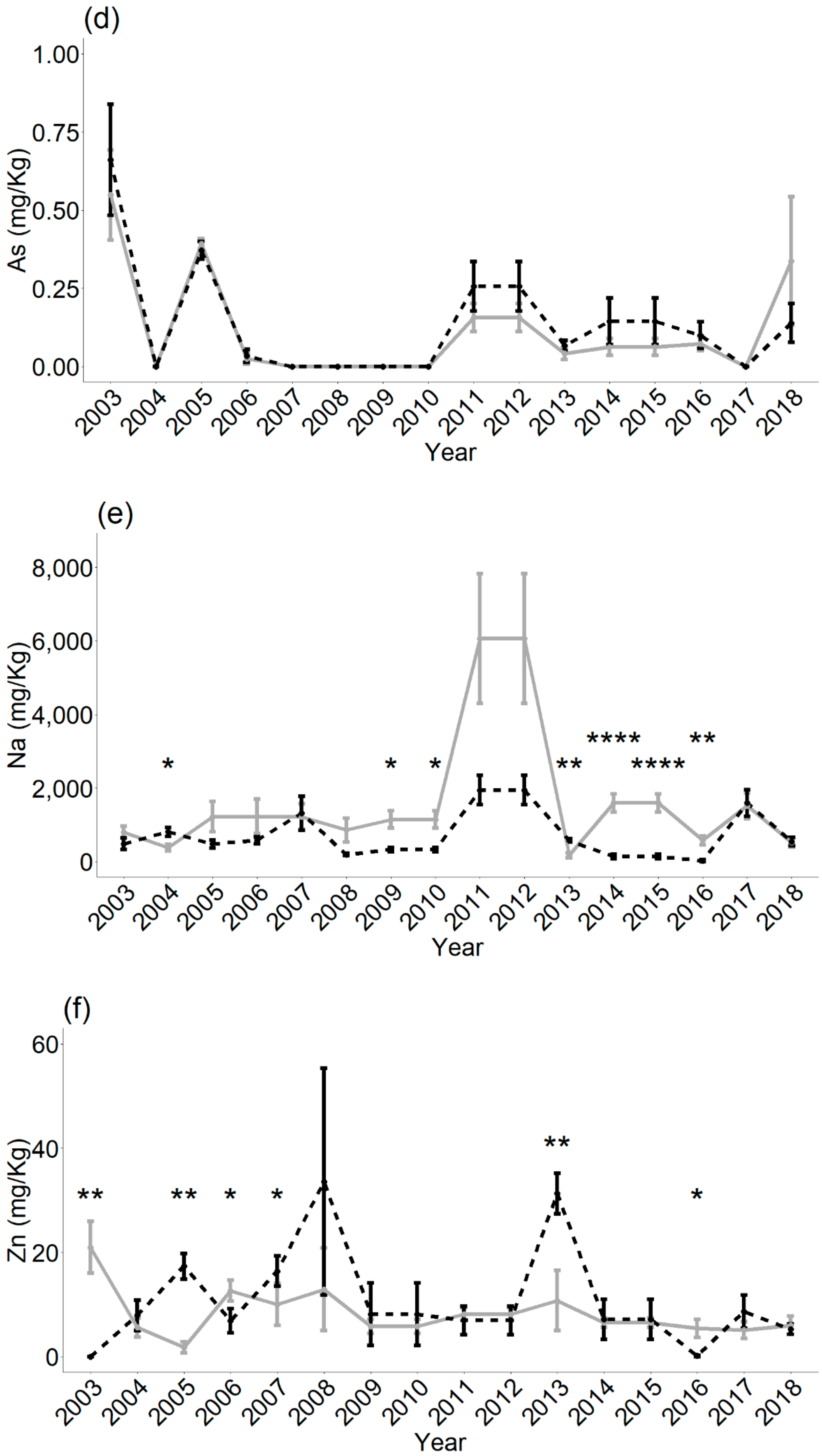
Year


(d)
As (mg/Kg)
1.00
0.75
0.50
0.25
0.00
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
Year
(e)
Na (mg/Kg)
8,000
6,000
4,000
2,000
0
*
*
*
**
****
****
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2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
Year
(f)
Zn (mg/Kg)
60
40
20
0
**
**
*
*
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*
2003
2004
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2009
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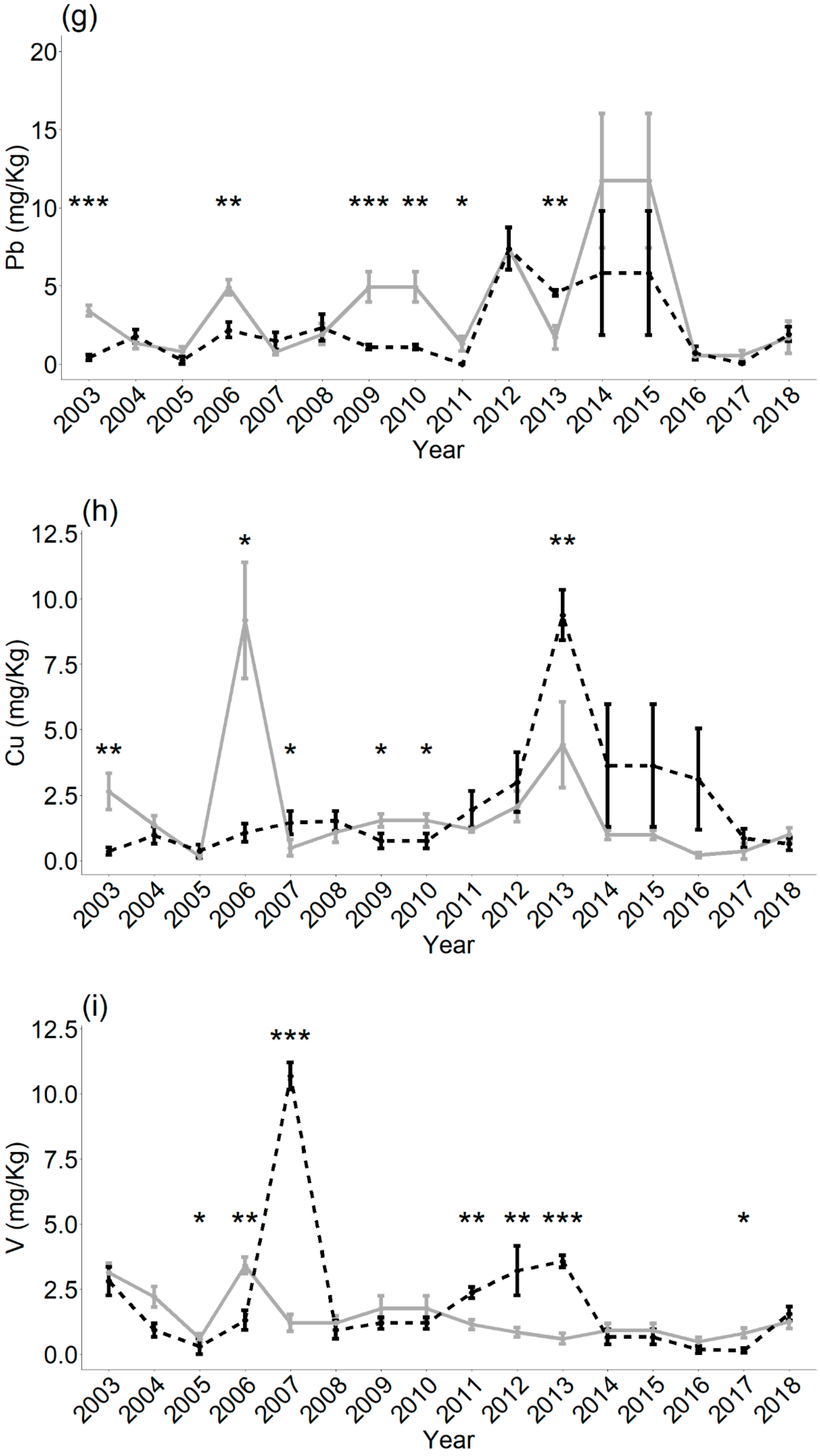
2018
Year


(g)
Pb (mg/Kg)
0
5
10
15
20
*** ** *** ** * **
2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018
Year
(h)
Cu (mg/Kg)
0.0
2.5
5.0
7.5
10.0
12.5
** * * * * **
2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018
Year
(i)
V (mg/Kg)
0.0
2.5
5.0
7.5
10.0
12.5
* ** *** ** ** *** *
2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018
Year

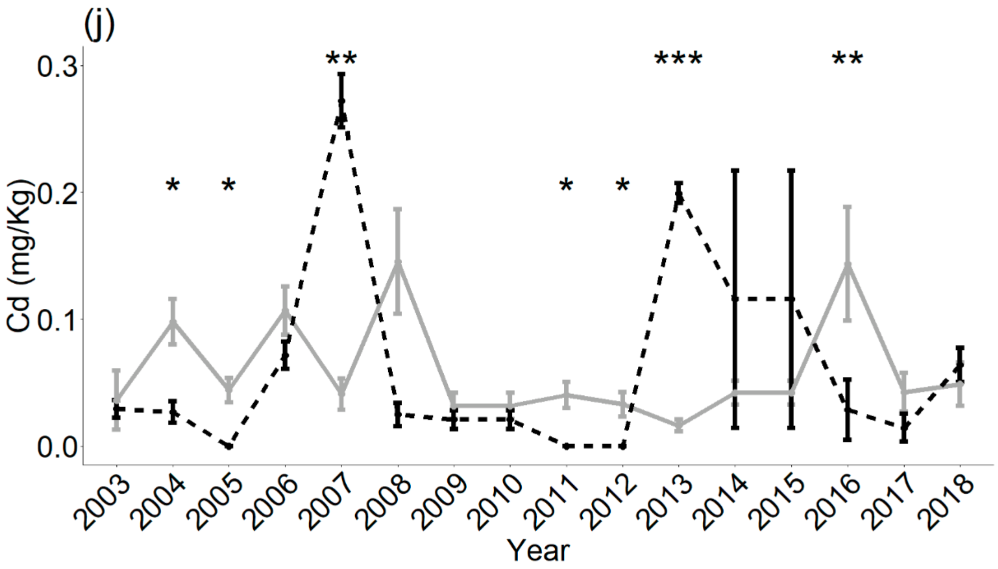


Fig. 2: In graphs 2a–d, values measured near and far from the source of contamination are represented by a black-dashed line and by a grey-continuous line respectively. In graphs 2e–j, values measured in the dry and in the humid exposure conditions are represented by a black-dashed line and by a grey-continuous line respectively. Statistical significance of differences between compared sets of data (Wilcoxon test *p*-values, significance level alfa at 0.05) are reported as asterisks. (*, *p*-value less than 0.05; **, *p*-value less than 0.01; ***, *p*-value less than 0.001).

4. Discussion

Data resulting from the biomonitoring activity have been analysed in the present study. The resulting analyses show that the two considered parameters – rainfall and distance from the source of contamination – influenced element accumulation in bags in different ways, and that differences in accumulation values between different exposure conditions are rarely significative. Nevertheless, elements accumulation seemed to have been influenced by anthropic activities carried out in the study area during the analysed time frame, as well as by ecological features of the area, such as vicinity of the sea and exposed lithology. Therefore, analysis of the data presented in this study, enable several considerations on the features of airborne pollutants present in the study area, and on how the use of *H. cupressiforme* as a biomonitor permitted the characterization of atmospheric pollution in the vicinity of the oil refinery. Among the assessed elements, as reported in [20], As, Cd, Hg, Ni and Pb are included in EU directives for air quality, Al, Cr, Cu, V and Zn are identified as indicators of industrial emissions and Ca, Fe, K, Mg, and Na are elements whose presence is mainly linked to the lithology of the area, in particular to granite and metamorphic rocks, and to the immediate vicinity of the sea. In the next paragraphs we will discuss the presence of these elements in the study area.

4.1. Air Quality and Industrial Emissions

As regards the analysis of elements' accumulation values in bags, the distinction between macro and trace elements appears clearly in Tables 1–3 in all the exposure conditions: Presence of Na, Ca, Fe, Mg and K reaches, in fact, thousands of mg per kg of moss, while trace elements such as Zn, Pb, Ni, Cu, V, Cr, As, Cd and Hg accumulation values range between 16.00 (Zn) and 0.01 (Hg) mg per kg of moss. Results of this study were compared with results of similar works, assessing airborne pollutants via moss bags. Ares et al. [23], studied a polluted area in the vicinities of an oil refinery in the city of Santa Cruz de Tenerife

(Spain), exposing transplants of the moss *Pseudoscleropodium purum* (Hewd.) M. Fleisch for two months. After-exposure, element concentration values (of Na, Fe, Pb, Ni, V, Cr, As, Cd and Hg) results were higher than those measured in this study, except for Na. In [24], accumulation values of several elements (Fe, Pb, Cu, Cr and As) are reported, measured via *H. cupressiforme* moss bags in urban and industrial areas in Turkey and in different regions of Europe (Czech Republic, Finland, Germany, Hungary, Netherlands, Norway, Poland and Spain). As a result, accumulation values measured in the study area in Turkey were always higher than those measured in the study area of the present work, as well as the majority of values measured around Europe, except for Ni values. Ni values in the present study are higher than those reported in [24] for Czech Republic, Finland, Germany, Hungary, Netherlands, Norway and Poland; Fe values in the present study exceeded those reported in [24] for Czech Republic and Finland; Cu values exceeded values measured in Finland and Galicia (Spain); and Pb values exceeded values assessed in Finland. As it regards trace elements, their presence in bags is obviously lower if compared with the macro elements, nevertheless as they are known to have a negative impact on human and environmental health [1,3,5], assessing their presence in the atmosphere is crucial in air quality studies. In the 2019 report on air quality by the European Environment Agency (EEA) [25], southwestern Sardinia was enlisted with those areas in which As and Ni presence is very low and does not exceed the 1 and 5 ng m^{-3} threshold values, respectively, while Pb and Cd atmospheric concentration values were reported to be quite high ranging between the 0.10 and the 0.50 μg m^{-3} and the 5 and 8 ng m^{-3}, respectively [25]. Concentration values measured in the present study (Tables 1–3) partially confirm evaluations made in the EEA report for southwestern Sardinia, in fact, As and Cd are among the least present in bags, ranging between 0.15 and 0.10 mg kg^{-1}, and 0.05 and 0.08 mg kg^{-1} (depending on exposure condition), respectively, while Pb and Ni present mid-range values, ranging between the 1.63 and the 4.63 mg kg^{-1} for Pb, and the 0.91

and the 5.05 mg kg^{-1} (depending on the exposure condition) for Ni. As this study was concerned with the influence of seasonality and distance from the source of contamination on accumulation of elements in bags, both macro- and trace elements present in moss bags seem, in a certain way, to be influenced by these two parameters (Tables 1 and 2). Nevertheless, the influence of the two parameters could be more or less pronounced and differences between accumulation values measured at different exposure conditions are rarely significative (Table 3). In what follows, accumulation values of the most representative elements will be discussed. Concentration of Na in bags increases in the humid condition of exposure, both near and far from the source of contamination, while its content increases near the source of contamination, and in both cases differences between values are significative. Presence of this element is generally not linked to industrial activity, but rather to the lithology of the area and sea spray [26,27], so that its higher concentration values in the vicinities of the refinery in the present case study could be explained by the scarce vegetal coverage around the industrial plant and by the proximity of the sea. Concentration values of Mg, Zn, Ni, Cu, V and As increase near the refinery showing higher concentration values in the dry condition of exposure. As it concerns the origin of these elements (except Mg, a macro-element whose presence is linked to the influence of the sea [23]), it could be attributed to dry deposition of anthropogenic airborne pollutants, generally linked to industrial activity [20], that is intensively carried out in the monitored refinery and in similar industrial plants in the study area. As it concerns the year-by-year analysis of data (figure 2a–j), this does not seem to point out any increasing or decreasing general trend in pollutants' content during the analysed time frame in the study area (only Fe content appears to decrease in the most recent years). The absence of any appreciable decreasing trend in accumulation data of the present study does not respect the decreasing trend depicted in the 2019 report on air quality by the EEA for As, Cd, Hg, Pb and Ni, though the EEA report refers to rural background measurements. Reported line charts also provide additional information about the influence of rainfall and distance on the accumulation of elements by *H. cupressiforme* moss bags. Finally, Table 5 shows how similar exposure condition (as it concerns rainfall and distance from the source of contamination) reduced dispersion of accumulation values in some of the analysed elements. Coefficient of variation is a dimensionless measure of dispersion of data around the mean value, and in the present case study, it is possible to notice how similar ecological condition of exposure reduced dispersion of accumulation values of Na, Ca, Zn and Hg. This suggests that *H. cupressiforme* moss bags are more sensitive to the influence of the two parameters considered in the present study, as it concerns the accumulation of these elements, allowing for more accurate estimations of their presence if the ecological conditions of exposure are more uniform.

4.2. Ecological Factors Influencing Biomonitoring Results

H. cupressiforme is a moss commonly used as biomonitor [3,28–33], in the present study *H. cupressiforme* moss bags proved to be a very sensitive biomonitor to ecological features of the monitored area. The role of ecological conditions of exposure in the accumulation of airborne pollutants will be briefly discussed. High accumulation values of macro-elements (Table 1) must be attributed to the vicinity of the sea and to the lithology of the area. More precisely, high levels of Na and Mg could be explained by the deposition of sea salt and marine aerosol [23] transported inland from the sea (moss bags distance from the sea ranges from ~1 to ~15 km) by the S-E-wind dominant in the area. Ca, Fe, K and Na are very common elements in Earth’s crust, so they can easily accumulate in moss bags via dry deposition of soil particulate matter. This form of deposition strongly contributes to the total concentration of elements in mosses, in fact elements originating from the soil readily accumulate in mosses [24], especially in arid regions with sparse vegetal coverage and exposed lithology [25], as is the study area. Reduced distances of bags by the source of contamination increase accumulation of pollutants in bags [1,9,26]. Tables 1 and 2 also show that in the present case study, vicinity of the source of contamination causes an increase in concentration of the anthropogenic elements Zn, Ni, Cu, V and As, while background areas seem to be less concerned by the presence of these elements [20]. K and Hg accumulation values are not in line with this trend, showing higher concentration levels in bags positioned far from the industrial plant. Nevertheless, the reason for this could lie in the vicinity of the sea and in sea-salt cations, transported by marine aerosol competing with K and Hg [1,10,14] or in unexpected sources of contamination, increasing the content of these elements in bags positioned far from the refinery. Regarding the influence of precipitation in elements' accumulation, Tables 1 and 2 show how, in humid conditions of exposure (corresponding to autumn and spring in the studied area), concentration of Na, K and Hg in bags increase when compared to the dry conditions of exposure (summer). This was expected as wet deposition is highly responsible for the accumulation of pollutants in mosses [9] and in several studies pollutants' accumulation in moss bags increased during humid conditions of exposure [18]. Exceptions to this general rule emerge in the present study in Ca, Mg, Zn, Ni, Cu, V and As. In fact, these elements reached higher accumulation values in the biomonitor during the dry period of the year, likely via dry deposition, though differences between accumulation values measured in dry and humid periods of the year are rarely significative. As this study concerns the influence of anthropic activity on airborne pollutants present in the area, element contents in bags appear to be influenced not only by the activity of the refinery, but also by construction works. More precisely, line charts point out a peak in dry deposition after 2010 in almost all the monitored trace elements. The explanation for this peak in pollutant content after 2010 could be the start of construction works of a great dam in the vicinity of the study area, that probably led to the mobilization of a great amount of particulate, that likely influenced trace elements' content in bags. Table 4 quantifies the variation in accumulation values before and after 2010 in some of the most representative elements. In this case study, *H. cupressiforme* moss bags provided stable accumulation values during the analysed time frame, as cv values reported in Table 5 appear to be sufficiently low, considering the feature of the study. In Table 5, it is also possible to observe that values measured in similar conditions of exposure are less dispersed, showing the influence of ecological parameters in the features of pollutants accumulation values in the biomonitor [1,9,18]. This study analyses a subset of data resulting from an ongoing technical survey, in which the moss bag technique has been normally used since the early 1990s as a tool to assess air quality in the surroundings of an oil refinery. The application of this approach, also in a technical, industrial field, confirms the validity of this biomonitoring technique as it is cost- effective and permits prolonged and highly spatially and temporally detailed assessments. Nevertheless, the influences of ecological parameters such as rainfall, lithology and the proximity of the sea emerge clearly from the analysis of bag element content, underlining how this approach in biomonitoring of airborne pollutants needs a carefully planned experimental design and a critical evaluation of results on the basis of the ecological context.

5. Conclusions

The moss bag technique is known to be a valid biomonitoring approach, in this case study, the reliability of this approach in the long-term monitoring of airborne pollutants has been proven by the low level of variation of accumulation values measured in the monitored time frame. Nevertheless, analysis of data demonstrate that bags were able to register environmental episodes that occurred during the biomonitoring campaign (*e.g.*, construction works in the study area), and that accumulation values are affected by ecological factors such as the presence of the sea, underlining the importance of a critical approach in interpreting accumulation data, and their relationship with ecological conditions of exposure. With that said, it is possible to consider the integration of the moss bag technique in the quantitative and qualitative characterization of airborne pollutants presence in contexts strongly influenced by human activity, such as industrial plants, cities, airports and similar, in order to guarantee time and spatial extensive, and low-priced monitoring of the presence of pollutants and the impacts on nature, in combination with other measurements achieved by more high-tech monitoring devices.

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Chapter 3: Heavy metal tolerance of orchid populations growing on abandoned mine tailings: A case study in Sardinia Island (Italy)

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ABSTRACT

Understanding how environmental pollutants influence plant occurrence, growth, and development is key for effective management plans and potential bioremediation. Rare plants, such as orchids, may occur in modified habitats and on soils containing heavy metals, yet their ecological and physiological responses to heavy metals is poorly understood. We investigated the influence of heavy metal pollution on orchid growth rates and interactions with soil fungal mutualists by comparing a large population of the orchid *Epipactis helleborine* (L.) Crantz subsp. *tremolsii* (Pau) E. Klein that grows on mine tailings in south-west Sardinia (Italy) with a population that grows on non-contaminated soils in central Sardinia. Soils of the contaminated site had high levels of heavy metals and low organic matter and nutritive elements content. We performed a morphological analysis on twenty individuals that have been subjected to measurement of bioaccumulation and translocation of heavy metals. Fungi associated with the roots of plants from the contaminated and uncontaminated site were grown and identified by DNA barcoding approach. Plants from the contaminated site were smaller than the ones growing in the uncontaminated site and were found to be able to tolerate heavy metals from the soil and to accumulate and translocate them into their organs. Fungi belonging to the genus *Ilyonectria* (Ascomycota) were found both in contaminated and uncontaminated sites, while an unidentified fungus was isolated from roots in the contaminated site only. These results are discussed in terms of orchids' tolerance to heavy metals and its physiological and ecological mechanisms. The role of contaminated habitats in harbouring orchids and peculiar *taxa* is also discussed.

Keywords: *Heavy metals; Orchids; Mycorrhiza; Epipactis; Soil pollution.*

1. Introduction

Due to its high geological and ecological complexity, Sardinia is considered a hot spot of biodiversity (Fenu et al., 2014). However, biodiversity in Sardinia has been under threat from human activities, such as mining, over the past two centuries. Mining activity in Sardinia reached a peak intensity in the first half of the nineteenth century, and in many cases, it was carried out without an appropriate management of mining by-products. For this reason, numerous abandoned mining areas are still today sources of environmental pollution (Bacchetta et al., 2018; Jiménez et al., 2011; Vacca and Vacca, 2001), since they are characterized by very high concentrations of heavy metal (HM) and metalloid pollutants including copper (Cu), lead (Pb), zinc (Zn), cadmium (Cd), chromium (Cr), arsenic (As) and antimony (Sb) (Cidu et al., 2014; Fanfani et al., 2000; Frau, 2000; Vacca and Vacca, 2001). Generally, the sources of contamination originating from previous mining activities are represented by extended impoverished heaps and tailing dumps (Bacchetta et al., 2015; Vacca and Vacca, 2001). The pollutants in those matrices are very reactive due to their chemical nature of sulphides and sulphates and are characterized by a great mobility in the environment due to their very fine dimensions, from the gravel to the silt size (De Waele and Pisano, 1998). Generally, the contaminated heaps in abandoned mining areas are not suitable for the colonization by the majority of the vascular flora because pollutants are present in high levels, the soils are poor and non-consolidated with very low organic matter content, and the vegetation canopy is absent or very rare (Bacchetta et al., 2018; Jiménez et al., 2011). Nevertheless, metal-tolerant, or metallophyte, *taxa* are able to colonize and grow in very harsh environments. Several species are able to successfully colonize strongly anthropogenically-influenced habitats such as abandoned mining areas (Shefferson et al., 2008), cemeteries and roadsides (Fekete et al., 2017; Löki et al., 2019). The Orchidaceae often occur in disturbed, anthropogenically modified habitats. There are 68 orchid species on

Sardinia (Lai, 2008) of which five are endemic (Gögler et al., 2015; Lussu et al., 2018). In the early stages of their development, orchids establish a mycorrhizal symbiosis with free living soil fungi to supply nutrients to the embryo (Arditti and Ghani, 2000). Symbiosis with soil fungi plays several fundamental ecological roles in adult individuals too, among the others, fungal symbiosis is one of the factors involved in the plant protection when environmental pollutants (*e.g.*, heavy metals and metalloids) are present in the soil (Jurkiewicz et al., 2001; Shefferson et al., 2008). However, whether similar fungi occur, and whether orchids have similar growth rates, in contaminated and uncontaminated soils remains unclear. Here we investigate *Epipactis helleborine* (L.) Crantz subsp. *tremolsii* (C. Pau) E. Klein (1979), a Eurasian orchid *taxon*, extent at south till Maghreb. We investigate its occurrence on the mining site of "Barraxiutta" in Domusnovas (south-west Sardinia), which contains almost one hundred individuals growing on an area ~4500 m^2. In the past two decades there has been an intense extraction of Zn, Cu, and argentiferous Pb in this area (De Waele and Pisano, 1998). To investigate the ability of *E. helleborine* subsp. *tremolsii* to accumulate heavy metals in its organs, all individuals within this population were characterized through morphometric and ecological approaches. Data obtained from the population of *E. helleborine* subsp. *tremolsii* growing on mine tailings dump were compared to those obtained from a population (control) collected in a non-contaminated site. The aims of this study were to: *i*) quantify morphological differences between orchids growing in contaminated and non-contaminated areas and evaluate if they are indicators of a physiological stress incurred by growing on heavy metal polluted soils; *ii*) estimate the concentration and compartmentation of heavy metals in different organs of the orchid; *iii*) investigate the role of soil fungi and symbiotic interactions in tolerance towards soil pollution and heavy metals.

2. Materials and methods

2.1. Study area

The abandoned mining site of “Barraxiutta” occurs in the municipality of Domusnovas (South-West Sardinia, Italy, Fig. 1; 39°22′05.82″ N, 8°36′28.46″ E − WGS84), where a mineralization of Sphalerite and Galena was exploited by mining. The uncontaminated control population occurs in the Province of Nuoro (40°12′39.13″ N, 8°41′14.43″ E − WGS84). This was chosen as control site because of its natural ecological conditions and for the remarkable distance separating it from the main communication routes and industrial areas.

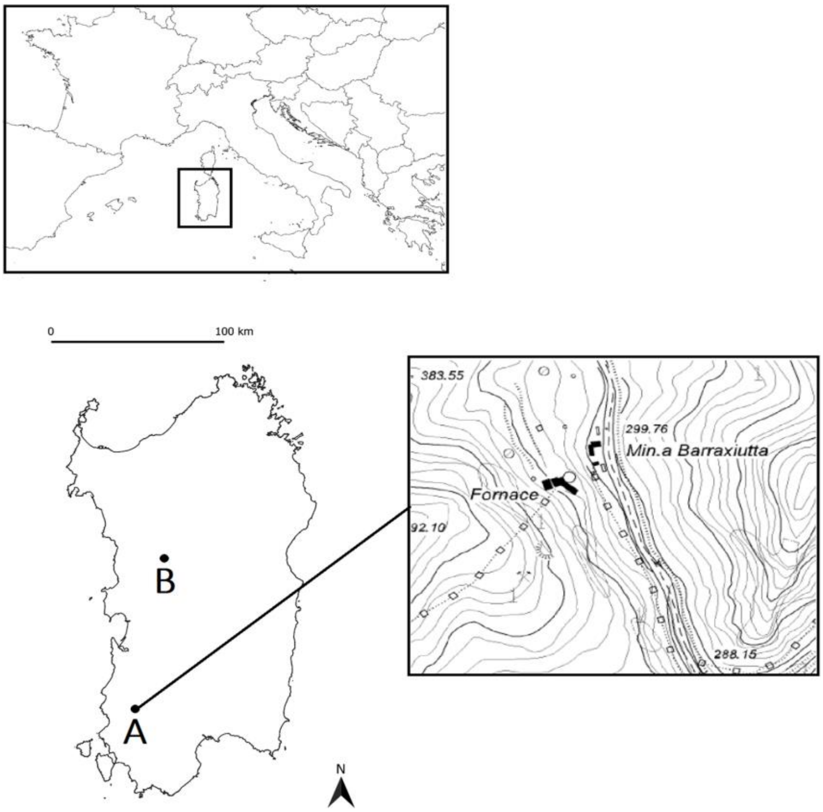

Fig. 1: Sardinia island (Italy). In detail, the collection sites: the contaminated mining area of Barraxiutta (A), and the control site (B).

2.2. Substratum collection and analysis

In different sites of the study area (a flotation tailings dump), three selected topsoils (0–25 cm) were described according to standard procedures of soil description (Schoeneberger et al., 2012). Soils were sampled for physical and chemical analyses. A similar procedure was adopted in the control area. The bulk soil samples were air dried and crushed to pass a 2 mm sieve. Sand (2.00–0.05 mm), silt (0.050–0.002 mm) and clay (< 0.002 mm) fractions were separated by the sieve and pipette methods after the removal of organic matter by H_2O_2 treatment and dispersion aided by Na-hexametaphosphate. The organic carbon content was determined by C elementary analyser (Leco, USA). Total nitrogen was determined by the Kjeldahl method, total phosphorus was determined by spectrophotometry after treatment with H_2SO_4, H_2O_2 and HF, available phosphorus was determined by the Olsen method, total potassium as determined by spectrophotometry after treatment with H_2SO_4, HCl and HF, and available potassium was determined by spectrophotometry after treatment with HCl and $BaCl_2$. Soil pH was measured by potentiometry in soil/solution suspensions of 1:2.5 H_2O. The sieved samples for the determination of the total metal content [iron (Fe), As, Cd, Cu, Cr, Pb, Zn, nickel (Ni) and manganese (Mn)] were digested in concentrated HNO_3 according to the EPA 3050-B method. For the determination of the metal bioavailable fractions, the Community Bureau of Reference (BCR) extraction method (acetic acid 0.11 M) was used. The soil extracts were analysed by an Inductively Coupled Plasma (ICP-OES 5110 Agilent).

2.3. Plant sample collection and analysis

The sampling of plant material was performed during the late spring 2018 from two populations of *E. helleborine* subsp. *tremolsii*: the population on the tailing heap was sampled in early April, while the control population at the end of May. This time shift in sampling allowed us to collect individuals showing the same phenological state (a fully developed inflorescence). We randomly selected 20% of each population for comparison from both the contaminated (20 individuals) and noncontaminated (5 individuals) site, to minimise the damage to these populations. Three morphometric parameters namely, plant total height, inflorescence dimensions (as the distance between the first flower and the apex of the inflorescence), length and width of the largest leaf were measured in vivo with metric tape. Subsequently, the individuals, including their rhizomes, were harvested. Roots, leaves, and stems from each individual were separated, and dried in oven at 75 °C up to complete dehydration (24 h). Subsequently, dried samples were weighed, pulverized with liquid nitrogen, and then digested with an acid mixture of 65% nitric acid (HNO_3) and 50% fluorhydric acid (HF) in a 2:1 ratio (v/v). Digestion was enhanced in a microwave oven (Ethos, Milestone). Fe, Cu, Zn, Cd and Pb concentrations were determined by an Inductively Coupled Plasma – Optical Emission Spectrometry (ICPOES) on an Optima 7000DV (PerkinElmer) and data were compared to a standard reference material (Thomas et al., 2004) to verify the accuracy. Standard solutions of each metal were also used to generate calibration curves of emission readings vs concentrations.

2.4. Bioaccumulation and translocation factors

The Bioaccumulation Factor (BAF) was calculated as the ratio between the concentration of a given metal species in the roots and the total or bioavailable fraction of the same metal species in the soil. The values of this index indicate the capacity of the plant whether it can accumulate (BAF > 1) a given metal or not (BAF < 1) in its roots. The Translocation Factor (TF) was also estimated to evaluate the ability of the plant to translocate metals from the roots to different epigeal parts. The TF is the ratio between the metal concentration in the epigeal portion of the plant and the concentration of the same metal in the roots. This index indicates the ability of the plant to translocate metals from the roots to the different epigeal parts such as stem, leaves, fruits etc. (TF > 1).

2.5. Mycorrhizal fungi isolation, cultivation, and DNA barcoding

Fungi were isolated from the roots of different plants: roots were collected, washed with sterile physiological solution (NaCl 0.9%), and sterilized with a solution of sodium hypochlorite (1.15%) for 5 min. These steps allowed to remove any possible contamination due to microorganisms present in the soil. After the treatment, roots were longitudinally sectioned, and the cut-exposed surface was put on agar growth media. For this first step of fungal isolation, a Potato Dextrose Agar media, added with antibiotic (chloramphenicol 200 mg mL^{-1}), was used. Mycorrhizal fungi were grown for 7 days at 25 °C before transferred on fresh culture media. Fungi were inoculated and grown on a Sabouraud Dextrose Agar media added with antibiotic (chloramphenicol 200 mg mL^{-1}). After 7–10 days of growth at 25 °C, the plates were stored at −20 °C. The three isolated and cultivated mycorrhizal fungi were analysed trough DNA barcoding (Hebert et al., 2003) analysis. Genomic DNA was isolated starting from 20 mg of culture medium from each sample using Chelex® 100 Molecular Biology Grade Resin. Amplification of the nuclear internal transcribed spacer region (ITS) was performed using puReTaq Ready-To-Go PCR beads (Amersham Bioscience, Italy) in a 25-µL reaction according to the manufacturer's instructions and primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4R (TCCTCCGCTTAT TGATATGC) from Luo and Mitchell, (2002). PCR cycles consisted of an initial denaturation of 5 min at 95 °C followed by 32 cycles of denaturation (30 s at 95 °C) annealing (30 s at 58 °C) and extension (60 s at 72 °C) and a final extension at 72 °C for 10 min. The obtained amplicons were isolated trough agarose gel electrophoresis (1.5%) and purified from agarose using MinElute PCR Purification Kit (Qiagen, Germany). Sequencing was performed by Macrogen Inc., Korea. Sequences were edited manually and taxonomically assigned using blastn algorithm on GenBank (NCBI). Each sequence was taxonomically assigned to the fungal *taxon* considering the nearest match (maximum identity>99% and query coverage of 100%) according to Bruni et al. (2015). In case of multiple matches with the same threshold values, the sequence was assigned to the genus level.

2.6. Photosynthetic measurements

The photosynthetic activity on both contaminated and control individuals was assessed by using a Mini PPM 100 fluorometer (EARS, The Netherlands). The maximum fluorescence (F_m), the fluorescence in ambient light (F_0, a relative reference of conditions where all reaction centres of the photosystem II are open), and the photosynthesis yield (Y, a direct indicator of the photosynthetic efficiency) were used as a discriminant of photosynthetic activity between contaminated and control individuals.

2.7. Statistical analysis on plant morphometric data and metal concentration

A preliminary test to assay the Gaussian distribution, homogeneity variance and homoscedasticity were performed on data in R (R Core Team, 2019) through Shapiro-Wilk test, Levene's test and Bartlett's test, respectively. After that, morphometric data, metal concentration, and element accumulation in different plant organs (in relation to the treatments) were tested in R by Kruskal and Wallis one-way analysis of variance by ranks, followed by post hoc Nemenyi test (pgirmess package).

3. Results

3.1. Pedological and physicochemical soil features

The analyses on topsoils of dump revealed that they were characterised by a ^A horizon with sandy texture (87.2% sand, 10.8% silt, and 2% clay), weak very fine and fine subangular blocky structure with a tendency to single grain, soft, nonsticky, and nonplastic consistency, strong effervescence after 1 NHCl application, organic carbon content of 0.53%, and pH (H_2O) equal to 7.8. Total nitrogen (N) is generally below 0.1 g kg^{-1}, total phosphorus (P) ranges between 388 and 410 mg kg^{-1}, available P ranges between 3.48 and 3.91 mg kg^{-1}, total potassium (K) is 500 mg kg^{-1}, and available K is around 10 mg kg^{-1}. Since they have formed on materials created by humans, as part of a mining process (mine spoils), these topsoils belong to soils that are classified as Spolic Technosols (IUSS Working Group WRB, 2015).

In the control area, the A horizon has a sandy loam texture (61.6% sand, 34.4% silt, and 4.0% clay), moderate fine subangular blocky structure, is soft with a slightly sticky and non-plastic wet consistency, and an organic carbon content of 6.83% and pH (H_2O) equal to 5.8. Total N is below 5.8 g kg^{-1}, total P is 1249 mg kg^{-1}, available P is 10.5 mg kg^{-1}, total K is 1846 mg kg^{-1}, and available K is around 207 mg kg^{-1}. The soils in the area are classified as Cambic Umbrisols (IUSS Working Group WRB, 2015). As it concerns the total and the bioavailable concentration of Fe, As and heavy metals of the collected topsoils, Zn and Pb are the elements with higher absolute values (Table 1). Concerning the total content, 60% of Cd, 49% of Pb, and 37% of Zn are bioavailable.

3.2. Morphometric parameters

Plant height and largest leaf length and width of individuals collected in the non-contaminated site resulted significantly greater than those measured in individuals sampled in the contaminated area (Table 2), while the differences regarding the inflorescence size resulted to be non-significant.

3.3. Heavy metals content in plant organs, accumulation, and translocation

Fe, Cu, Zn, Cd, and Pb concentrations were detected in the organs of orchids grown on the tailing dump and control soils. Although metal concentration values were not very high, metal concentrations, mainly Fe and Zn, was higher in the organs of plants grown on dump than those grown on the control soil (Table 3). Furthermore, the highest concentration of Fe and Zn, was measured in the roots of orchids grown on polluted soils. In general, metal concentrations in organs of plants grown in the contaminated soils were significantly greater (even one order of magnitude higher) of those collected from the non-contaminated soil (Table 3). Bioaccumulation factors were calculated for plants collected in the contaminated area considering both the available metal soil concentration (BAF_{bioav}), or the total one (BAF_{tot}) (Table 4). In the case of BAF_{tot} the values were lower than 1 for all the detected metals, except Zn. Considering BAF_{bioav} the values were all greater than 1, except Cd. The Fe BAF_{bioav} was extremely high because of the very low Fe availability in the contaminated soils. TF values for plants collected in the mine tailings, was shown to be > 1 if we consider the epigeous portion of individuals (except Fe) (Table 5). Also in plants collected from the non-contaminated soil certain TF values were higher than 1, in particular in the case of Zn and Pb, whilst TF of Fe was lower than 1.

Table 1: Soil metal concentrations and bioavailability. Data were expressed as mean ± SD. l.o.d. = limit of detection.

Elements	Concentration (mg g^{-1})	Bioavailability (mg g^{-1})
[Cr]	0.010 ± 0.001	l.o.d.
[Mn]	1.24 ± 0.06	0.22 ± 0.02
[Fe]	55.98 ± 7.44	0.05 ± 0.03
[Ni]	0.020 ± 0.002	l.o.d.
[Cu]	0.79 ± 0.08	0.010 ± 0.002
[Zn]	13.10 ± 2.71	4.87 ± 0.08
[Cd]	0.15 ± 0.03	0.09 ± 0.01
[Pb]	5.21 ± 0.69	2.57 ± 0.88
[As]	0.19 ± 0.04	l.o.d.

Table 2: Morphometric data and comparison among orchids grown on contaminated or not- contaminated soils. Data were expressed as mean ± SD.

Morphometric parameters	Contaminated soil (cm)	Control soil (cm)	*p*-values
Height	20.65 ± 4.56	37.7 ± 5.42	<0.05
Inflorescence size	3.57 ± 1.66	5.04 ± 2.03	>0.05
Leaf length	6.53 ± 0.99	7.66 ± 1.12	<0.05
Leaf width	3.36 ± 0.71	4.58 ± 1.01	<0.05

Table 3: Concentrations of Fe, Cu, Zn, Cd, and Pb in plant organs. Data were expressed as mean mg g^{-1} ± SD. R = root; S + L = stem + leaves; l.o.d. = limit of detection. * (t-test, Welch approx.)

Elements	Metal concentration in epigeal organs			Metal concentration in ipogeal organs		
	S + L (Contaminated)	S + L (Control)	*p*-values	R (Contaminated)	R (Control)	*p*-values
Fe	15.36 ± 4.83	2.71 ± 7.47	<0.05*	43.08 ± 23.19	17.09 ± 16.08	<0.05
Cu	0.26 ± 0.84	0.02 ± 0.79	<0.05*	23.28 ± 14.15	0.25 ± 13.46	<0.05
Zn	8.52 ± 3.62	0.23 ± 4.52	<0.05*	23.28 ± 14.15	0.25 ± 13.46	<0.05
Cd	0.01 ± 0.01	l.o.d.	//	0.09 ± 0.07	l.o.d.	//
Pb	1.66 ± 1.43	l.o.d.	//	4.10 ± 2.50	l.o.d.	//

Table 4: BAF values considering total (BAF_{tot}) and bioavailable (BAF_{bioav}) fractions of metals in the soil. Data were expressed as mean ± SD.

Elements	BAF_{tot}	BAF_{bioav}
Fe	0.77 ± 0.41	783.25 ± 421.68
Cu	0.12 ± 0.08	9.16 ± 6.35
Zn	1.78 ± 1.08	4.77 ± 2.91
Cd	0.58 ± 0.44	0.97 ± 0.73
Pb	0.79 ± 0.48	1.59 ± 0.97

Table 5: Translocation of metals in contaminated and control individuals. Data were expressed as mean ± SD. Epigeous = stem + leaves.

Elements	TF					
	Epigeous		Leaves		Stem	
	Contaminated	Control	Contaminated	Control	Contaminated	Control
Fe	0.67 ± 0.96	0.15 ± 0.06	0.69 ± 1.57	0.06 ± 0.02	0.33 ± 0.53	0.10 ± 0.05
Cu	1.56 ± 4.46	1.10 ± 0.27	0.65 ± 1.72	0.56 ± 0.12	0.83 ± 2.48	0.54 ± 0.15
Zn	1.21 ± 2.86	2.20 ± 1.64	1.00 ± 2.26	0.95 ± 0.66	0.34 ± 0.35	1.25 ± 0.99
Cd	1.45 ± 4.13	//	0.69 ± 1.78	//	0.73 ± 2.11	//
Pb	1.93 ± 5.35	3.59 ± 2.07	1.60 ± 4.29	1.49 ± 0.81	0.32 ± 0.52	2.10 ± 1.35

3.4. Fungal DNA barcoding

DNA extraction was carried out for all the samples and the whole amplification products showed a clear single band after electrophoresis (min-max length 502–550 bp). All the PCR products were sequenced, and high-quality bidirectional sequences were obtained. One of the sequences was taxonomically assigned to an unidentified endophyte fungus of the Valsaceae family identified by Girlanda et al. (2002) (99.45% of similarity with the GenBank accession number AF373050.1) with no supported assignment at lower taxonomic rank. Another sequence was assigned to the Ascomycota Whittaker (1959) *phylum* identified by Vu et al. (2019) (GenBank reference sequence accession number MH863168.1), while the last sequence was assigned to the genus *Ilyonectria* Chaverri and Salgado (2011).

3.5. Photosynthetic rates as indicators of plants' stress

Individuals from the contaminated population were subjected to assessments of their photosynthetic rates and compared with controls. In particular, the photosynthetic yield (Y), the maximum fluorescence (F_m), and minimum fluorescence (F_0) were measured in a statistically relevant sample of 10 individuals per population (control and contaminated). Values of Y correspond to 47.34 ± 1.44 (±3.05%; s: 4.57) and to 62.44 ± 0.96 (±1.53%; s: 3.024) for the contaminated population and controls, respectively; similarly, values of F_m equal to 633.1 and of F_0 to 382.2 were found for the contaminated population, while values of F_m equal to 998 and F_0 equal to 373.5 were found for the control population. Eventually, the F_0/F_m ratio was evaluated. This parameter is an indication of fluorescence not related to photochemistry and that increases when photosynthetic efficiency decreases, hence upon stress. For the same pool of individual, the F_0/F_m ratio was found to be equal to 0.53 ± 0.01 (±2.72%; s: 0.045) for the contaminated population and 0.37 ± 0.01 (±2.55%; s: 0.03) for the control population, and this difference clearly indicated stressed individuals. Moreover, by comparing the two groups, the Y in contaminated plants decreased by 24.18% with respect to controls, and the F_0/F_m ratio in contaminated plants increased (hence the stress increased) by 40.52% with respect to controls.

4. Discussion

We investigated a population of the orchid *E. helleborine* subsp. *tremolsii* growing on soils derived from mining activity (characterized by low content of organic matter and nutritive elements and high concentration of metals) and compared this to a population growing in an uncontaminated area. We found that the population of *E. helleborine* subsp. *tremolsii* that occurs on the mine produces smaller individuals and that there is presence of metal pollutants in organs of plants. These two features could indicate a condition of stress in the orchid population growing on the tailing dump, to be attributed both to the heavy metal contamination and to the lack of essential elements such as N, P and K. However, the same fungal mutualist was found in both the contaminated and uncontaminated sites. While species belonging to the *Epipactis* can often be found in anthropogenically disturbed habitats, growing in parks, city gardens and in areas interested by a previous mining activity (Jurkiewicz et al., 2001; Richards and Swan, 1976; Shefferson et al., 2008; Szarek-Łukaszewska, 2009) the results shown here indicate that there might be physiological differences between plants that occur in disturbed habitats compared with un-modified habitats. The contamination of the studied site can be attributed to the previous mining activity that was carried out for about one hundred years, reaching production rates of 130 tons of tout venant with 60% in Pb (De Waele and Pisano, 1998). The waste material and the flotation tailings produced during the mining activity were not properly managed and still today present relevant contents of heavy metals and are characterized by high environmental mobility (De Waele and Pisano, 1998). The total and the bioavailable content of Fe, As, and heavy metals of the studied topsoils reflects the origin of the parent material. Zn and Pb are very abundant in the area, the former is derived from sphalerite and the bulk of oxidised products called “calamine”, and the latter is derived from both galena and from oxidation minerals, such as anglesite and cerussite. Cd is mostly related to Zn-minerals and follows its abundance, with particular enrichments related to treatment plants and mine tailing areas. As is a common element in some of the pyrites, especially those of the orebodies at the base of the Cambrian carbonates. Consequently, as to be expected, total contents of Zn, Pb, and Cd in topsoil samples are much higher than limits imposed by the Italian law (GURI, 2006, D.lgs. 152) for sites of commercial and industrial use (Zn = 1.5 mg g^{-1}, Pb = 1.0 mg g^{-1}, and Cd 0.015 mg g^{-1}). Higher total values, with respect to the law limits, set at 0.05 mg g^{-1} and 0.6 mg g^{-1}, respectively, are also found for As and Cu. It should be noted that total values of Zn and Pb in the studied topsoils are also higher than the median values of stream sediments in the district (Zn = 1.2 mg g^{-1} and Pb = 0.95 mg g^{-1}) (Boni et al., 1999), that can be taken as an indication of the local post-mining geochemical baseline. Zn was found to be the most bioavailable metal in soils followed by Pb, and Mn. On the contrary, Fe, although the most abundant metal in soils, is one of the less bioavailable. Some of the metals present in soils, such as Fe, Mn, Ni and Cu are essential micronutrients for plants metabolism. However, concentrations detected in the studied soils are significantly higher than in unpolluted soils, and this feature could represent an environmental filter for some plant species (Laghlimi et al., 2015). Nevertheless, plants often react to soil chemical composition in different ways, and several *taxa* are adapted to colonizes polluted sites to take advantages of, for example, reduced competition with other plants for nutrients and space (Barrutia et al., 2011; Faucon et al., 2011; Shefferson et al., 2008). Other metals that we detected, Al, Cd, Pb, and Cr are known only to be toxic for plants. They are frequently phytotoxic and negatively affect photosynthesis, respiration, nutrient uptake, gene expression, and membrane integrity (Laghlimi et al., 2015; Rascio and Navari-Izzo, 2011). Besides, the high presence of cations in soils could cause the saturation of the radical cation exchange sites determining a reduced efficiency in the uptake of other important non-metal cations, such as Ca^{2+} and Na^{+}. This fact, in combination with the low content of organic matter, drastically reduces the ability of a balanced nutrient uptake for the plant. The analysis of metal content in the individuals revealed the presence of Fe, Zn and Pb in roots, while in stems and leaves Fe, Zn and Pb were mainly accumulated. Fe and Zn are micronutrients (known to be toxic only at high levels) and were detected in the organs of the orchids grown on polluted soils together with Pb, known for its phytotoxicity (Amari et al., 2017; Lamhamdi et al., 2011). Given that, we assume that *E. helleborine* subsp. *tremolsii* cannot avoid the uptake of soil metals present at high concentrations, and that their concentrations in the organs are, to some extent, proportional to the concentration in the soil. Similarly, Jurkiewicz et al., (2001) analysed the content of several heavy metals in the individuals of a population of *Epipactis atrorubens* (Hoffm.) Besser (1809) (a species taxonomically closely related to *E. helleborine* subsp. *tremolsii*), colonizing a Zn mine tailing heap in southern Poland. Also in that case heavy metals were accumulated in plants' organs, and surprisingly Fe, Zn and Pb were the elements mostly accumulated in roots and epigeal portion of plants as in the present study (concentrations detected in the individuals of the present study are approximately an order of magnitude greater). Tolerance to heavy metal pollution is common in different plant *taxa*, such as *Helianthus annuus* L., (1753) (Cicatelli et al., 2017; Lin et al., 2003), *Zea mays* L., (1753) (Li et al., 2011; Moccia et al., 2017; Tanyolaç et al., 2007; Vigliotta et al., 2016), *Populus* spp. (Cicatelli et al., 2010; Di Lonardo et al., 2011; Krpata et al., 2008), *Dittrichia* spp. (Buscaroli et al., 2017; Guarino et al., 2018). However, those *taxa* can be more or less tolerant of soil heavy metal pollution depending on several ecological and autecological factors as their genotype, the bioavailability of the pollutants, the occurrence of radical symbiosis with fungi and bacteria and other pedo-climatological, physical, and chemical growth conditions. Similarly, we need to know more about how individual *E. helleborine* subsp. *tremolsii* plants respond to variation in soil chemical composition and whether this influences its interactions with mutualistic fungi. BAF values were highly variable considering the BAF_{tot} or the BAF_{bioav}. BAF_{bioav} is more relevant as it considers the fraction of elements available to the plant. BAF_{bioav} values showed that all the analysed elements (except Cd) are accumulated in roots. Generally, plants hold heavy metals in roots to protect the photosynthetic tissues from the toxic effects of pollutants (Rascio and Navari-Izzo, 2011). However, translocation up to the epigeal portion of the plant, showed by values of TF greater than one for Pb, Cu, and Zn (in particular Pb and Zn are translocated to the leaves), suggests that *E. helleborine* subsp. *tremolsii* is in some cases able to translocate heavy metals in its epigeal organs. Considering that *E. helleborine* subsp. *tremolsii* is a geophyte that, as the other mediterranean orchids, loses stems and leaves after seeds dispersion (G.I.R.O.S., 2016), the translocation of metal pollutants to the epigeal portion could be an active process carried out with the aim of detoxifying the organism across the vegetative season, and storing heavy metals in the perennial part of the plant, the hypogeal one. Soil fungi are known to establish symbiotic relationships with the roots of several plant species. The ecto- and endo- mycorrhizal symbiosis plays a key role in facilitating nutrients uptake, but it can also protect from different kinds of stresses, including heavy metal stress. The ecological role of soil fungi in the mitigation of heavy metal stress in plants has been largely investigated and characterized with ultrastructural and taxonomical approaches (Bradley et al., 1982; Gonzales- Chavez et al., 2002; González-Guerrero et al., 2008; Joner et al., 2000; Martino et al., 2000; Perotto et al., 2002; Turnau and Dexheimer, 1995) showing how soil fungi and mycorrhizal symbiosis can reduce the bioavailability of pollutants protecting plants by their negative effects (Cordero and Casadevall, 2017; Gadd and De Rome, 1988; Turnau and Dexheimer, 1995; Zheng et al., 2009). Because of those properties, the root endophytes of the orchids growing in the polluted and control areas have been identified by mean of DNA barcoding. Sequence analysis allowed the identification of the genus *Ilyonectria* in both contaminated and control individuals and of an unidentified fungus isolated from *Rosmarinus officinalis* roots (Girlanda et al., 2002), associated to roots of orchids on contaminated site and of an endophyte belonging to the Ascomycota phylum on both sites. Due to the absence, in the present study, of any kind of germination or in-vitro experiment involving the isolated fungi and the studied orchid, we cannot verify the presence of mycorrhization in the studied case. Still the genus *Ilyonectria* has been described as symbiont in different orchids' genera like *Paphiopedilum* Pfitzer (1887) (Han et al., 2016), *Pterostylis* R. Br., (1810) (Obase and Matsuda, 2014), *Microtis* R.Br., (1810) (Frericks, 2016), *Calanthe* R. Br. (1821) (Park et al., 2018) and also in *Epipactis* Haller (Obase and Matsuda, 2014). Nevertheless, Shefferson et al. (2008), found different fungal symbionts (*Trichophaea woolhopeia*, *Geopora cooperi*, *Chalara dualis*) associated with different *Epipactis* sp. populations that occurred on heavy metal polluted soils. Hence, *Epipactis taxa* might be generalist in their fungal associates, but the extent to which fungal associates can tolerate soil heavy metal contamination is poorly understood.

5. Conclusion

This study revealed that *E. helleborine* subsp. *tremolsii* can grow in the presence of high concentrations of heavy metals and metalloids in the substratum, and that they can also accumulate and translocate those elements in their organs. The same fungal *taxa* were found in the roots of plants grown on polluted and unpolluted soils. Control and contaminated populations differ in size and photosynthetic rates, suggesting heavy metal polluted soils inhibit plant growth. Despite the tolerance to such extreme conditions and the ability to accumulate and translocate pollutant of the soil, this species should not be considered a *taxon* for phytoremediation and phytostabilization plans, due to its low biomass productivity. Nevertheless, the mechanisms of tolerance should be better studied in this species, to understand if metal-tolerance of the studied population is due to a specific fungal association, rather than to specific features of members of *Epipactis*.

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Chapter 4: Epipactis tremolsii seed diversity in two close but extremely different populations: just a case of intraspecific variability?

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ABSTRACT

Analysis of the seed morphology is a widely used approach in ecological and taxonomic studies. In this context, intraspecific variability with respect to seed morphology (size, weight, and density) was assessed in two close *Epipactis tremolsii* Pau. populations sharing the same ecological conditions, except for the soil pollution distinguishing one of them. Larger and heavier seeds were found in plants growing on the heavy metal polluted site, while no differences in seed density were detected between seeds produced by plants growing on the contaminated and the control site. Moreover, seed coats and embryos varying together in their dimensions were described in the control population, while coats varying in their size independently from embryos were described in plants growing on the polluted site. Seeds from the two studied populations significantly differed in several parameters suggesting that intraspecific seed variability occurred in the case study.

Keywords: *Epipactis; intraspecific variability; seed ecology; seed morphometry; heavy metals.*

1. Introduction

Though studies on orchid reproduction usually focus on plant-pollinator interactions and on the role of flowers in reproductive fitness [1–5], reproduction in Orchidaceae is also peculiar because of the unique structure of their seeds. The development of seeds in orchids requires a very low energetic investment, as they lack endosperm (or present a very limited amount of it) and are only constituted of a small embryo contained in a membranous coat. Consequently, seeds can be produced in large amounts, reaching thousands of units in each fruit [6]. As the fruit dehisces, thousands of seeds are dispersed in the environment, mainly transported by wind, until they reach a substrate suitable for their germination. The above-mentioned, extremely simplified seed structure, together with its small size (the average length ranging from 500 to 900 µm, the smallest measuring around 100 µm) [7], could be the reason why orchid seed traits and their relationship with the environment have been overlooked in different species. To our knowledge, studies focusing on orchid seed structure and diversity generally address taxonomic questions (which comparative seed morphology could help solve) [8,9], describe the role of seeds in orchid reproduction and fungal symbiosis [10], and assess the variability of seeds in relation to growth conditions and growth habits [9,11]. Results show how seeds may vary considerably in some genera of Orchidaceae, in relation to both growth habits and taxonomy [8–11]. Nevertheless, the key role of seeds in orchid reproduction and species dispersal suggests the urgency to deepen research on their inter- and intraspecific variability, their functional aspects, and their variability in relation to ecological growth conditions. In this sense, seed morphometry often represents a cheap and simple approach [12] to collect a great amount of data that can be used to address taxonomic, functional, and ecological issues [9–11]. Seed traits have been used in both ecological and taxonomic studies, especially in the genus *Epipactis* Zinn [13–16]. Species boundaries in *Epipactis* are difficult to define [13,17] and its taxonomy is controversial at the point that the taxonomic treatment of some taxonomic units may vary depending on different authors [17]. The present study investigates the intraspecific seed size variability in *E. tremolsii* Pau. *Epipactis* is a peculiar orchid genus because, although its ecological optimum is represented by shady forests and mature soils [18], species belonging to the genus *Epipactis* can also colonize harsh ecological contexts such as heavily anthropized habitats, thus exhibiting phenotypic plasticity [15,16,19–23]. Our study principally focuses on a wild population of *E. tremolsii*, which has colonized an extreme and restricted ecological context, a tailing dump, characterized by a strong heavy metal (HM) soil pollution (PPS hereafter, which stands for "plants growing on the HM polluted site") [19]. PPS present general morphological traits in common with the surrounding populations, except for the seed features, which are not easily observable in the field. Therefore, our objective was to characterize seed morphology and weight, considering their ecological and functional importance. Preliminary microscopical observations, together with the scientific literature on the topic, suggested that seeds may differ among the two studied populations depending on the peculiar growth conditions characterizing one of them (PPS) [16,24–27]. To characterize seeds produced by PPS, a second population of the same species geographically close, and sharing the same climatic and ecological conditions (except for the soil pollution), was chosen for comparison (PCS hereafter, which stands for "plants growing on the control site"). Given this framework, the present study aims to investigate the intraspecific variability in seed size and weight between two close populations of *E. tremolsii*. Specifically, the aims of this research are; *i*) to determine the number of seeds fitting in a fixed volume unit as a proxy for seed size, *ii*) to characterize seeds in terms of length, width and area, and *iii*) to determine differences in seed weight and density in relationship to the population of origin.

2. Results

Volumetric measurements pointed out a difference in seed size between the two studied populations (Figure 1a). In fact, a significantly different number of seeds (p-value ≤ 0.01 by t-test) fitted in the volume unit used in the measurements (0.05 mm^3): 240.4 ± 6.81 (mean ± SE) seeds from PPS, whereas 286.0 ± 6.88 seeds from PCS fitted in the same volume unit. The weight (± SE) of the seeds fitting in the 0.05 mm^3 volume unit was 1.28 ± 0.05 mg in PPS and 1.13 ± 0.03 mg in the PCS population. The mean single-seed weight was 5.32 ± 0.23 µg in PPS and 3.94 ± 0.11 µg in PCS (Figure 1b): heavier seeds were produced by PPS (p-value ≤ 0.01 by t-test). The average volume of the single seed consisted of 2.08 × 105 µm^3 and 1.75 × 105 µm^3 in PPS and PCS, respectively. The single-seed density remained relatively constant in the two populations, showing a mean value (± SE) of 2.56 × 10−5 ± 0.061 × 10−5 µg/µm^3 in PPS and 2.25 × 10−5 ± 0.11 × 10−5 µg/µm^3 in PCS. The measurements of coats and embryos (Table 1; Figure 2) pointed out that coat areas vary depending on the population from which seeds come from; conversely, embryos maintained a relatively constant size. Average (± SE) coat areas were of 0.24 ± 0.005 mm^2 and 0.19 ± 0.006 mm^2 in PPS and PCS, respectively, with strong statistical support (p-value ≤ 0.0001 by t-test; Figure 2a), whereas average values for embryo areas were 0.02 ± 0.001 mm^2 in PPS and 0.03 ± 0.001 mm^2 in PCS, whereby the latter parameter did not present statistical significance, supporting the observed size differences (p-value > 0.05 by t-test; Figure 2b). In addition, a correlation matrix per population was drawn considering all measured parameters. Measurements taken on seeds from PPS (Figure 3a) were significantly correlated only when they belonged to the same structure (coat embryo), (embryo width *vs* embryo area, R = 0.89, p-value < 0.01; coat length *vs* coat area, R = 0.71, p-value < 0.01; embryo length *vs* embryo area, R = 0.65, p-value < 0.01; coat width *vs* coat area, R = 0.63, p-value < 0.01). On the contrary, several coat and embryo measurements from PCS (Figure 3b) were also correlated to each other (coat length *vs* embryo length, R = 0.82, *p*-value < 0.01; coat width *vs* embryo width R = 0.63, *p*-value <0.01; coat length *vs* embryo area, R = 0.68, *p*-value <0.01; coat area *vs* embryo area, R = 0.57, *p*-value < 0.01).

Table 1: Measurements taken on seeds sampled from PPS and PCS (PPS indicating plants growing on the polluted site; PCS indicating plants growing on the control site). Values are reported as mean ± SE mm or mm^2 for linear (lengths and widths) or bidimensional (areas) measurements respectively. Statistical significance of differences is reported by asterisks as follows: * = *p*-value < 0.05; ** = *p*-value < 0.01; *** = *p*-value < 0.001; **** = *p*-value < 0.0001; ns = non-significant.

Measurements	PPS	PCS	Test	Results
Coat length	1.13 ± 0.02	0.94 ± 0.02	Mann–Whitney U test	W = 8846; $p = 1.06 \times 10^{-5}$; ****
Embryo length	0.24 ± 0.002	0.23 ± 0.004	Mann–Whitney U test	W= 6281.5; $p = 0.13$; ns
Coat width	0.27 ± 0.004	0.25 ± 0.004	Mann–Whitney U test	W = 7204.5; $p = 3.61 \times 10^{-4}$; **
Embryo width	0.13 ± 0.002	0.14 ± 0.003	Mann–Whitney U test	W = 3904.5; $p = 1.21 \times 10^{-4}$; ***
Coat area	0.24 ± 0.005	0.19 ± 0.006	t-test	t = 6.07; $p = 5.79 \times 10^{-4}$; ****
Embryo area	0.02 ± 0.001	0.03 ± 0.001	t-test	t = -1.83; $p = 0.07$; ns

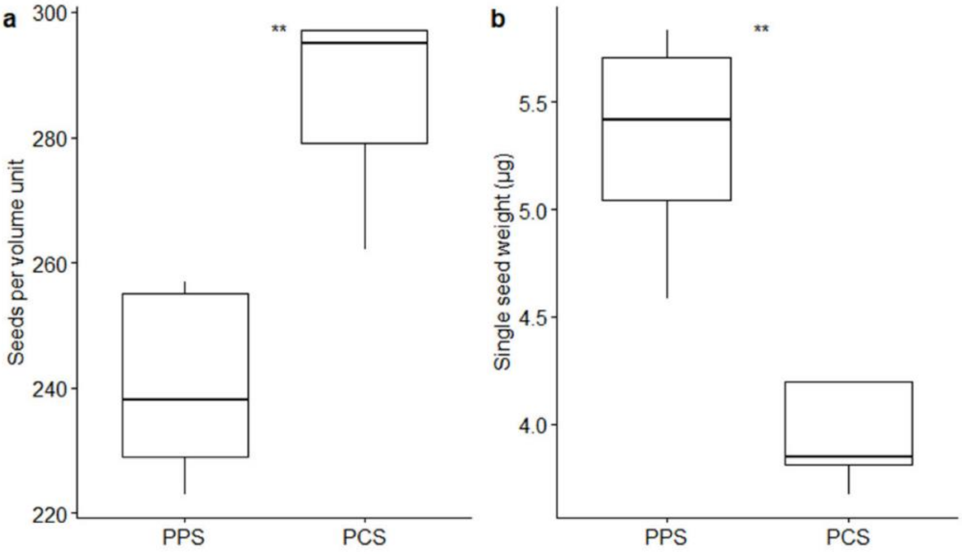

Fig. 1: Boxplots describing differences between the number of seeds fitting in the fixed volume of 0.05 mm^3 (a) and between the single-seed weight (b) in relation to the two different populations (PPS indicating plants growing on the polluted site; PCS indicating plants growing on the control site). Statistical significance of differences by t-test is reported by asterisks as follows: * = *p*-value < 0.05; ** = *p*-value < 0.01; *** = *p*-value < 0.001; **** = *p*-value < 0.0001; ns = non-significant.

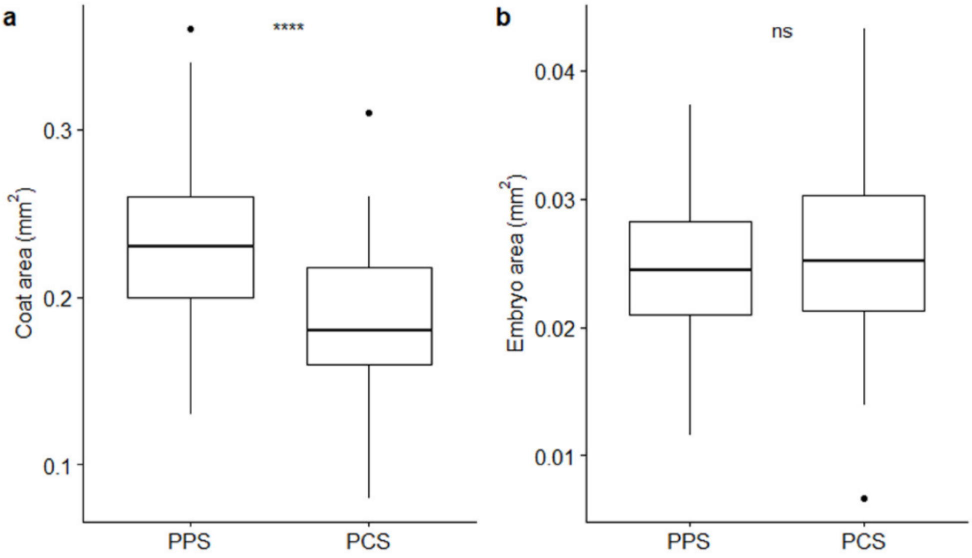

Fig. 2: Boxplots describing differences between coat (a) and embryo (b) areas in relation to the two different populations (PPS indicating plants growing on the polluted site; PCS indicating plants growing on the control site). Statistical significance of differences (t-test) is reported by asterisks as follows: * = *p*-value < 0.05; ** = *p*-value < 0.01; *** = *p*-value < 0.001; **** = *p*-value < 0.0001; ns = non-significant.

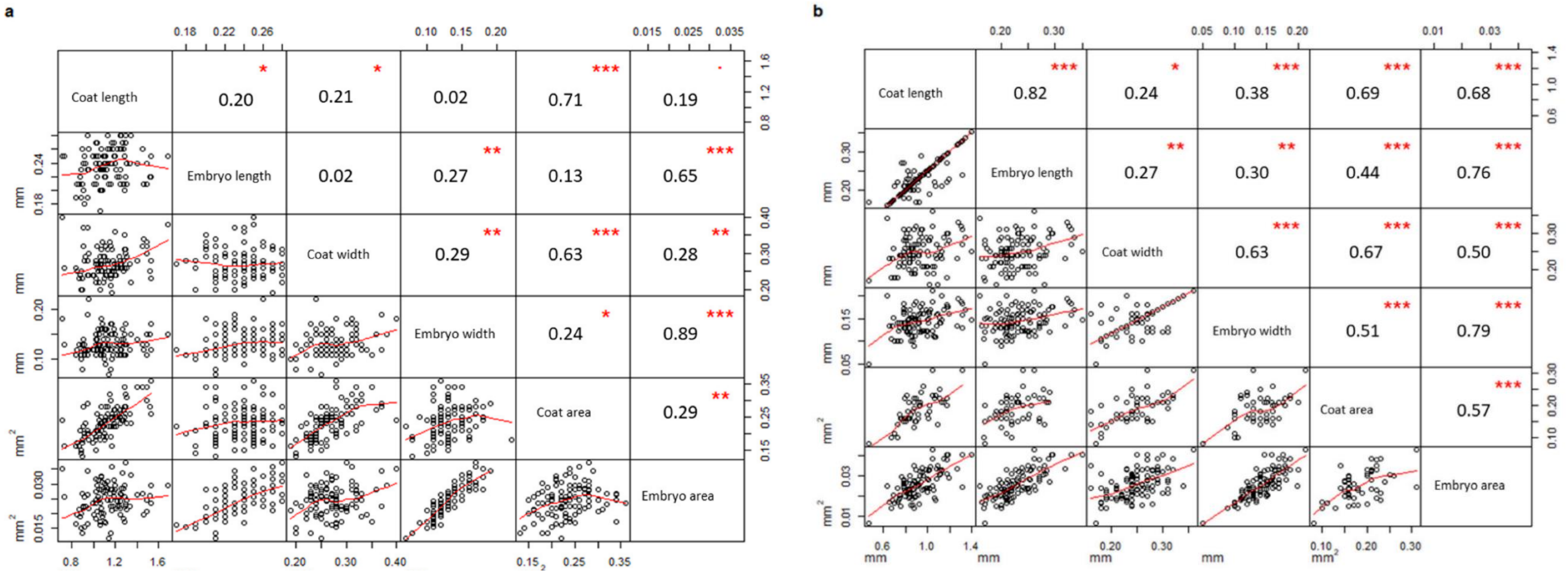

Fig. 3: Correlation matrices describing the correlation between variables measured in PPS (a) and PCS (b) respectively (PPS indicating plants growing on the polluted site; PCS indicating plants growing on the control site). The diagonal shows the variable names, the lower half of the panel reports scatterplots between pairs of variables; the higher portion of the panel shows correlation values and their significance levels reported as asterisks. The significance of the correlations is reported by asterisks as follows: * = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.001; . = p-value < 0.1; (not significant correlations are not marked).

3. Discussion

In the present study, preliminary volumetric analyses were carried out on seeds sampled from our target populations, highlighting larger seeds in PPS. A more detailed analysis focused on seed size and weight and on the relative size of embryo and coat structures pointed out that PPS produced heavier seeds with larger coats, whereas embryo size and seed density remained relatively constant in the two populations. Correlation matrices pointed out that coat and embryo sizes covaried in PCS, while coats and embryos varied independently in PPS. More precisely, embryos in PPS maintained a constant size, unlike coats, which were larger compared to PCS. Species belonging to the genus *Epipactis* can grow in a variety of natural habitats, preferring shady and humid ones, nevertheless, they can often be found settling in habitats with a strong anthropogenic disturbance, such as parks, city gardens, and areas interested by past mining activities [15,16,19–23]. Therefore, HM pollution of the growth substrate does not appear as a limiting factor for the distribution of this *taxon* in new ecological niches as a pioneer. Seed morphometry is a widely used approach to taxonomic issues and allowed us to detect several significant differences between the two studied populations. Results obtained in the present study described intraspecific variability (regarding the analysed seed traits), depending on the population of origin of the sampled *E. tremolsii* plants. Larger coats and embryos were found in populations inhabiting habitats with anthropogenic pressure in Rewicz et al. [16], and the authors proposed that phenotypic plasticity may have a key role in *Epipactis*, which tends to successfully colonize a wide variety of habitats. Miura et al. [10] reported the role of orchid seed coat in protecting the embryo against pathogens and non-symbiotic fungi and in harbouring symbiotic fungi so that embryo infection, required for its germination, could happen in a proper way. On the other hand, the role of soil fungi in the mitigation of HM-related stress in plants has been largely investigated [28–34] demonstrating how soil fungi and mycorrhizal symbiosis could reduce pollutant bioavailability and protect plants against the detrimental effect of inorganic pollutants [34–36]. HM phytotoxicity could be extremely high in the first stages of the life cycle (*i.e.*, seed germination and protocorm formation) [37–41], given the lack of physical, physiological, and ecological mechanisms adopted by plants to prevent damage caused by soil pollutants. A potential role of the here described larger seed coats produced in PPS could be shielding embryos in their early development stages from HM pollution by harbouring fungi able to reduce pollutant bioavailability to the embryo. Nevertheless, to assess whether seed coats could accomplish an adaptive ecological role protecting embryos from soil pollution, further microstructural investigations are needed. In this sense, it will be crucial to investigate the possibility of a differential pollutants' location between seeds coats and embryos and, contextually, the role of the coat in shielding embryos from soil pollutants. The taxonomy in *Epipactis* is still controversial, and the taxonomic treatment of entities belonging to this genus results is often difficult [17]. Nevertheless, seed size diversity alone does not allow to propose a taxonomic split nor the identification of an *E. tremolsii* ecotype corresponding to PPS. To address this issue, further studies are needed to evaluate whether observed seed intraspecific variability is a common feature of the *Epipactis* species, or it is an indication that PPS could be identified as a different taxonomic unit. In this sense, it will be crucial to extend this approach based on seed morphology studies to more *Epipactis* populations in an extensive taxonomic investigation on the genus in Sardinia. If seed variability observed in the present study should remain confined to the extreme ecological context in which PPS grew, the peculiar *E. tremolsii* population settling on the "Barraxiutta" mining tailings dump would deserve to be preserved as a genetic diversity reservoir (as reported in other research on small plant populations worldwide [42–44]), and as an example of adaptive strategies put in place by orchids to cope with extreme HM pollution

4. Materials and Methods

4.1. Species Description

In Sardinia, the genus *Epipactis* is represented by six entities among species and subspecies [45]. We refer here to [46], which considers the study species as *E. tremolsii*. *E. tremolsii* is a perennial (rhizomatous) herbaceous plant, producing 1 to 6 aerial stems up to 60 cm tall [18]. The inflorescence can host up to 50 opening, cross-pollinating flowers per plant. The above-described features may vary considerably in terms of size and general appearance due to the species' tolerance to different ecological conditions [18]. Mid-shady to shady contexts on moist, deep substrates represents typical habitats of the species that frequently occur on dense woodlands edges and clearings up to 900 m a.s.l. [18].

4.2. Data Collection

The present study was carried out in the municipality of Domusnovas (South-West Sardinia, Italy) that hosts the two studied populations. PPS were located on a strongly HM-polluted mining tailings dump (55.98 ± 7.44 mg g^{-1} of Fe; 13.10 ± 2.71 mg g^{-1} of Zn; 5.21 ± 0.69 mg g^{-1} of Pb, data reported as mean values ± standard deviation in mg g^{-1} [19], Table S1) called "Barraxiutta", which originated during the intense mining activity carried out in the area from 1871 to 1971 aiming to extract Pb and Zn [47]. Soil pollution characterizing the tailing dump resulted in a pollutant content in plants' epigeal organs of 15.36 ± 4.83 mg g^{-1} of Fe, 8.52 ± 3.62 mg g^{-1} of Zn, and 1.66 ± 1.43 mg g^{-1} of Pb (data reported as mean values ± standard deviation in mg g^{-1} [19], Table S2). Conversely, PCS grew in a forested site, geographically closest to the contaminated one (c. 1.00 km as the crow flies) and was characterized by pristine conditions since no mining activity that could have generated soil pollution was ever carried out there nor in its vicinity. Moreover, the control site was chosen on a different hydrographic basin so that pollutants' washout from the mining area could be excluded. In addition, the condition of high naturalness characterizing the control site, together with the absence of evident signals of phytotoxicity affecting the other plants growing in this site, were considered as indicative of the absence of HM soil contamination. A preliminary survey was conducted to choose the control population, which was selected so that it was certainly ascribable to the same taxon inhabiting the contaminated site (Figure S1). The control and contaminated population were located a small distance apart (the control population was the *E. tremolsii* population that settled closer to the contaminated one) and shared the same ecological and climatic conditions, a shady Mediterranean *Quercus ilex* L. forest presenting mature and deep soils. Despite the fact that the tailing dump presented a scarce vegetal coverage due the soil pollution, PCS grew in a forest clearing, so that light conditions were also comparable in the two studied populations. Seed-containing fruits were collected in the early summer (July) 2020. One mature fruit per individual, presenting early signs of dehiscence, was selected, and carefully removed from the inflorescence of twenty individuals per studied population that were randomly selected. Each collected fruit was preserved in separate 50 mL-Falcon tubes and stored under controlled laboratory conditions until performing the analyses. Seed measurements were carried out separately on mixtures of seeds coming from different fruits. At first, to broadly assess differences in seed volume depending on the population of origin, volumetric measurements were carried out on the collected seeds of both populations. The number of seeds that fitted in a fixed volume of 0.05 mm^3 (a laboratory micro spoon) was counted using a stereomicroscope. The count was repeated five times, each time using different seeds to obtain results in five replicates per population. Single-seed volumes were obtained by dividing the volume of 0.05 mm^3 by the amount of seeds fitting in it. The single-seed weight was obtained by calculating (in 5 replicates, each one consisting of 0.05 mm^3 × 10 volumes of seeds) the weight of 0.05 mm^3 of seeds weighed using the Gibertini Europe 60 (Italy) analytical scale, and then dividing the obtained value by the average amount of seeds fitting in the same volume, as previously calculated. Single-seed density was consequently obtained by dividing the average single-seed weight by the average single-seed volume, as previously determined for both populations. Several linear and bidimensional seed traits were measured: Length (corresponding to the major axis), width (corresponding to the minor axis), and area (automatically calculated by the measurement software on the basis of the manually selected perimeter of the structure) of both coats and embryos (Figure 4). These measurements allowed us to assess how the two structures (coat and embryo) varied with respect to each other. These measurements were carried using a stereomicroscope connected to a monitor by mean of the HD cam TiEsseLab TrueChrome HD IIS (Tiesselab, Italy) and using the software TiEsseLab IS CAPTURE Rel. 3.6.7 (Tiesselab, Italy). A total of 100 seeds from each population were used to carry out these measurements. All measurements were carried out during the same day, to prevent dehydration or hydration of samples from affecting measurements.

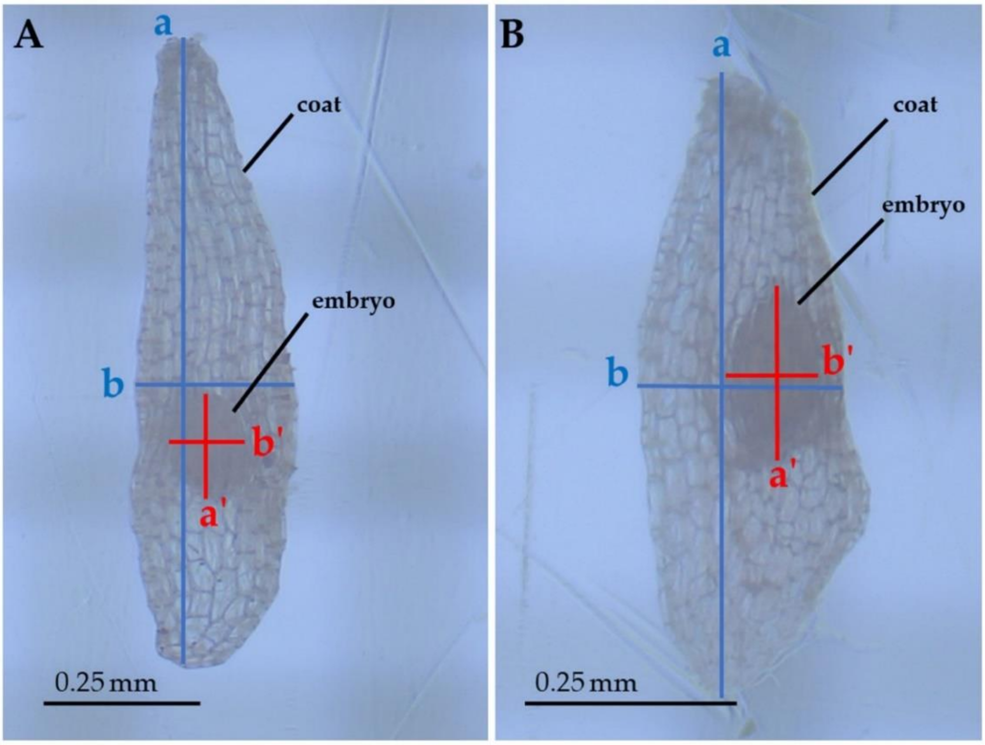

Fig. 4: Seeds of *E. tremolsii* collected from PPS and PCS (A and B, respectively), magnified ≈ 70 times. The axes show the measurements carried out on seeds: a, coat length; b, coat width; a’, embryo length; b’, embryo width (areas were automatically calculated by the measurement software on the basis of the manually selected perimeter of the structure).

4.3. Data Analysis

Prior to performing any statistical analysis, we checked whether data resulting from seed measurements were normally distributed by using the Shapiro–Wilk's test (data presenting Shapiro–Wilk's test *p*-value > 0.05 or <0.05 were considered normally distributed or not-normally distributed, respectively), combined with a visual inspection of data distribution in case of small-sized samples (Q-Q plot). Once proved the normal or not-normal distribution of data, parametric t-test, and non-parametric Mann–Whitney U test were used, respectively, to assess the significance of mean differences between measurements taken on seeds coming from the two studied populations.

Correlation matrices were used to better understand the relationship between the coat and embryo sizes (length, width, and areas) in relation to the two populations. Descriptive statistics were obtained using the 1.2.1335 base version of the R-Studio software [48]. The same version of the software was used to assess data distribution and test the statistical significance of the difference between paired datasets (base version), to draw plots ("ggpubr" package [49]) and to perform and plot the correlation matrix of data ("PerformanceAnalytics" package [50]), later modified concerning the font size, to improve its readability.

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Supplementary material

Fig. S1: Plants belonging to the two studied populations.

Table S1: Heavy metal concentration levels in the tailing dump. Data are reported as mean values (mg g^{-1}) ± standard deviation.

Element	**Concentration**
Fe	55.98 ± 7.44
Zn	13.10 ± 2.71
Pb	5.21 ± 0.69
Mn	1.24 ± 0.06
Cu	0.79 ± 0.08

Table S2: Heavy metal concentration levels in stem, leaves and root. Data are reported as mean values (mg g^{-1}) ± standard deviation.

Element	**Stem + Leaves**	**Root**
Fe	15.36 ± 4.83	43.08 ± 23.19
Zn	8.52 ± 3.62	23.28 ± 14.15
Pb	1.66 ± 1.43	4.10 ± 2.50
Cu	0.26 ± 0.84	0.09 ± 0.06

Chapter 5: Seed bank conservation and early stages of seed development in metallicolous and non-metallicolous orchid populations: results of a field pilot experiment

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ABSTRACT

The reclamation of polluted abandoned mines is becoming increasingly urgent due the threats their represent to environmental and human health. This could be the case of the heavy metal polluted tailing dump of Barraxiutta (Domusnovas, South-Western Sardinia, Italy), which hosts a metallicolous population of *Epipactis tremolsii* (Orchidaceae). This pilot study evaluated the suitability of seed packet-based field experiments to explore different seed-based conservation options for this population. Four different sowing treatments (full-crossed experimental design) were set up in the tailing dump and a nearby unpolluted site (control site). The field phase of the experiment lasted 12 months; in this experiment, the experimental seed bank preservation and incipient seed development were observed and tested by parametric or non-parametric test corrected for multiple comparisons and by ANOVA. The results highlighted the following. *i*) The experimental seed bank does not deteriorate during the pilot study. *ii*) An incipient and differential seed development was observed. *iii*) A plant–soil fungi interaction at the seed level was observed. The suitability of the seed packet technique, even in extreme environments (polluted mine wastes), was proven in the present study. The use of this field technique will be a valuable tool to evaluate the best *E. tremolsii* metallicolous population conservation options via translocation and *quasi in-situ* approaches.

Keywords: *Epipactis tremolsii; Mining areas; Heavy metals; Translocation; Seed packet.*

Abbreviations: *Ctl*, control population; *Ctl in Ctl*, control seeds sowed in the control site; *Ctl in Ctm*, control seeds sowed in the tailing dump; *Ctm*, metallicolous population; *Ctm in Ctl*, metallicolous seeds sowed in the control site; *Ctm in Ctm*, metallicolous seeds sowed in the tailing dump.

Implications for practice

- The seed packet technique is a suitable approach to explore the seed bank conservation and incipient seed development of orchids in extreme environments (*i.e.* heavy metal polluted mining by-products).
- The one-year duration of the experiment is appropriate to test seed bank conservation in the tailing dump and describe initial, non-mycorrhizal seed developmental stages. To explore seed–soil fungi interactions in the study site a longer experiment than the one here described would be required.
- Incipient seed development dynamics are site-specific. Therefore, the management of the tailing dump should consider adequate conservation options for the population adapted to this metalliferous environment and future seed-based conservation approaches to the metallicolous *E. tremolsii* population should employ metallicolous seeds.

1. Introduction

Mining has been considered one of the principal sources of environmental pollution worldwide (Thornton 1996; Rieuwerts et al. 2009; Beane et al. 2016). Mines have a substantial impact on local and regional environments during the production phase; even after their closure and abandonment, mines may continue to degrade surrounding lands, water bodies and air (Mannu et al. 2020). The management of abandoned mines is challenging yet urgent and represents an unresolved environmental problem (Caboi et al. 1993; Thornton 1996; Damian et al. 2019). However, the reclamation of abandoned mines could endanger the conservation of peculiar and rare metallicolous flora settlements. Heavy metals (HMs hereafter) are known for their detrimental effects on the biosphere; however, some plants (metal-tolerant or metallophyte *taxa*) evolved to thrive despite the occurrence of HMs (Baker et al. 2010). The existing peculiar biota in natural or anthropogenic metalliferous areas should be preserved and studied to investigate those adaptation mechanisms that allowed them to live in such harsh contexts (Whiting et al. 2004; Baker et al. 2010). The Barraxiutta tailing dump (Sardinia, Italy) is contaminated by Pb, Zn and Fe (amongst others) and is formed by fine particles (De Agostini et al. 2020a; De Agostini et al. 2020b). The reclamation of this area is urgent and becoming increasingly imminent due to its features. Orchids can often be found growing in naturally metalliferous environments (van der Ent et al. 2015; Mikavica et al. 2020) as well as in disturbed, anthropogenically polluted habitats, including abandoned mines (Jurkiewicz et al. 2001; Esfeld et al. 2008; Shefferson et al. 2008). The Barraxiutta tailing dump is not an exception because it hosts a population of *Epipactis tremolsii* Pau (Orchidaceae), accounting for approximately 500 individuals. Previous studies described the orchids in the tailing dump bioaccumulating and translocating soil pollutants in their organs and presenting reduced size and photosynthetic efficiency. In addition, metallicolous orchids produce larger and heavier seeds if compared to seeds produced by non metallicolous individuals (De Agostini et al. 2020a; De Agostini et al. 2020b). Orchids in the tailing dump complete their biological cycle, reach the flowering stage and produce fruits and thousands of viable seeds per plant despite the extreme ecological growing context.

Given this framework, the following ecological issue is delineated as follows: the reclamation of the Barraxiutta tailing dump would inevitably result in the loss of the peculiar metallicolous population of *E. tremolsii*, its genetic heritage and its potential as an evolutionary model.

The suitability of the seed packet technique to explore different conservation options (based on translocation) in the extreme ecological framework of a polluted tailing dump was tested in this study for the first time. The validation of this technique in such an extreme germination environment would provide a valuable tool in the conservation of the metallicolous orchid population of Barraxiutta and similar conservation cases. Several conservation approaches failed due to the lack of knowledge regarding the species-specific and population-specific recruitment mechanisms and dynamics (Turner et al. 2017; Phillips et al. 2020). Experimental *E. tremolsii* seed bank preservation and early seed developmental stages were followed during a year in a seed packet experiment (full-crossed design) conducted in the field in a polluted tailing dump and an unpolluted control site to address the aims of the current study. We hypothesized that the seed packet technique would possibly be proven valid even in the extreme study area; moreover, the two studied populations would demonstrate different seed development dynamics given the extreme soil pollution featuring one of them.

2. Methods

2.1. Plant species

Epipactis tremolsii Pau (syn. *E. helleborine* subsp. *tremolsii* (Pau) E. Klein. for other authors) is a perennial rhizomatous orchid that frequently occurs in dense woodland edges and forest clearings up to 900 m a.s.l. The species thrives in mid-shady to shady contexts on mature soils. *E. tremolsii* plants produce 1 to 6 aerial stems up to 60 cm tall and inflorescences hosting up to 50 cross-pollinating flowers during the vegetative season (middle spring) (De Agostini et al. 2020b). The flowering season occurs in late spring in Sardinia (De Agostini et al. 2020b). The studied species is not subjected to any protection or evaluation by the IUCN in addition to CITES (Sardinia has no law that protects the floristic heritage).

2.2. Contaminated and control site

This study was conducted in the field. Seeds were sampled and sowed in the tailing dump *E. tremolsii* population (*Ctm* hereafter) and in a control *E. tremolsii* population unaffected by mining pollution (*Ctl* hereafter). The Barraxiutta mining dump (Domusnovas, Sardinia, Italy) covers c. 3000 m^2 and originates from the flotation process of rocks containing Galena and Sphalerite. Consequently, high contents of Pb (up to 5 $mg\ g^{-1}$), Zn (up to 13 $mg\ g^{-1}$) and Fe (up to 56 $mg\ g^{-1}$) were measured in the dump. The mining by-products in the dump are formed by fine particles and the plant coverage is almost absent in the area (De Agostini et al. 2020a; De Agostini et al. 2020b). *Ctl* is located near (around 1 Km as crow flies) to the Barraxiutta tailing dump and the growth conditions between the two populations are comparable, except for soil features. Both populations settle in forest clearings, but while the tailing dump has sandy soil, poor organic matter and is extremely polluted by HMs, the control site has mature soil rich in organic matter and is not contaminated by HMs. *Ctl* hosts c. 50 adult individuals, whilst *Ctm* has c. 500 adult individuals. An extensively detailed *Ctm* and *Ctl* description is presented in De Agostini et al. (2020a, 2020b).

2.3. Seed collection and seed packet construction

Seeds were collected from *Ctm* and *Ctl* on the same day (23 July 2020). Mature fruits were collected and individually preserved in 1.5 mL Eppendorf tubes. Ten plants per population were randomly selected and two fruits per plant were collected (20 fruits sampled in each population) to ensure that seed samples were representative of the seed diversity of the two populations. Seeds from the two populations were excerpted from the fruits, cleaned and carefully evaluated under the presence of well-developed embryos by microscopy in the laboratory. *Ctm* and *Ctl* seed vitality was comparable, revealing over 95% in both populations. Seed packets were prepared as described in Rasmussen & Whigham (1993) (Figure S1 for details), with only minor modifications. Squares of 5 cm × 5 cm 20 μm mesh size plankton net (SCUBLA S.r.l., Remanzacco, Italy) were framed in 24 mm × 36 mm glassless plastic slide mounts (GEPE Geimuplast GmbH, Farchant, Germany). A total of 60 packets were prepared and an identification code was reported on each one. A consistent and known (estimated) number of seeds was enclosed in each packet by using a laboratory micro spoon of 0.05 mm^3. The mean number (± standard error, SE) of *Ctm* and *Ctl* seeds in each packet was estimated through five repeated counts. Consequently, *Ctm* packets contained c. 240.40 ± 6.81 (mean ± SE) seeds, whilst *Ctl* packets contained c. 286.00 ± 6.88 (mean ± SE) seeds.

2.4. Experimental design

Ctm and *Ctl* seed packets were sown in a full-crossed experimental design, thus resulting in the following four sowing treatments: *Ctl in Ctl, Ctl in Ctm, Ctm in Ctm* and *Ctm in Ctl* (Figure 1). A total of 15 *Ctl* and 15 *Ctm* packets were buried in experimental parcels of 5 m × 5 m and horizontal position at c. 5 cm in depth in each sowing site. The packets were secured in groups of five to a camping peg by utilising a fish line. Seed development was monitored during a 12-month experiment. Packets were buried during July (2020) when *E. tremolsii* seed dispersion naturally occurs in the wild. Packet retrieval started in November 2020 and was divided into five retrieval campaigns (t1 to t5) distanced 45 days each other. A total of 12 packets were collected (3 packets per sowing treatment as replicates) during each retrieval campaign. Packet retrieval ended in May 2021.

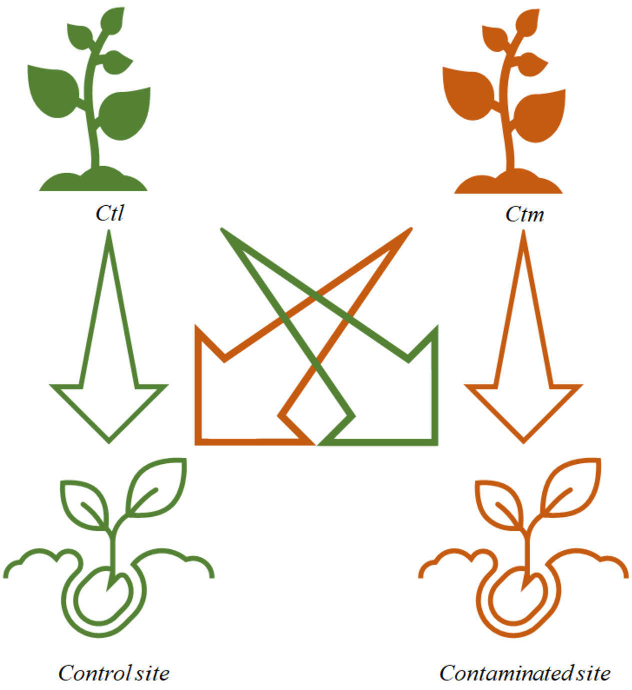

Figure 1: Experimental design scheme. *Ctl* and *Ctm* indicates the control and the metallicolous *E. tremolsii* population respectively.

2.5. Seed observation and measurements

Packets were transported to the laboratory under controlled conditions (dark and constant humidity) after each retrieval campaign and all observations were conducted within two days. Seed analysis was conducted by using a stereomicroscope connected to an HD cam (TiEsseLab TrueChrome HD IIS, Tiesselab, Italy) implemented by a measurement software (TiEsseLab IS CAPTURE Rel. 3.6.7, Tiesselab, Italy). The number of intact seeds at t1 in each sowing treatment was compared with that of t5 to detect any significant seed loss. Seeds were then assigned to a developmental stage ranging from S1 (unmodified seed) to S2 (swollen, non-mycorrhizal seedling) (Figure S2) according to the scheme reported in Těšitelová et al. (2012). A seed that showed early signs of development represented by a marked swelling was measured and photographed. Seeds were measured in the following parameters: coats and embryos width, height and area, as described in De Agostini et al.

(2020b). Any swollen seeds plus ten unmodified seeds per packet were measured in each retrieval campaign.

2.6. Statistical analysis

Variations in the number of seeds amongst the different retrieval campaigns were assessed by t-test after checking for its assumptions. Changes in seed morphological traits during the experiment were tested by ANOVA or Kruskal–Wallis ANOVA depending on whether ANOVA assumptions were satisfied. *Post-hoc* Tukey's or Wilcoxon signed-rank tests were performed depending on whether ANOVA or Kruskal–Wallis ANOVA was used. One-sample t-test or one-sample Mann–Whitney U test (depending on applicability criteria) was used to test S2 seeds against S1 seeds collected in the same retrieval campaign and sowing treatment (*p*-values were adjusted for multiple testing by Bonferroni correction). All statistical analyses were conducted using the 1.2.1335 version of the R-Studio software (R Foundation for Statistical Computing, 2018) implemented by 'ggpubr' package (Kassambara, 2020).

3. Results

3.1. Seed conservation during the experiment and morphometric parameters

Seed content in each packet after the retrieval ranged from 201 seeds to 359 seeds (SE = 4.56) and no difference was observed between t1 and t5 in any of the sowing treatments (Figure 2). Packet observation under the stereo microscope permitted to observe since t1, especially in packets retrieved from the control site, dense networks of fungal hyphae invading the packets (Figures 3 and 4). ANOVA on morphometric parameters showed that the only sowing treatment in which parameters varied amongst the retrieval campaigns was the non-mixed sowing treatment *Ctm in Ctm* (Figure 5). Overall, embryo width increased with time and coat width and area reached a peak at t3. Morphometric data and ANOVA results are extensively reported in Tables S1 and S2, respectively.

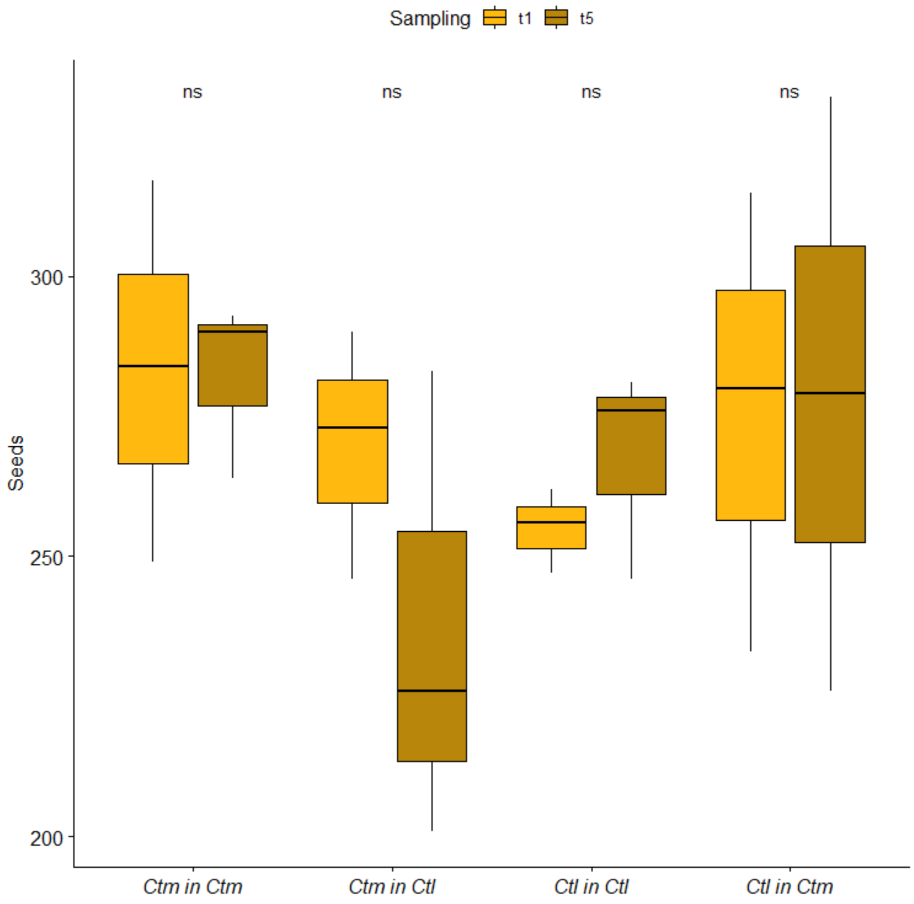

Figure 2: Number of seeds is reported on the y axis, sowing treatments are reported on the x axis (*Ctm in Ctm* indicates metallicolous seeds sowed in the tailing dump; *Ctm in Ctl* indicates metallicolous seeds sowed in the control site; *Ctl in Ctl* indicates control seeds sowed in the control site; *Ctl in Ctm* indicates control seeds sowed in the tailing dump). First and last retrieval campaigns (t1 and t5 respectively) are indicated by different colours. t-test results are reported above each series of boxplots as follow: ns = non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Level alfa at 0.05.

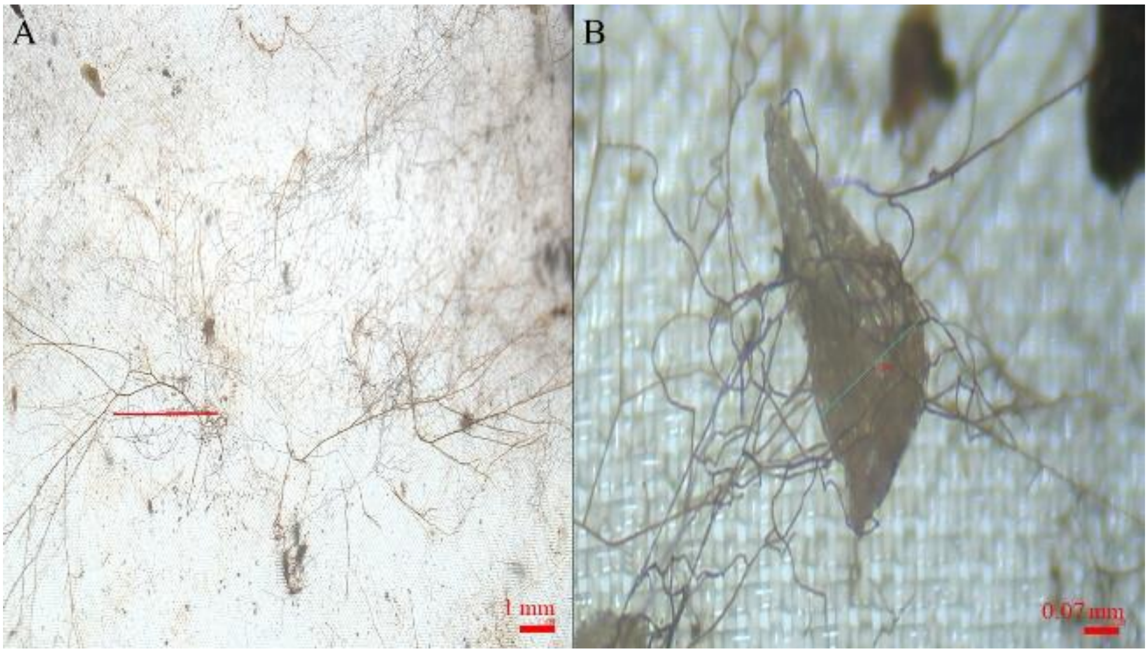

Figure 3: Stereomicroscope images of fungal hyphae invading the packets (panel A), and enveloping seeds (panel B). In panel B a developing (S2) control seed retrieved from the control site.

Figure 4: Fungal hyphae (400X magnified in panel A, 100X magnified in panel B). In panel B a fungal hypha enters a *E. tremolsii* seed through its operculum.

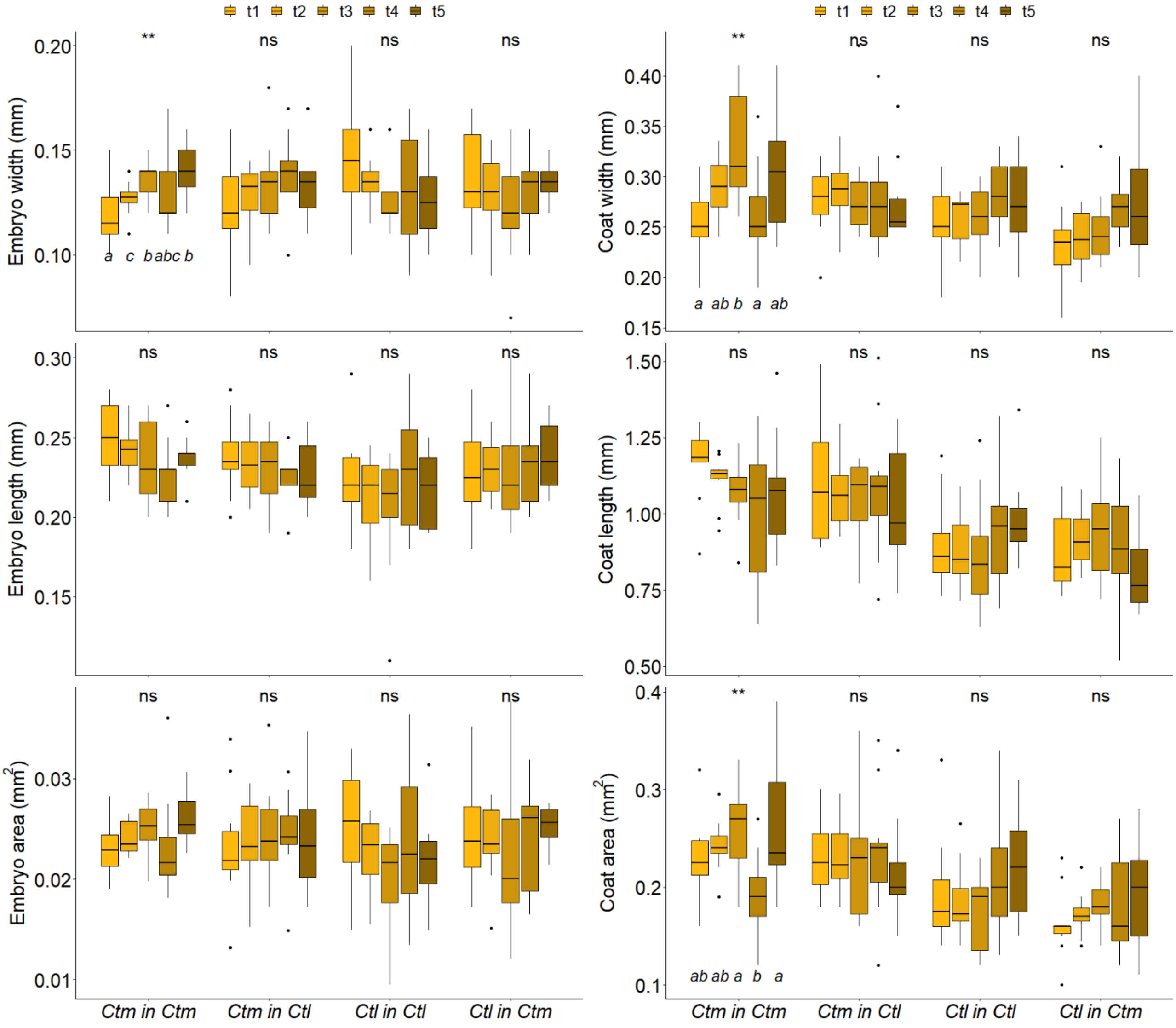

Figure 5: Morphometric parameters are reported on the y axis, sowing treatments are reported on the x axis (*Ctm in Ctm* indicates metallicolous seeds sowed in the tailing dump; *Ctm in Ctl* indicates metallicolous seeds sowed in the control site; *Ctl in Ctl* indicates control seeds sowed in the control site; *Ctl in Ctm* indicates control seeds sowed in the tailing dump). Retrieval campaigns from t1 to t5 are indicated by different colours. ANOVA or Kruskall-Wallis test results are reported above each series of boxplots as follow: ns = non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Post-hoc results are reported by compact letter display under each boxplot. Level alfa at 0.05.

3.2. Developing embryos

Eleven S2 seeds were measured. S2 seeds (Figure S3) were observed at t3 and t4 (10 in non-mixed sowing treatment (*Ctl in Ctl* and *Ctm in Ctm*) and one in a mixed sowing treatment (*Ctl in Ctm*). S2 seeds were observed in 40% of packets in the *Ctl in Ctl* sowing treatment and in 6.67% of packets in *Ctm in Ctm* and *Ctl in Ctm*. No packets in the *Ctm in Ctl* sowing treatment contained S2 seeds. One-sample test results are reported in Table S3.

4. Discussion

Translocation could represent a way to maintain endangered populations (Phillips et al. 2020; Bell 2021, Bell et al. 2021), such as the metallicolous population of *E. tremolsii*. The imminent reclamation (2020–2024 according to the Sardinian Industrial Plan guidelines) of the abandoned mine would endanger the conservation of the metallicolous orchid population. Translocations or the *quasi in-situ* conservation *sensu* Volis & Blecher (2010) are conservation approaches based on the controlled placement of plant material into a (semi-) natural area to preserve the genetic diversity of peculiar, endangered populations (Bell 2020; Bell et al. 2021). Similar approaches to plant conservation are increasing worldwide (Fenu et al. 2019; Fenu et al. 2020; Abeli et al. 2021). However, any orchid conservation plan based on translocations should consider the importance of specific germination niches to realise successful interventions (Bell 2020; Bell 2021). Germination of orchid seeds requires their symbiotic association with soil fungi, and the complex of abiotic and biotic factors necessary for this to happen it is called a germination niche (Rasmussen et al. 2015; Whigham et al. 2021). Consequently, the conservation and management of endangered orchid populations via translocation is a challenging task in which knowledge of the species-specific and population-specific germination and recruitment mechanisms is crucial (Rasmussen et al. 2015; Turner et al. 2017; Phillips et al. 2020). This study reports the results of a pilot experiment conducted to evaluate the feasibility of the seed packet technique to explore population dynamics and translocation options for the metallicolous *E. tremolsii* population.

A long-lived seed bank and delayed germination are typical in forest orchids, such as *Epipactis* (Rasmussen & Pedersen 2012; Tešitelová et al. 2012; Jacquemyn et al. 2015). This feature was also observed in the present study. The seed bank in the two studied *E. tremolsii* populations showed no significant seed loss or degradation during the experiment in any of the sowing treatments. The seed packet-based experiment was performed for the first time in the present study in a polluted tailing dump and provided a unique look at the resistance of *E. tremolsii* seeds to extreme abiotic stressors. *E. tremolsii* seeds from metallicolous and non-metallicolous populations surrounding the tailing dump remained unaltered despite MHs, low pH levels and absence of vegetal coverage nor litter protection on the seed bank. This element is important for conservation purposes because it suggests the implementation of metallicolous *E. tremolsii* seed bank by surrounding non-metallicolous *E. tremolsii* populations. Moreover, future field experiments exploring seed translocation options should not focus on the issue of seed loss in the extreme environments of tailing dumps. Seed morphometry may be affected by the availability of nutrients, light and peculiar growth conditions affecting mother plants (Rasmussen et al. 2015; De Agostini et al. 2020b) as well as by the position and number of capsules on the inflorescence and differences in flower pollination (Buyun et al. 2015; Medrano et al. 2000). In the specific study case, seeds produced in the tailing dump are featured by larger coats and fixed-sized embryos compared with those produced by non-metallicolous populations (De Agostini et al. 2020b). On the contrary, the significant variations in seed morphometry observed during this experiment should be attributed to the sowing treatment because seed material was sampled to avoid the aforementioned factors that confound experimental results.

Seed coats were significantly larger at t3 than at t1 and t4. Considering potential evapotranspiration data in the study area (intended as the ratio between precipitation and temperatures, Table S4, data obtained from the database of the climatic monitoring authority of Sardinia), t3 was found to be the retrieval campaign featured by the moistest soil. Moist soils at t3 could have caused larger coats (passive imbibition) and swelling embryos (width) because soil moisture positively affects seed development in orchids (Rasmussen et al. 2015). Coherently, starting from t3, seeds began to develop from S1 to S2, demonstrating incipient and non-mycorrhizal developmental stage. Despite orchid realized niche is mostly determined by symbiotic fungi occurrence, the passage to a S2 non-mycorrhizal seed developmental stage is largely triggered by the abiotic features of the substratum (Tešitelová et al. 2012). Interestingly and coherently with the initial hypothesis, incipient seed development occurred mainly in non-mixed sowing treatment, suggesting site-specific seed development dynamics determined by the peculiar abiotic features of the substrate. The seed packet technique is suitable to explore seed development and germination in the study area because any variation in seed morphometry was promptly observed and registered.

Adequate germination niches amongst large and mature orchid populations may no longer exist or may be severely compromised (Rasmussen et al. 2015); thus, large population size should not be considered as a guarantee for good resilience after a disturbance in orchids. Therefore, the reclamation of the Barraxiutta tailing dump could have catastrophic outcomes considering the conservation of the metallicolous orchid population. Metallicolous *E. tremolsii* may struggle to settle outside of the tailing dump and *vice-versa* non-metallicolous seeds could struggle to settle on the tailing dump due to the presence of extreme abiotic stressors. Therefore, solid knowledge on the germination dynamics of the studied population should inform any future translocation plan.

Present work proved the viability of the seed packet technique in the investigated extreme environment and highlighted differential and site-specific seed development dynamics between the polluted and the control populations. Future studies directed to explore seed germination dynamics as well as translocation and *quasi in-situ* conservation options in the peculiar metallicolous orchid population are necessary. Longer duration of germination experiments as well as wider coverage of the study area would be necessary to better understand recruiting mechanisms and germination dynamics of the *E. tremolsii* metallicolous population. Thus, an adequate and crucial knowledge of the ecological needs of the tailing dump population will be obtained to prevent any thoughtless management intervention interesting the peculiar *E. tremolsii* metallicolous population.

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Supplementary material

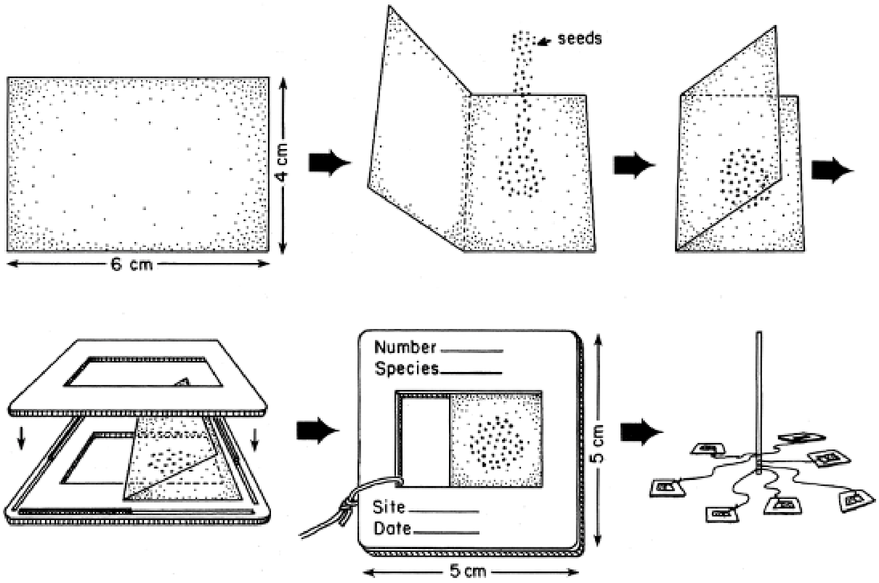

Figure S1: Packets used in the present study were built as reported in Rasmussen and Whigham (1993) with minor modifications.

Figure S2: Developmental stages of *E. helleborine* (a closely related species of E. *tremolsii*) seedlings, as reported in Těšitelová et al. (2012). From the stage of unmodified seed to that of branched seedling. *"Stage 1: ungerminated seed; stage 2: swollen, nonmycorrhizal seedling; stage 3: small, oval-shaped mycorrhizal seedling; stage 4: pear-shaped seedling longer than 0.5 mm, a protocorm; stage 5: seedling with leaf primordium longer than 1 mm; and stage 6: branched seedling"*.

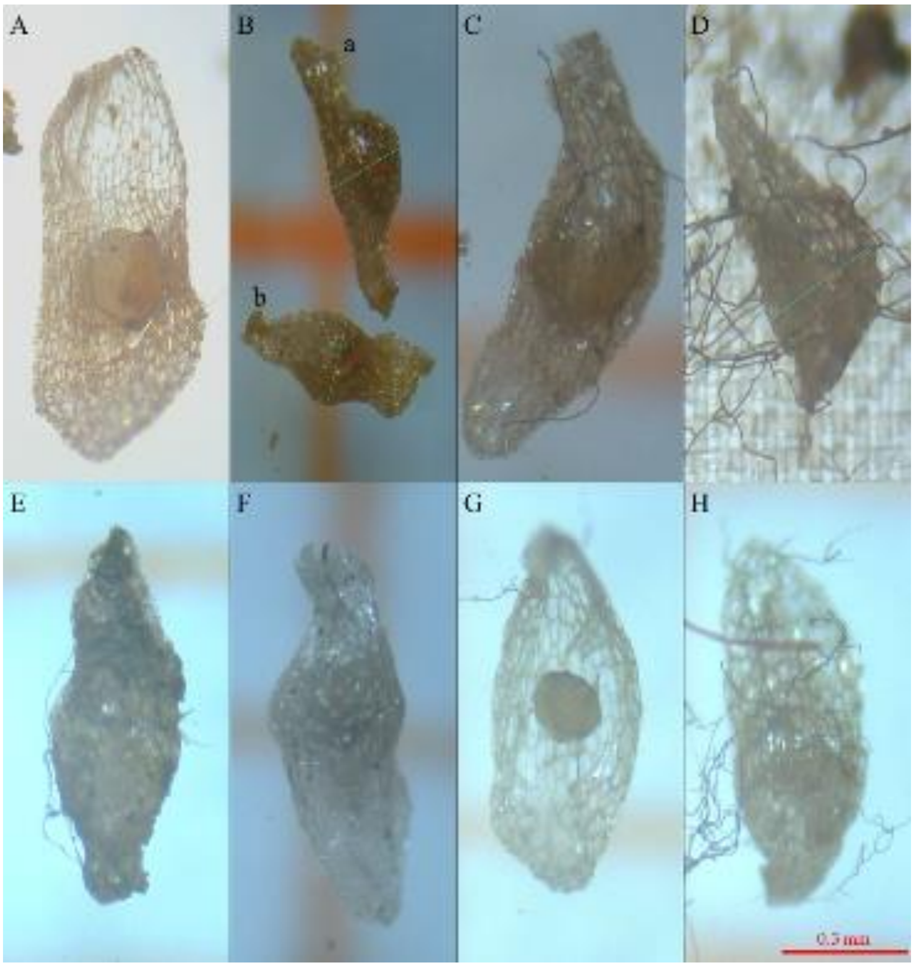

Figure S3: Some of the developing seeds observed during the experiment. A, B (a, b), C and D were collected on the third retrieval campaign while E, F, G and H were collected on the fourth.

Table S1: Morphometric parameters (first and fifth columns) measured in each sowing treatment (second and sixth columns) in the different retrieval campaigns (third and seventh columns) are reported as mean value ± SE (fourth and eighth columns). *Ctm in Ctm* indicates metallicolous seeds sowed in the tailing dump; *Ctm in Ctl* indicates metallicolous seeds sowed in the control site; *Ctl in Ctl* indicates control seeds sowed in the control site; *Ctl in Ctm* indicates control seeds sowed in the tailing dump.

Morphometric parameter	Sowing treatment	Retrieval campaign	Mean ± SE (mm/mm^2)	Morphometric parameter	Sowing treatment	Retrieval campaign	Mean ± SE (mm/mm^2)
Coat width	*Ctl in Ctl*	t1	0.26 ± 0.012	Embryo width	*Ctl in Ctl*	t1	0.145 ± 0.009
		t2	0.26 ± 0.008			t2	0.135 ± 0.004
		t3	0.26 ± 0.009			t3	0.125 ± 0.004
		t4	0.28 ± 0.010			t4	0.132 ± 0.008
		t5	0.28 ± 0.014			t5	0.127 ± 0.006
	Ctl in Ctm	t1	0.23 ± 0.013		*Ctl in Ctm*	t1	0.136 ± 0.007
		t2	0.24 ± 0.009			t2	0.129 ± 0.006
		t3	0.25 ± 0.011			t3	0.123 ± 0.008
		t4	0.27 ± 0.008			t4	0.132 ± 0.005
		t5	0.27 ± 0.02			t5	0.135 ± 0.003
	Ctm in Ctm	t1	0.25 ± 0.011		*Ctm in Ctm*	t1	0.119 ± 0.004
		t2	0.29 ± 0.009			t2	0.127 ± 0.003
		t3	0.33 ± 0.016			t3	0.135 ± 0.003
		t4	0.26 ± 0.012			t4	0.130 ± 0.004
		t5	0.30 ± 0.018			t5	0.141 ± 0.004
	Ctm in Ctl	t1	0.28 ± 0.011		*Ctm in Ctl*	t1	0.123 ± 0.007
		t2	0.29 ± 0.011			t2	0.128 ± 0.005
		t3	0.28 ± 0.018			t3	0.134 ± 0.007

		t4	0.28 ± 0.015			t4	0.138 ± 0.005
		t5	0.27 ± 0.013			t5	0.133 ± 0.005
Coat length	*Ctl in Ctl*	t1	0.90 ± 0.048	Embryo length	*Ctl in Ctl*	t1	0.22 ± 0.01
		t2	0.88 ± 0.040			t2	0.21 ± 0.008
		t3	0.86 ± 0.062			t3	0.20 ± 0.012
		t4	0.94 ± 0.055			t4	0.23 ± 0.01
		t5	1.00 ± 0.045			t5	0.22 ± 0.008
	Ctl in Ctm	t1	0.88 ± 0.042		*Ctl in Ctm*	t1	0.23 ± 0.01
		t2	0.92 ± 0.031			t2	0.23 ± 0.005
		t3	0.96 ± 0.058			t3	0.23 ± 0.01
		t4	0.90 ± 0.053			t4	0.23 ± 0.008
		t5	0.81 ± 0.040			t5	0.24 ± 007
	Ctm in Ctm	t1	1.16 ± 0.039		*Ctm in Ctm*	t1	0.25 ± 0.008
		t2	1.11 ± 0.026			t2	0.24 ± 0.005
		t3	1.07 ± 0.031			t3	0.23 ± 0.007
		t4	1.00 ± 0.056			t4	0.22 ± 0.006
		t5	1.07 ± 0.06			t5	0.24 ± 0.005
	Ctm in Ctl	t1	1.01 ± 0.065		*Ctm in Ctl*	t1	0.24 ± 0.008
		t2	1.08 ± 0.039			t2	0.23 ± 0.007
		t3	1.05 ± 0.041			t3	0.23 ± 0.007
		t4	1.09 ± 0.066			t4	0.22 ± 0.004
		t5	1.03 ± 0.059			t5	0.23 ± 0.007

Coat area	*Ctl in Ctl*	t1	0.19 ± 0.018	Embryo area	*Ctl in Ctl*	t1	0.025 ± 0.002
		t2	0.18 ± 0.013			t2	0.023 ± 0.001
		t3	0.17 ± 0.013			t3	0.020 ± 0.001
		t4	0.21 ± 0.019			t4	0.024 ± 0.002
		t5	0.22 ± 0.018			t5	0.022 ± 0.001
	Ctl in Ctm	t1	0.16 ± 0.011		*Ctl in Ctm*	t1	0.024 ± 0.002
		t2	0.17 ± 0.007			t2	0.024 ± 0.002
		t3	0.18 ± 0.008			t3	0.023 ± 0.003
		t4	0.19 ± 0.016			t4	0.024 ± 0.001
		t5	0.19 ± 0.018			t5	0.025 ± 0.001
	Ctm in Ctm	t1	0.23 ± 0.013		*Ctm in Ctm*	t1	0.023 ± 0.001
		t2	0.24 ± 0.009			t2	0.024 ± 0.001
		t3	0.26 ± 0.013			t3	0.025 ± 0.001
		t4	0.23 ± 0.011			t4	0.023 ± 0.001
		t5	0.23 ± 0.021			t5	0.026 ± 0.001
	Ctm in Ctl	t1	0.23 ± 0.012		*Ctm in Ctl*	t1	0.023 ± 0.002
		t2	0.23 ± 0.013			t2	0.024 ± 0.001
		t3	0.23 ± 0.019			t3	0.024 ± 0.002
		t4	0.23 ± 0.019			t4	0.024 ± 0.001
		t5	0.21 ± 0.017			t5	0.024 ± 0.002

Table S2: Analysis of variance results. Each morphometric parameter (first column) was tested in each sowing treatment (second column) to vary during the experiment. Analysis carried out, together with the *post-hoc* test (if implemented) are reported in the fourth column while fifth and sixth columns report test results. Seventh column reports the *post-hoc* results. The couples of retrieval campaigns where data were reported to significantly vary are reported in the seventh column linked by the ~ symbol and are accompanied by the *p*-values of the respective *post-hoc* test. *Ctm in Ctm* indicates metallicolous seeds sowed in the tailing dump; *Ctm in Ctl* indicates metallicolous seeds sowed in the control site; *Ctl in Ctl* indicates control seeds sowed in the control site; *Ctl in Ctm* indicates control seeds sowed in the tailing dump.

Morphometric parameter	Sowing treatment	Variance test/*post-hoc* test	Variance tests' F value / chi-squared	*p*-values	*Post-hoc* tests' results
Coat Width	*Ctl in Ctl*	ANOVA	0.99	0.42	###
	Ctl in Ctm	ANOVA	2.13	0.09	###
	Ctm in Ctm	ANOVA / Tukey's	5.37	0.001	t3~t1, *p*-value = 0.002 t4~t3, *p*-value = 0.005
	Ctm in Ctl	Kruskall	1.76	0.78	###
Coat length	*Ctl in Ctl*	Kruskall	4.9	0.3	###
	Ctl in Ctm	ANOVA	1.46	0.23	###
	Ctm in Ctm	ANOVA	1.81	0.14	###
	Ctm in Ctl	ANOVA	0.26	0.90	###
Coat area	*Ctl in Ctl*	ANOVA	1.37	0.25	###
	Ctl in Ctm	Kruskall	2.38	0.67	###
	Ctm in Ctm	ANOVA / Tukey's	4.51	0.003	t4~t3, *p*-value = 0.006 t5~t4, *p*-value = 0.007
	Ctm in Ctl	ANOVA	0.22	0.92	###
Embryo Width	*Ctl in Ctl*	ANOVA	1.37	0.26	###
	Ctl in Ctm	ANOVA	0.67	0.61	###
	Ctm in Ctm	Kruskall / Pairwise U test	15.01	0.005	t1~t3, *p*-value = 0.042 t1~t5, *p*-value = 0.042 t2~t5, *p*-value = 0.042
	Ctm in Ctl	ANOVA	0.96	0.44	###
Embryo Length	*Ctl in Ctl*	ANOVA	0.86	0.5	###
	Ctl in Ctm	ANOVA	0.19	0.94	###
	Ctm in Ctm	ANOVA	2.13	0.091	###
	Ctm in Ctl	ANOVA	0.56	0.69	###
Embryo Area	*Ctl in Ctl*	ANOVA	1.39	0.25	###

	Ctl in Ctm	ANOVA	0.27	0.89	###
	Ctm in Ctm	Kruskall	9.41	0.051	###
	Ctm in Ctl	ANOVA	0.12	0.97	###

Table S3: Observed swelling seeds were tested to present significantly greater size than non-swelling seeds observed in the same sowing treatment and retrieval campaign (first and second column respectively). Morphometric parameters are reported in the fourth column of the table, while fifth and sixth columns report test results (*p*-values are corrected by Bonferroni correction). *Ctm in Ctm* indicates metallicolous seeds sowed in the tailing dump; *Ctm in Ctl* indicates metallicolous seeds sowed in the control site; *Ctl in Ctl* indicates control seeds sowed in the control site; *Ctl in Ctm* indicates control seeds sowed in the tailing dump.

Sowing treatment	Retrieval campaign	Morphometric parameter	Test	Test results
Ctl in Ctl	t3 (n = 5)	Coat width	t-test	t = **-3.182;** *p*-value = **0.03**
		Coat length	t-test	t = 1.5151; *p*-value = 1.00
		Coat area	t-test	t = 1.8818; *p*-value = 1.00
		Embryo width	U test	W = NA; *p*-value = 0.02
		Embryo length	U test	W = **NA;** *p*-value = **0.02**
		Embryo area	t-test	t = **-32.573;** *p*-value < 0.001
Ctm in Ctm	t3 (n = 1)	Coat width	t-test	t = -2.4038; *p*-value = 0.11
		Coat length	t-test	t = 2.883; *p*-value = 1.00
		Coat area	t-test	t = -1.4646; *p*-value = 0.52
		Embryo width	U test	W = **NA;** *p*-value = **0.01**
		Embryo length	t-test	t = -1.9907; *p*-value = 0.22
		Embryo area	t-test	t = **-17.394;** *p*-value < 0.001
Ctl in Ctl	t4 (n = 4)	Coat width	t-test	t = **-2.7748;** *p*-value = **0.06**
		Coat length	t-test	t = 2.1719; *p*-value = 1.00
		Coat area	t-test	t = 1.5471; *p*-value = 1.00
		Embryo width	t-test	t = **-14.252;** *p*-value < 0.001
		Embryo length	t-test	t = **-5.567;** *p*-value < 0.001
		Embryo area	t-test	t = **-16.911;** *p*-value < 0.001
Ctl in Ctm	t4 (n = 1)	Coat width	t-test	t = **-15.103;** *p*-value < 0.001
		Coat length	t-test	t = 1.8914; *p*-value = 1.00
		Coat area	t-test	t = **-3.8097;** *p*-value = **0.01**
		Embryo width	t-test	t = **-9.8681;** *p*-value < 0.001
		Embryo length	t-test	t = 6.5998; *p*-value = 1.00
		Embryo area	t-test	t = **-3.7491;** *p*-value = **0.01**

Table S4: Potential evapotranspiration data (third column) of retrieval campaigns reference month (second column). Data were obtained from climatic monitoring authority of Sardinia.

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Agenzia Regionale per la Protezione dell’Ambiente della Sardegna (Arpas), 2020c. Riepilogo mensile meteorologico e agrometeorologico - Gennaio 2021.

Retrieval campaign	Reference month	Potential evapotranspiration (mm)
t1	November (2020)	31 – 35
t2	December (2020)	23 – 24
t3	January (2021)	21 – 25
t4	March (2021)	61 – 65
t5	May (2021)	111 – 120

General conclusions

The present thesis addressed the relationships between environmental heavy metals and plants in bryophytes and orchids. Research activity was conducted in the field in environments featured by anthropogenic heavy metals contaminating soil and atmosphere. The main part of the thesis (Chapters 1, 3, 4 and 5) focuses on the ecophysiological adaptations of metallicolous populations of mosses and orchids. This investigation was performed by working *in-situ* on naturally occurring metallicolous populations. Therefore, it was possible to observe and study the adaptive strategies of mosses and orchids to cope with environmental heavy metals. Consequently, the results reported in the aforementioned chapters should be considered as the microevolutionary outcomes of the strong selective pressure that acts in the studied extreme environments. Metallicolous orchids and mosses exhibited peculiar morphology, ecology and physiology when coping with environmental heavy metals compared with individuals of the same species growing in the absence of environmental pollution. In mosses, species-specific adaptations that are strongly dependent on growth forms and ecological attributes of species were found (Chapter 1). Metallicolous orchids accumulated and translocated soil pollutants in their organs, are smaller and less efficient in their photosynthesis than non-metallicolous orchids (Chapter 3). Moreover, metallicolous orchids produced peculiar seeds different from those produced by non-metallicolous individuals (Chapter 4). The suitability of the seed packet technique for exploring the seed bank conservation and incipient seed development of orchids in extreme environments was proved and site-specific seed development in the metallicolous orchid population was assessed (Chapter 5). These results confirm how plants can thrive in polluted environments despite heavy metal phytotoxicity by adjusting their ecology and physiology. The peculiar biodiversity due to the natural selection acting in the investigated extreme growth contexts can cope with heavy metal stress and should be consequently regarded as a resource in the management of abandoned mines and such critical environments. Moreover, metallicolous populations constitute ideal models to study stress tolerance mechanisms in plants, a knowledge that can be helpful in bioremediation approaches. By contrast, metallicolous biodiversity, which is generally deeply ecologically linked to its metalliferous environments, is also a fragile and threatened element in the ecosystem; thus, metallicolous biodiversity should be preserved. The reclamation of abandoned mine areas as well as the same mining activity could endanger the permanence of metallicolous flora. Further studies should continue to focus on naturally occurring metallicolous populations of orchids and mosses to characterise their autecology and adaptive strategies and protect them from the destruction of their habitat.

The second chapter (Chapter 2) discusses the use of plants (*i.e.* the moss *Hypnum cupressiforme*) in the biomonitoring of air quality and airborne pollutants. The moss bag technique has been used and implemented during the last decades; this technique is also utilised in the present thesis, were its reliability in the biomonitoring of inorganic airborne pollutants was proved. The study on accumulation values measured during an extended time frame (16 years) demonstrated some aspects to consider when interpreting biomonitoring results. In particular, the strong influence of exposure conditions (*i.e.* seasonality, distance from the source of contamination, anthropic activities in addition to the ones monitored and the ecological framework of the biomonitoring campaign) on accumulation values was observed. Therefore, a critical interpretation of the biomonitoring campaign results, which accounts for the aforementioned confounders, should always accompany the raw accumulation values provided by the biomonitor exposure in the environment.

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